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Inhibition of tumor-derived prostaglandin-E2 blocks the induction of myeloid-derived suppressor cells and recovers natural killer cell activity

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Disclosure of Potential Conflicts of Interest
The authors declare no competing financial interests.
Abstract

Purpose: Increased frequencies of myeloid-derived suppressor cells (MDSCs) correlate with poor prognosis in patients with cancers. Tumor-derived prostaglandin-E2 (PGE2) plays an important role in inducing MDSCs. However, the detailed mechanisms of this induction remain unknown. In order to develop targeted therapies for MDSCs, we sought to investigate the molecular basis of PGE2-regulated accumulation of MDSCs and their functional consequence on natural killer (NK) cell activity.

Experimental design: The effects of PGE2 in inducing phenotypic, signaling and functional alternations on monocytes were analyzed in vitro. Suppression of NK cell activity by PGE2-treated monocytes was compared with that of freshly isolated CD14+HLA-DRlow/- monocytic MDSCs (moMDSCs) from patients with melanoma. In addition, to explore the in vivo relevance of targeting PGE2 to reduce MDSC-mediated suppression of NK cells, we established a murine model, where tumor cells were disabled from cyclooxygenase-2 (COX-2) production.

Results: Patient-derived moMDSCs inhibited NK cell activity through the production of TGF-β. In vitro, binding of PGE2 to EP2 and EP4 receptors on monocytes activated the p38MAPK/ERK pathway and resulted in elevated secretion of TGF-β. Similar to moMDSCs, PGE2-treated monocytes potently suppressed NK cell activity through production of TGF-β. Furthermore, silencing COX-2 in murine 4T1 tumor cells reduced the accumulation of CD11b+Gr1+ MDSCs in the spleen, resulting in concomitant improved in vivo clearance of NK cell sensitive YAC-1 cells.
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Conclusions: Our results reveal an indispensable role of tumor-derived PGE2 in inducing MDSCs and suggest a favorable outcome of combining COX-2 targeted therapy and adoptive NK cell transfer in patients with cancer.

Translational Relevance

Myeloid-derived suppressor cells (MDSCs) accumulate in high frequencies in peripheral blood of cancer patients and mediate potent inhibition of adaptive anti-tumor immune responses. These cells are associated with the stage of cancers and have recently been shown to predict survival of patients with melanoma. However, it is less clear how MDSCs are induced in patients with cancers and how they affect the activity of human natural killer (NK) cells. In this study, we evaluated the importance and molecular property of tumor-derived PGE2 in enhancing the suppressive functions by MDSCs of NK cells. Collectively, our data suggest that a favorable clinical outcome may be achieved when COX-2 inhibition and NK adoptive transfer a combined in the treatment of cancers.
Introduction

Tumor progression is frequently associated with a severe impairment of the host innate and adaptive immune response. In addition to immune-regulatory mechanisms directly mediated by tumor cells, a variety of suppressive immune cells, such as regulatory T cells, M2-like tumor-associated macrophages (TAMs) and tolerogenic dendritic cells, have been reported to be expanded in tumor-bearing animals and cancer patients (1).

Recently, myeloid-derived suppressor cells (MDSCs) have been proposed as key immune regulators in various solid and hematologic malignancies (2). While murine MDSCs are characterized as monocytic (moMDSCs; CD11b+/Ly6G-/Ly6C^high) and granulocytic (grMDSCs; CD11b+Ly6G^high/Ly6C^low) (3), the phenotypes of MDSCs in cancer patients remains controversial. Distinct phenotypes of MDSCs are associated with different types of human cancers (2, 4). In advanced stage melanoma patients, an accumulation of circulating CD14^+HLA-DR^low/- moMDSCs were shown in peripheral blood (5-8), which demonstrated a potent suppression of autologous lymphocytes through production of TGF-β (5). In two separate studies, we showed that MDSC-derived reactive oxygen species (ROS), prostaglandin E2 (PGE2) and STAT-3 signaling contribute to the suppression of autologous T cells (6, 9). Other studies have highlighted a variety of suppressive mechanisms mediated by MDSCs, including indoleamine 2,3-dioxygenase (IDO), arginase-1, IL-10 and iNOS (10, 11).

Increased numbers of MDSCs have been correlated with the disease stage in breast cancer patients (8) and survival of ovarian cancer patients (12). In a recent study, Weide and colleagues demonstrated MDSCs, but not Tregs, predicted the survival of patients.
with advanced melanoma and were strongly correlated with the absence of antigen-specific T cells (13). In contrast, decreased frequencies of MDSCs were shown when melanoma patients were treated with BRAF inhibitors (14), or anti-CTLA4 blocking antibodies (15).

Although the detailed molecular mechanisms remain to be elucidated, a number of inflammatory modulators, including GM-CSF (5, 16-19), IL6 (17, 19) and TNF-α (19, 20) have been suggested to contribute to the induction and expansion of MDSCs. Recently, we and others proposed that PGE2 is indispensable for the induction and maintenance of MDSCs in cancer patients. Co-culture with tumor cells secreting PGE2 or treatment with PGE2 was shown to enhance the suppressive functions of monocytes on T cells. Furthermore, inhibition of cyclooxygenase-2 (COX-2), the enzyme catalyzing the synthesis of PGE2, potently rescued suppression of T cells mediated by MDSCs (9, 21).

Despite the recent advances in our understanding of how MDSCs suppress T cell responses, much less is known how NK cell responses are influenced by MDSCs. In tumor-bearing mice, MDSCs were shown to suppress NK cell activation and cytolytic capacity via membrane-bound TGF-β (22, 23). In contrast, it has been proposed that MDSCs may also exert a stimulatory function on NK cells through ligation of the NKG2D receptor (24). In human cancers, it is less documented how MDSCs interact with NK cells. A recent study showed that suppression of NK cells by moMDSCs was mediated through the ligand of NKp30 in patients with hepatocellular carcinoma (25).

To elucidate the role of tumor-derived PGE2 in the generation of MDSCs, we utilized freshly isolated moMDSCs from patients with advanced melanoma, as well as an in vitro
MDSC-induction model. Our results demonstrate that suppression of NK cell activity by CD14^+HLA-DR^{low/-} moMDSCs from patients with advanced melanoma is mediated via TGF-β. Furthermore, treatment with PGE2 conferred monocytes with an MDSC-like phenotype and intracellular signaling pattern enabling them to suppress NK cell responses in a TGF-β-dependent manner. Finally, we demonstrate that shRNA-targeting of COX-2 in murine tumor cells result in a significant reduction of MDSC accumulation in spleen, with concomitant enhanced lysis of YAC-1 cells in vivo. To our knowledge, these observations for the first time reveal the direct involvement and molecular mechanism of PGE2 in stimulating the production of TGF-β from MDSCs and its importance in suppressing human NK cells. Given the prevalence of COX-2/PGE2 over-expression in tumors, these findings could provide fundamental knowledge on how MDSC-mediated immune suppression is initiated and provide targets for limiting the induction of MDSCs in various human cancers.
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Materials and Methods

Patient material and cell isolation

Stage III and IV metastatic melanoma patients were accrued under the guidance of Declaration of Helsinki and written consent forms were signed prior to the material collection. Ethical permission of the study was granted by the review board of Karolinska Institutet (#2011/143-32/1). NK cells and moMDSCs (CD14⁺HLA-DR<sup>low/−</sup>) were isolated from whole blood of the patients as follow. CD³⁺ T cells were removed using CD3 depletion reagents (RosetteSep, StemCell) during Ficoll gradient centrifugation (CD³⁺ cells < 1%, Ficoll-Paque Plus, GE Healthcare). Next, HLA-DR positive cells were sorted with anti-human HLA-DR microbeads (Miltenyi Biotech) and moMDSCs were thereafter purified from the HLA-DR negative fraction using a human CD14⁺ monocyte isolation kit (CD56⁺ cells < 1%, Miltenyi Biotech). NK cells were enriched from the CD³⁻CD14⁻HLA-DR⁻ fraction (Miltenyi Biotech). Cells were thereafter seeded for functional assays or frozen at -80 °C for western blot analysis. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy individuals by Ficoll gradient centrifugation as stated above. NK cells and monocytes were purified with a human NK cell or a CD14⁺ monocyte isolation kit, respectively (purity > 95%, Miltenyi Biotech).

Mice

All animal experiments were conducted under ethical approved study (N603/12, Karolinska Institutet). BALB/c mice (8 to 10 weeks old) were purchased from Charles River Laboratory.
Mouse tumor cell line

Murine breast cancer cell line 4T1 stably expressing scrambled or shRNAs targeting COX-2 enzyme was provided by Dr. William Schiemann (Case Comprehensive Cancer Center, USA) and maintained in DMEM supplemented with 10% FBS. YAC-1 cells were cultured in RPMI1640 supplemented with 10% FBS.

Proliferation assays

Purified monocytes were seeded in triplicates in 96-U-bottom well plates at 1:1 ratio with autologous NK cells in IMDM medium (Gibco) supplemented with 10% heat-inactivated human AB serum (referred as ‘medium’ below), in absence or presence of 50 nM PGE2 and 200 IU/ml IL2. Next, reagents targeting different suppressive mechanisms were added, including 10 µg/ml neutralizing antibodies for PGE2 (Cayman Chemical) or TGF-β (R&D systems), 20 µM selective antagonists for PGE2 receptors AH6809 (EP2), AH23848 (EP4) and L798106 (EP3) (all from Sigma-Aldrich). Following 2 days of co-culture, ³H-thymidine (25 µCi per well) was added to the cells and harvested after additional 12 to 14 hours. Incorporation of ³H-thymidine in the harvested cells was analyzed by a Micro-Beta scintillation counter (TRILUX 1450, PerkinElmer).

Chromium release assays

Following 48 hours of co-culture with monocytes in presence or absence of PGE2, NK cell cytotoxicity was analyzed by chromium (⁵¹Cr) release assays (7 or 18 hours) against K562 (STR fingerprint in Table S1) cells at a 5-to-1 effector-target ratio, as previously described (26). For the ex vivo studies, patient-derived moMDSCs and resting or IL2-activated NK cells were co-cultured at 1:2 ratio, with the presence of 10 µg/ml
neutralizing antibodies for TGF-β or PGE2, 200 IU/ml of the ROS scavenger catalase (Sigma-Aldrich) or superoxide dismutase (Sigma-Aldrich), 500 µM arginase inhibitor N(ω)-hydroxy-nor-L-arginine (nor-NOHA, Calbiochem) or iNOS inhibitor NG-Monomethyl-L-arginine (L-NMMA, Sigma-Aldrich). Following over-night incubation, chromium release assays were performed as mentioned above.

**Cytokine production assays**

Levels of IFN-γ released from patient-derived NK cells were quantified by ELISA assay after 20 hours (MabTech) after addition of K562, in presence of moMDSCs or inhibitors indicated above. Alternatively, patient-derived NK cells were activated with 200 IU/ml IL2, and co-cultured with autologous moMDSCs at 1:1 ratio and in the presence of TGF-β or PGE2 neutralizing antibodies and IFN-γ release was analyzed after 48 hours.

Co-cultures of NK cells and control or PGE2-treated monocytes were harvested and TGF-β concentration was measured using ELISA (R&D systems). For the patient-derived moMDSCs, cells were seeded in medium only, or in presence of 10 µM SB203580 (p38MAPK inhibitor, Sigma-Aldrich), 10 µM H89 (protein kinase A inhibitor, Cayman Chemical), 10 µM PD98059 (MEK1/2 inhibitor, Sigma-Aldrich) or DMSO (Sigma-Aldrich). Following 48 hours of co-culture, supernatants were collected and evaluated for concentrations of TGF-β by ELISA. Moreover, IFN-γ production during IL2 activation in the NK-moMDSC co-cultures was quantified by ELISA (MabTech).

**Western blot**

Monocytes treated with PGE2 (50 nM) for 30 minutes, or freshly isolated moMDSCs were washed in PBS, and frozen at -80 °C for protein analysis by Western Blot. Protein
lysates were prepared from pellets and the blots were developed as previously described (27). In brief, equal amount of protein (50 μg) were loaded and detections of each protein were performed using primary monoclonal antibodies (mAb), including anti-p38MAPK, anti-phospho(Thr180/182)-p38MAPK, anti-ERK, anti-phospho(Thr202/Tyr204)-ERK, anti-AKT, anti-phospho(Ser473)-AKT (Cell Signaling). Beta-actin was used as loading control (Sigma-Aldrich). The membrane was then stained with horse-radish-peroxidase-conjugated secondary antibodies (Cell Signaling), incubated in LumiLight Western Blotting Substrate™ (Roche Diagnostics), and recorded in a LAS3000 system (Fuji).

Flow cytometry and fluorochrome-conjugated antibodies

Detailed information of antibodies utilized in this study is summarized in Table S2. Phenotypic changes of healthy donor-derived monocytes were compared to PGE2-treated monocytes (1 μM). Moreover, surface molecules associated with NK cell functions were also analyzed when cultured with control or PGE2-treated monocytes. In brief, cells were harvested, washed and stained in FACS buffer (PBS containing 1% human serum albumin) (HSA, Octapharma) for 20 minutes at 4 °C. For measuring the intracellular expression of COX-2 enzyme, 4T1 tumor cells were fixed and permeabilized by BD CytoFix/CytoPerm buffer, followed by washing and staining steps using BD Perm/Wash buffer (BD Biosciences).

In experiments where murine Tregs were evaluated, extra-cellular molecules were first stained, and intracellular expression of FoxP3 was measured with BD transcription factor buffer set (BD Biosciences), according to the manufacturer’s protocol. Cells were
acquired on a BD LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Treestar).

**NK cell *in vivo* cytotoxicity**

Since COX-2 silencing in 4T1 cells results to a delay of tumor growth *in vivo* (28), inoculated cell numbers were adjusted and all experiments were performed in mice bearing comparable tumor burdens. In brief, control, COX-2 silenced 4T1 cells or PBS was injected subcutaneously (s.c.) into the right flank of the mice. When average tumor size reached 300 mm$^3$ (calculated by length×width×height×0.5 (29)), murine NK cell sensitive target YAC-1 cells were labeled with DiR near infra-red dye, (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine iodide, Invitrogen) for 10 minutes, followed by washing and intravenous (i.v.) injection into all mice, to evaluate the cytolytic activity of NK cells *in vivo*. The fluorescence intensity of YAC-1 cells was examined by *in vivo* live imaging using an IVIS camera platform (Caliper Life Sciences Benelux & Nordic) 1 hour and 5 hours after the injection. Lungs of mice bearing size-matched tumors were resected and intensity of YAC-1 cells was evaluated after 5 hours. Splenocytes were isolated from mice that did not receive YAC-1 injection, and measured for percentages of CD11b$^+$Gr1$^+$ MDSCs, CD3$^-$CD49b$^+$ NK cells and CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells by flow cytometry as described above.

**Statistics**

All data were first analyzed in the software mentioned above and summarized by Prism Version 6 software (GraphPad). All data were first tested for normal distribution, thereafter differences among groups were analyzed by student t-test or non-parametric,
Mann-Whitney U tests (as indicated in the figure legends). Unless otherwise stated, multiple inputs from separate experimental and control groups were presented as means and SD. Representative histograms or images were chosen based on the average values.

**Results**

Melanoma-derived CD14\(^+\)HLA-DR\(^{low/-}\) moMDSCs potently inhibit NK cell functions

To analyze the influence of MDSC on NK cells, patient-derived CD14\(^+\)HLA-DR\(^{low/-}\) moMDSCs and HLA-DR\(^+\) mononuclear cells were isolated and co-cultured with autologous non-activated NK cells. In the presence of moMDSCs, cytolytic capacity and production of IFN-\(\gamma\) by NK cells were significantly inhibited (\(p<0.01\)) ([Figure 1A](#) and [B](#)). Similar to non-activated NK cells, the presence of moMDSCs during IL2-activation of NK cells resulted in significantly impaired cytotoxicity and IFN-\(\gamma\) production by NK cells (\(p<0.01\) and \(p<0.05\), respectively) ([Figure 1C](#) and [D](#)). In contrast, HLA-DR\(^+\) cells did not show suppressive functions against NK cells ([Figure S1A](#)).

TGF-\(\beta\) is the major suppressive mechanism employed by patient-derived moMDSCs

We next investigated the molecular basis of suppression mediated by patient-derived moMDSCs. As shown in [Figure 2A](#) and [B](#), blocking moMDSC-derived TGF-\(\beta\) significantly rescued the cytotoxicity (\(p<0.05\)) and production of IFN-\(\gamma\) by non-activated NK cells (\(p<0.01\)). Moreover, addition of the hydrogen peroxide scavenger catalase led to significant improvement of NK cell-mediated cytotoxicity ([Figure 2A](#)). Similarly, when TGF-\(\beta\) blocking antibody was present in co-cultures of IL2-activated NK cells and moMDSCs, NK cell cytotoxicity (\(p<0.01\)) ([Figure 2C](#)) and IFN-\(\gamma\) released by NK cells (\(p<0.05\)) ([Figure 2D](#)) were significantly improved. However, catalase did not show
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functional restoration of IL2-activated NK cells (data not shown). Of note, addition of neutralizing antibodies did not modulate cytolytic functions of IL2-activated NK cells in presence of HLA-DR+ cells (Figure S1A).

PGE2-treated monocytes resemble patient-derived moMDSCs

We and others have previously shown that PGE2 contributes to the conversion of monocytes into MDSC-like cells (9, 21). In order to validate the similarities of PGE2-induced MDSCs and patient-derived moMDSCs, we evaluated the phenotypic alternations and PGE2-induced intracellular signaling pathways. The results demonstrated that PGE2 increased the expression of CD14 on monocytes while the expression of HLA-DR was down-regulated (Figure 3A and B). Expression of other myeloid cell markers including; CD16, CD80, DC-Sign, CD33 and CD137 (4-1BB), were not altered between control-cultured and PGE2-treated monocytes (Figure S2A).

Intracellularly, p38MAPK and ERK pathways were phosphorylated in monocytes, as a result of PGE2 treatment (Figure 3C). This was also the case in freshly isolated moMDSCs, in comparison to control HLA-DR+ cells (Figure 3C). However, neither of the samples demonstrated phosphorylation of AKT proteins, even though total expression of AKT was up-regulated in monocytes subsequent to in vitro culture (Figure 3C). Due to these similarities with moMDSCs, PGE2-treated monocytes were regarded as ‘MDSC-like’ cells.

MDSC-like cells suppress NK cells through TGF-β

To validate the suppressive functions of MDSC-like cells, we measured the proliferation, phenotype as well as cytolytic capacity of NK cells, when co-cultured with MDSC-like
cells or control monocytes. Since PGE2 directly suppressed proliferation of IL2-activated NK cells in a dose-dependent manner (Figure S2B), a non-inhibitory level of PGE2 (50 nM) was chosen for all subsequent assays. At this concentration, MDSC-like cells significantly \((p<0.05)\) reduced NK cell killing capacity when present at a 1-to-1 ratio during NK cell activation (Figure 4A and S2C). Moreover, MDSC-like cells significantly inhibited the proliferation of NK cells \((p<0.01)\) (Figure 4B). Notably, the expression of NKG2D, NKp46 and NKp44 were significantly reduced \((p<0.05)\) on IL2-activated NK cells, when MDSC-like cells were present. Although to a lower extent, co-culture with monocytes down-regulated the expression of these molecules (Figure 4C).

Next, we asked what mechanisms were responsible for the suppressive actions mediated by MDSC-like cells. Similar to patient-derived moMDSCs, neutralization of TGF-\(\beta\) significantly restored NK cell-mediated cytotoxicity \((p<0.01)\) and proliferation \((p<0.05)\) to similar levels to that of NK cells cultured alone (Figure 4D and E). Importantly, neutralization of TGF-\(\beta\) or PGE2 had no direct impact on the cytotoxicity of NK cells (Figure S1B).

To assess which PGE2 receptor on monocytes contributes to the PGE2-mediated induction of NK cell-suppressive MDSC-like cells, antagonists of EP receptors or PGE2 blocking antibody were added to NK-MDSC-like co-cultures. Interrupting the activity of EP2 and EP4, but not of EP3, significantly blocked the suppressive capacity of the MDSC-like cells on NK cells \((p<0.01)\) (Figure 4D). In addition, significant recovery of NK cell proliferative ability was observed by antagonizing the EP2 receptor \((p<0.05)\) (Figure 4E).
PGE2 enhances the production of TGF-β by monocytes

Since TGF-β plays a major role in suppression of NK cell responses by MDSC-like cells and moMDSCs, we next evaluated if monocytes differ in their ability to produce TGF-β in presence or absence of PGE2. Compared with untreated monocytes, MDSC-like cells produced significantly higher levels of soluble TGF-β \((p<0.05)\) (Figure 5A), which was abolished when cultured in presence of neutralizing antibodies to PGE2 \((p<0.05)\) (Figure 5A). These observations were confirmed in the NK-MDSC-like co-cultures, excluding the possibility of TGF-β-driven auto-regulation by PGE2-treated NK cells (Figure 5B). In contrast, monocytes expressed low levels of membrane-bound TGF-β, which remained unchanged after PGE2 treatment (data not shown).

Protein kinase A is known to be one of the major intracellular regulators of EP2/EP4 signaling (30) and contribute to the activation of p38MAPK pathway in myeloid cells (31). Therefore, chemical inhibitors of p38MAPK (SB203580), MAP or ERK kinase (MEK1/2, PD98085), protein kinase A (H-89), or DMSO were utilized. Indeed, interruption of these pathways resulted in significant attenuation of TGF-β release from patient-derived moMDSCs (Figure 5C), confirming the functional contribution of these pathways to MDSC-mediated suppression of NK cells in humans.

Over-expression of COX-2 in tumor cells regulates MDSC accumulation and NK cell cytotoxicity in vivo

To assess the functional consequences of COX-2 expression in inducing MDSCs in vivo, we used a murine model, where control or COX-2-silenced 4T1 mammary carcinoma cells were inoculated subcutaneously (Figure S3A).
Inoculation of control 4T1 cells resulted in a significant accumulation of CD11b+Gr1+ MDSCs \((p<0.001)\) (Figure 6A), but a decrease in the percentage of NK cells in the spleen of BALB/c mice \((p<0.05)\) (Figure 6B). Notably, significantly fewer CD11b+Gr1+ MDSCs \((p<0.01)\) (Figure 6A) and restored numbers of NK cells \((p<0.01)\) (Figure 6B) were found in spleens when COX-2 was not expressed by tumor cells. Although significantly higher percentages of regulatory T cells \((CD4^+CD25^+FoxP3^+, p<0.01)\) (Figure 6C and Figure S3B) and lower percentages of CD3+ T cells \((p<0.001)\) (Figure S3C) were observed in mice inoculated with control 4T1 tumor cells, these cell populations were not regulated by tumor-derived COX-2 production (Figure 6C and S3C). Moreover, the percentage of CD4+ T cells was comparable in all groups (Figure S3D).

To evaluate the cytolytic capacity of NK cells in vivo, naive or mice bearing similar tumor burdens were challenged with DiR-labeled YAC-1 cells and the signal was evaluated by live imaging. Along with the decrease in MDSCs and increased NK cell percentages in mice bearing COX-2-silenced 4T1 tumors, a significantly improved ability to reject NK cell sensitive YAC-1 cells were observed \((p<0.05)\) (Figure 6D). Resection of lungs from YAC-1 infused mice bearing COX-2-silenced 4T1 tumor cells showed a pronounced reduction of YAC-1 cells after 5 hours, compared with the control group (Figure 6E). No difference in the fluorescence intensity of inoculated YAC-1 cells was observed within one hour after inoculation, showing that the fluorescence labeling intensity was similar among the groups (Figure S3E).
Discussion

In the recent years, there is an increasing interest in understanding the immune-regulatory role of MDSCs in cancers. NK cell responses are negatively regulated by several immunosuppressive mechanisms employed by tumor cells (32), regulatory T cells (33, 34) and MDSCs (23, 25). While the mechanisms of how MDSCs suppress T cells have been extensively studied, much less is known how MDSCs regulate the activity of human NK cells. Here we provide evidence that support a direct involvement of PGE2 during the initiation and maintenance of NK cell suppression by MDSCs.

Co-culture of moMDSCs isolated from melanoma patients with NK cells resulted in reduced cytolytic function and IFN-γ release of resting and IL2-activated NK cells. Since neutralization of moMDSC-derived TGF-β restored NK cell activity, it demonstrated the importance of this cytokine in MDSC-mediated inhibition of NK cells. Moreover, ROS scavenger catalase significantly rescued the cytolytic activity of resting NK cells in presence of moMDSCs. In contrast, production of IFN-γ from resting NK cells was not improved in presence of catalase. This implies that while the cytotoxic function of resting NK cells is sensitive to ROS-mediated suppression, it is not the case for IFN-γ production.

It is in line with earlier observations, where cytotoxic CD56\textsuperscript{dim} NK cells are more susceptible to ROS-induced apoptosis, compared to cytokine-producing CD56\textsuperscript{bright} NK cells (35, 36). The distinction in sensitivity to ROS between the two NK cell subsets does not apply to IL-2-activated NK cells, where TGF-β, rather than ROS or other mechanisms, dominated the suppression of both cytotoxicity and cytokine production of NK cells.
Although strongly suggested by previous studies (5, 7), blockade of TGF-β secretion of CD14⁺HLA-DR^{low/-} moMDSCs did not result to rescued proliferation of purified human T cells (6, 9, 37). In the earlier studies, thymidine incorporation was evaluated in PMA-activated lymphocytes, when moMDSCs were present at different ratios. Therefore, TGF-β mediated suppression of NK cells or their production of T cell-stimulating cytokines in these experimental settings, as demonstrated in our study, may contribute to explain the existing discrepancies in the literature.

Production of arginase I and iNOS are two well-documented mechanisms that MDSCs employ to suppress tumor-reactive T cell responses. We here find moMDSC-mediated suppression of NK cells to be independent of these factors, concurring with previous observations in patients with hepatocellular carcinoma (25). This can be explained by the heterogeneity of human MDSC population, in which granulocytic MDSCs are potent producers of arginase I, whereas suppression mediated by the monocytic subset is largely dependent on other factors, i.e. TGF-β (5) or ROS (38). Thus, due to the multi-faceted functions of MDSCs, it is reasonable to propose that multiple suppressive factors need to be targeted to abolish MDSC-induced immune suppression.

In agreement with Lechner et al., we have previously shown that co-culture with a variety of tumor cell lines converted monocytes into MDSC-like cells, which mediated suppression of T cells through COX-2 production (9, 19). To elucidate the molecular basis of this induction, recombinant PGE2 was used as a surrogate for monocyte-tumor cell co-culture. Treatment of PGE2 maintained an immature status of monocytes, including high expression levels of CD14, but reduced expression of HLA-DR. Culture of monocytes in presence of PGE2 suppressed NK cell responses via production of TGF-
β and blockade of the EP2 or EP4 receptor on monocytes reduced this suppression. Notably, PGE2 treatment also enabled the suppressive functions of HLA-DR<sup>+</sup> cells isolated from advanced stage melanoma patients (data not shown). This provides a novel mechanistic insight, in addition to the previously reported positive feedback loop of PGE2 production mediated by the EP2 and EP4 receptors on human moMDSCs (21).

Phosphorylation of p38MAPK and ERK proteins has previously been shown to be associated with MDSCs in tumor-bearing animals (39, 40) and cancer patients (41). However, the functional implications and molecular basis of these pathways remain unknown. Here, we provide a novel perspective, where elevated PGE2 levels in cancer patients could activate these pathways in myeloid cells through the EP2 or EP4 receptor. Consequently, these cells are conferred with an immature MDSC phenotype as well as the ability to produce TGF-β. In addition, this may lead to a functional resistance to apoptosis of MDSCs, for example by Fas-FasL interactions (40), or a prolonged survival in tumor microenvironment, in comparison to other HLA-DR<sup>+</sup> cells. Thus, targeting similar pathways in MDSCs with BRAF or MEK1/2 inhibitors may contribute to the clinical responses in cancer patients (14).

Further studies of the role of PGE2 in inducing NK-suppressive MDSCs was addressed by an in vivo 4T1 mammary carcinoma model, which induced extensive accumulation of MDSCs in the spleen of the tumor-bearing mice (3). Remarkably, when these tumor cells were disabled from COX-2 production, we observed a significant reduction of MDSCs with a concomitant recovery of NK cell numbers. Live imaging confirmed an enhanced in vivo clearance of NK cell sensitive YAC-1 cells in mice bearing COX-2-silenced
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tumors. Notably, COX-2 silencing didn’t influence the induction of Tregs, indicating that MDSCs were the major contributors of the peripheral NK suppression in this model.

Previous studies using dietary supplement of COX-2 inhibitors have resulted to decreased frequencies of MDSCs in the tumor microenvironment and in the periphery (42). In comparison, our approach provides selective inhibition of tumor-derived COX-2, where the essential role of COX-2/PGE2 during the in vivo accumulation of MDSCs was confirmed (43). To correlate results acquired from patient-derived moMDSCs, MDSCs were only evaluated in the spleens of the tumor-bearing mice.

Recently, others have proposed GM-CSF to be the major factor of MDSC induction in a Kras/p53-mutated pancreatic cancer model (16). Even though tumor-derived PGE2 was not evaluated in that study, a number of reports have demonstrated the over-expression of COX-2 or PGE2, and their tumor-promoting roles in spontaneous pancreatic cancer model (44), or human pancreatic cancer tissues (45, 46). Thus, it is likely that GM-CSF, PGE2, as well as other tumor-derived inflammatory mediators, collaborate in promoting the induction of MDSCs in cancers.

Taken together, our data demonstrated a novel role of PGE2 in inducing suppressive functions of MDSCs on NK cells, primarily by activating the production of TGF-β in myeloid cells through EP2/4 receptor and p38MAPK/ERK pathway. Given the prevalence of COX-2 over-expression and the central immune-regulatory role of TGF-β, these mechanisms might be relevant in several types of human cancers. Therefore, it is suggested that combinational therapy of COX-2 inhibition and NK adoptive transfer may lead to a favorable clinical outcome in cancer patients.
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Figure Legends

Figure 1. Melanoma-derived CD14⁺HLA-DR<sub>low/-</sub> moMDSCs potently inhibit NK cell activity.

Freshly isolated NK cells from advanced stage melanoma patients were co-cultured with autologous moMDSC (CD14⁺HLA-DR<sub>low/-</sub>) overnight and analyzed for A) cytolytic activity against K562 cells (n=7) and B) production of IFN-γ induced by K562 (n=7). Alternatively, NK cells were co-cultured with autologous moMDSC during IL2 activation (200 U/ml, 48 hours) and analyzed for C) cytolytic activity against K562 cells (n=6) and D) IFN-γ produced during IL2 activation (n=3). Data are shown as mean±SD, Mann-Whitney non-parametric U test, *, p<0.05; **, p<0.01.

Figure 2. TGF-β is the major suppressive mechanism employed by patient-derived moMDSCs

Freshly isolated patient-derived NK cells were cultured alone or together with autologous moMDSC overnight in presence of different blocking reagents and analyzed for their ability to A) lyse K562 cells (n=6) and B) produce of IFN-γ induced by K562 (n=6). Alternatively, NK cells were cultured alone or with autologous moMDSC during IL2 activation (200 U/ml, 48 hours), in presence of neutralizing antibodies against TGF-β or PGE2 and C) cytolytic activity of NK cells against K562 cells (n=6) and D) IFN-γ produced during IL2 activation were analyzed (n=6). Data are shown as mean±SD, Mann-Whitney non-parametric U test, *, p<0.05; **, p<0.01.
Figure 3. PGE2-treated monocytes resemble patient-derived moMDSCs.

A) Surface expression of CD14 and HLA-DR on monocytes cultured in presence or absence of PGE2 (1 µM, 48 hours). Data is presented as mean±SD from three individual experiments. B) Representative histograms of CD14 and HLA-DR staining. C) Representative micrograph of expression and phosphorylation of p38MAPK, ERK, and AKT in monocytes from healthy donors cultured in presence or absence of PGE2 (50 nM, 30 minutes) or freshly isolated patient-derived HLA-DR⁺ cells or moMDSCs (n=3).

Figure 4. MDSC-like cells suppress NK cells through TGF-β

NK cells isolated from healthy individuals were activated with IL-2 (200 U/ml, 48 hours) alone or together with monocytes in presence or absence of PGE2 (50 nM) and their A) cytotoxicity against K562 (n=8), B) proliferation (n=5), and C) phenotype (n=4) were analyzed. Further, ability of IL2-activated NK cells to D) lyse K562 cells (n=6) and E) proliferate, when co-cultured with monocytes in presence or absence of PGE2, neutralizing antibodies against TGF-β, PGE2 or antagonists of EP2 (AH6809), EP3 (L798106) or EP4 (AH23848) (all at 20 µM, n=5) were demonstrated. Data are presented as mean±SD, Mann-Whitney non-parametric U test, *, p<0.05; **, p<0.01; n.s., not significant.

Figure 5. PGE2 enhances the production of TGF-β on monocytes.

A) Monocytes isolated from healthy donors were cultured alone or in the presence of PGE2 (50 nM, 48 hours) or together with a neutralizing antibody against PGE2 and analyzed for production of TGF-β (n=3). B) Production of TGF-β in cultures of NK cells alone or when co-cultured with monocytes, in the presence or absence of PGE2 (50nM,
n=4). C) Patient-derived moMDSCs (CD14^{+}HLA-DR^{low/-}) were cultured in the presence of DMSO, SB203580 (p38MAPK), PD98085 (MEK1/2) or H-89 (protein kinase A) (all used at 10 µM) and analyzed for production of TGF-β (n=5) relative to non-treated moMDSCs. Data are shown as mean±SD, student T-test, *, p<0.05; **, p<0.01.

Figure 6. Over-expression of COX-2 in tumor cells regulates MDSC accumulation and NK cell cytotoxicity in vivo.

Splenocytes were isolated from naïve BALB/c or mice bearing similar tumor burden (control or COX-2 silenced 4T1 cells). A) Percentages of MDSCs (CD11b^{+}Gr1^{+}), B) NK cells (CD3^{+}CD49b^{+}) or C) regulatory T cells (CD3^{+}CD4^{+}CD25^{+}FoxP3^{+}) were determined by flow cytometry. D) Quantitative analysis of YAC-1 cells in mice bearing control 4T1 or COX-2-silenced 4T1 tumors were examined by in vivo live imaging using the IVIS camera platform. E) Lungs were resected and remaining YAC-1 cells were measured after 5 hours. Numbers of the dots in each group correspond to numbers of mice utilized in the experiments. Data are presented as mean±SD, Mann-Whitney non-parametric U test, *, p<0.05; **, p<0.01; ***, p<0.001; n.s., not significant.
References


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Figure 1

A 
Fresh NK cells

B 
Fresh NK cells

C 
IL2-activated NK cells

D 
IL2-activated NK cells
Figure 3

A

Fold changes to fresh monocytes

CD14

HLA-DR

No PGE2

with PGE2 (1 uM)

B

CD14

HLA-DR

no PGE2

with PGE2 (1 uM)

C

Patient moMDSCs

MDSC-like cells

phospho-p38MAPK

40 kDa

42/44 kDa

phospho-ERK

60 kDa

phospho-AKT

p38MAPK

40 kDa

42/44 kDa

ERK

60 kDa

AKT

β-actin

42 kDa

monoMDSCs

HLA-DR+

Mono

Mono+PGE2

(50 nM)
Figure 4

Panel A: Bar graphs showing the percentage of lysed K562 cells with and without PGE2 in NK only and NK + mono conditions.

Panel B: Bar graphs showing 3H incorporation in NK only, NK + mono, and no NK conditions with and without PGE2.

Panel C: MFI of NK cells with and without PGE2 for NKG2D, NKp46, and DNAM-1.

Panel D: MFI of NK cells with and without PGE2 and with different antagonists.

Panel E: Relative changes of proliferation with and without PGE2 and with different antagonists.
Figure 5

A

B

C

relative changes of TGF-β

DMSO  SB203580  PD98059  H-89

0.4  0.6  0.8  1.0  1.2

NK only  NK + Mono
Figure 6

A) % of MDSCs in splenocytes

B) % of NK cells in lymphocytes

C) % of Tregs in CD3 T cells

D) YAC-1 intensity (1x10^4)

E) Lungs

Legend:
- Naive
- Ctrl 4T1
- shCOX-2 4T1

Significance levels:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001

Note: This figure compares the effects of Ctrl 4T1 and shCOX-2 4T1 on the percentage of MDSCs, NK cells, and Tregs in splenocytes and lymphocytes, as well as YAC-1 intensity in the lungs.
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Yumeng Mao, Dhifaf Sarhan, Andre Steven, et al.

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