

Inhibition of Tumor-Derived Prostaglandin-E2 Blocks the Induction of Myeloid-Derived Suppressor Cells and Recovers Natural Killer Cell Activity

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Abstract

Purpose: Increased frequencies of myeloid-derived suppressor cells (MDSC) 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. 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 alterations on monocytes were analyzed *in vitro*. Suppression of NK-cell activity by PGE2-treated monocytes was compared with that of freshly isolated CD14⁺HLA-DR^{low/-} monocytic MDSCs (moMDSC) 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 TGFβ. *In vitro*, binding of PGE2 to EP2 and EP4 receptors on monocytes activated the p38MAPK/ERK pathway and resulted in elevated secretion of TGFβ. Similar to moMDSCs, PGE2-treated monocytes potently suppressed NK-cell activity through production of TGFβ. 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.

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. *Clin Cancer Res*; 20(15); 4096–106. ©2014 AACR.

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 (TAM), and tolerogenic dendritic cells, have been reported to be expanded in tumor-bearing animals and patients with cancer (1).

Recently, myeloid-derived suppressor cells (MDSC) have been proposed as key immune regulators in various solid and hematologic malignancies (2). Although 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 patients with cancer remains controversial. Distinct phenotypes of MDSCs are associated with different types of human cancers (2, 4). In patients with advanced stage melanoma, 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 TGFβ (5). In 2 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, IL10, and iNOS (10, 11).

Increased numbers of MDSCs have been correlated with the disease stage in patients with breast cancer (8) and

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Translational Relevance

Myeloid-derived suppressor cells (MDSC) accumulate in high frequencies in peripheral blood of patients with cancer 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 are combined in the treatment of cancers.

survival of patients with ovarian cancer (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 patients with melanoma 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 patients with cancer. Coculture 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 overexpression 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.

Materials and Methods

Patient material and cell isolation

Patients with stage III and IV metastatic melanoma were accrued under the guidance of Declaration of Helsinki and written consent forms were signed before 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^{low/-}) were isolated from whole blood of the patients as follows. CD3⁺T cells were removed using CD3 depletion reagents (Rosette-Sep, StemCell) during Ficoll gradient centrifugation (CD3⁺ cells <1%; Ficoll-Paque Plus, GE Healthcare). Next, HLA-DR⁺ 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^{neg}CD14^{neg}HLA-DR^{neg} fraction (Miltenyi Biotech). Cells were thereafter seeded for functional assays or frozen at -80°C for Western blot analysis. Peripheral blood mononuclear cells (PBMC) 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 (8–10 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. W. Schiemann (Case Comprehensive Cancer Center, Cleveland, OH) and maintained in DMEM supplemented with 10% FBS. YAC-1 cells were cultured in RPMI-1640 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 the absence or presence of 50 nmol/L 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 TGFβ (R&D systems), 20 µmol/L selective antagonists for PGE2 receptors AH6809 (EP2), AH23848 (EP4), and L798106 (EP3) (all from Sigma-Aldrich). Following 2 days of coculture, 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 MicroBeta scintillation counter (TRILUX 1450, PerkinElmer).

Chromium release assays

Following 48 hours of coculture with monocytes in the presence or absence of PGE2, NK-cell cytotoxicity was analyzed by chromium (⁵¹Cr) release assays (7 or 18 hours) against K562 (STR fingerprint in Supplementary 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 cocultured at 1:2 ratio, with the presence of 10 µg/mL neutralizing antibodies for TGFβ or PGE2, 200 IU/mL of the ROS scavenger catalase (Sigma-Aldrich) or superoxide dismutase (Sigma-Aldrich), 500 µmol/L arginase inhibitor N(ω)-hydroxy-nor-L-arginine (nor-NOHA; Calbiochem) or iNOS inhibitor NG-monomethyl-L-arginine (L-NMMA; Sigma-Aldrich). Following overnight incubation, chromium release assays were performed as mentioned above.

Cytokine production assays

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

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

Western blot analysis

Monocytes treated with PGE2 (50 nmol/L) for 30 minutes, or freshly isolated moMDSCs were washed in PBS, and frozen at -80°C for protein analysis by Western blot analysis. 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). β-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 Supplementary Table S2. Phenotypic changes of healthy donor-derived monocytes were compared with PGE2-treated monocytes (1 µmol/L). 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, extracellular 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 (Tree Star).

NK-cell *in vivo* cytotoxicity

Because 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 into the right flank of the mice. When average tumor size reached 300 mm³ (calculated by length × width × height × 0.5; ref. 29), murine NK-cell sensitive target YAC-1 cells were labeled with DiR near infra-red dye (1,1'-dioctadecyl-3,3,3',3'-tetra-methylindotri-carbocyanine iodide; Invitrogen) for 10 minutes, followed by washing and intravenous 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 and

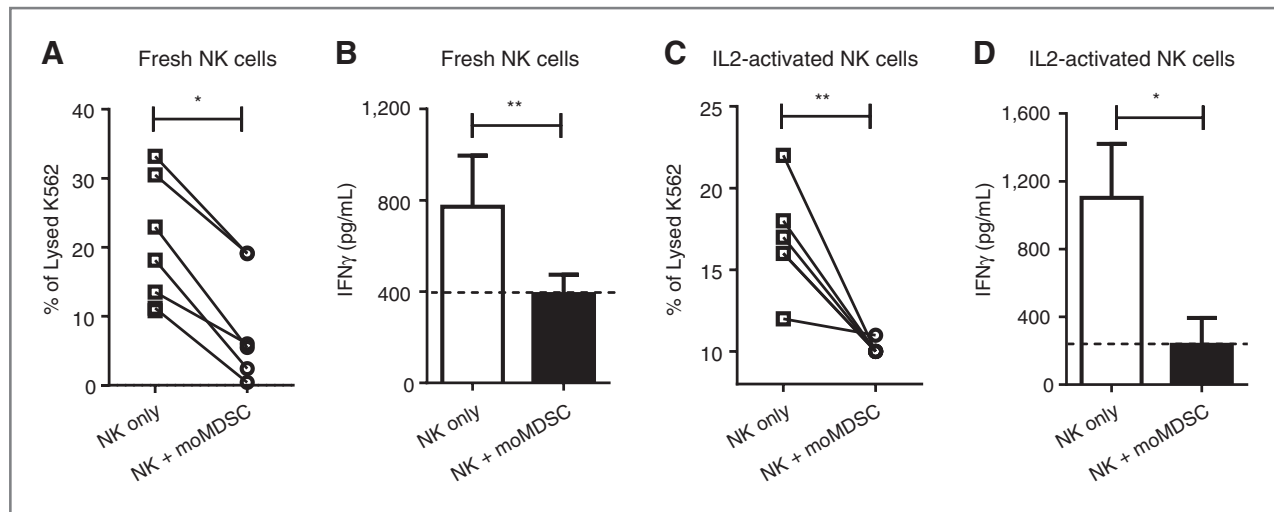


Figure 1. Melanoma-derived CD14⁺HLA-DR^{low/-} moMDSCs potently inhibit NK-cell activity. Freshly isolated NK cells from patients with advanced stage melanoma were cocultured with autologous moMDSCs (CD14⁺HLA-DR^{low/-}) overnight and analyzed for cytolytic activity against K562 cells ($n = 7$; A) and production of IFN γ induced by K562 ($n = 7$; B). Alternatively, NK cells were cocultured with autologous moMDSCs during IL2 activation (200 U/mL, 48 hours) and analyzed for cytolytic activity against K562 cells ($n = 6$; C) and IFN γ produced during IL2 activation ($n = 3$; D). Data are shown as mean \pm SD; Mann-Whitney nonparametric U test; *, $P < 0.05$; **, $P < 0.01$.

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.

Statistical analysis

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 nonparametric, 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 cocultured with autologous nonactivated NK cells. In the presence of moMDSCs, cytolytic capacity and production of IFN γ by NK cells were significantly inhibited ($P < 0.01$; Fig. 1A and B). Similar to nonactivated NK cells, the presence of moMDSCs during IL2-activation of NK cells resulted in significantly impaired cytotoxicity and IFN γ production by NK cells ($P < 0.01$ and $P < 0.05$, respectively; Fig. 1C and D). In contrast, HLA-DR⁺

cells did not show suppressive functions against NK cells (Supplementary Fig. S1A).

TGF β is the major suppressive mechanism used by patient-derived moMDSCs

We next investigated the molecular basis of suppression mediated by patient-derived moMDSCs. As shown in Fig. 2A and B, blocking moMDSC-derived TGF β significantly rescued the cytotoxicity ($P < 0.05$) and production of IFN γ by nonactivated NK cells ($P < 0.01$). Moreover, addition of the hydrogen peroxide scavenger catalase led to significant improvement of NK-cell-mediated cytotoxicity (Fig. 2A). Similarly, when TGF β blocking antibody was present in cocultures of IL2-activated NK cells and moMDSCs, NK-cell cytotoxicity ($P < 0.01$; Fig. 2C) and IFN γ released by NK cells ($P < 0.05$; Fig. 2D) were significantly improved. However, catalase did not show 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 the presence of HLA-DR⁺ cells (Supplementary Fig. 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). 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 whereas the expression of HLA-DR was downregulated (Fig. 3A and B). Expression of other myeloid cell markers, including

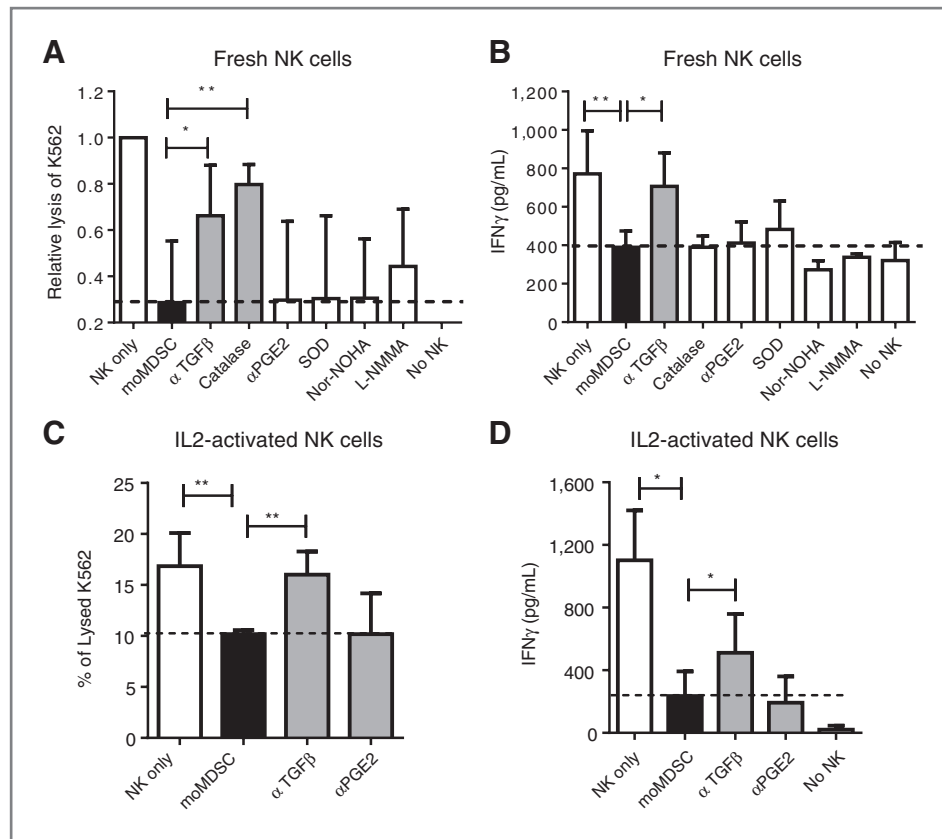


Figure 2. TGFβ is the major suppressive mechanism used by patient-derived moMDSCs. Freshly isolated patient-derived NK cells were cultured alone or together with autologous moMDSC overnight in the presence of different blocking reagents and analyzed for their ability to lyse K562 cells (*n* = 6; A) and produce of IFNγ induced by K562 (*n* = 6; B). Alternatively, NK cells were cultured alone or with autologous moMDSC during IL2 activation (200 U/mL, 48 hours), in the presence of neutralizing antibodies against TGFβ or PGE2 and cytolytic activity of NK cells against K562 cells (*n* = 6; C) and IFNγ produced during IL2 activation were analyzed (*n* = 6; D). Data, mean ± SD; Mann–Whitney nonparametric *U* test; *, *P* < 0.05; **, *P* < 0.01.

CD16, CD80, DC-Sign, CD33, and CD137 (4-1BB), was not altered between control-cultured and PGE2-treated monocytes (Supplementary Fig. S2A).

Intracellularly, p38MAPK and ERK pathways were phosphorylated in monocytes, as a result of PGE2 treatment

(Fig. 3C). This was also the case in freshly isolated moMDSCs, in comparison to control HLA-DR⁺ cells (Fig. 3C). However, neither of the samples demonstrated phosphorylation of AKT proteins, even though total expression of AKT was upregulated in monocytes subsequent to

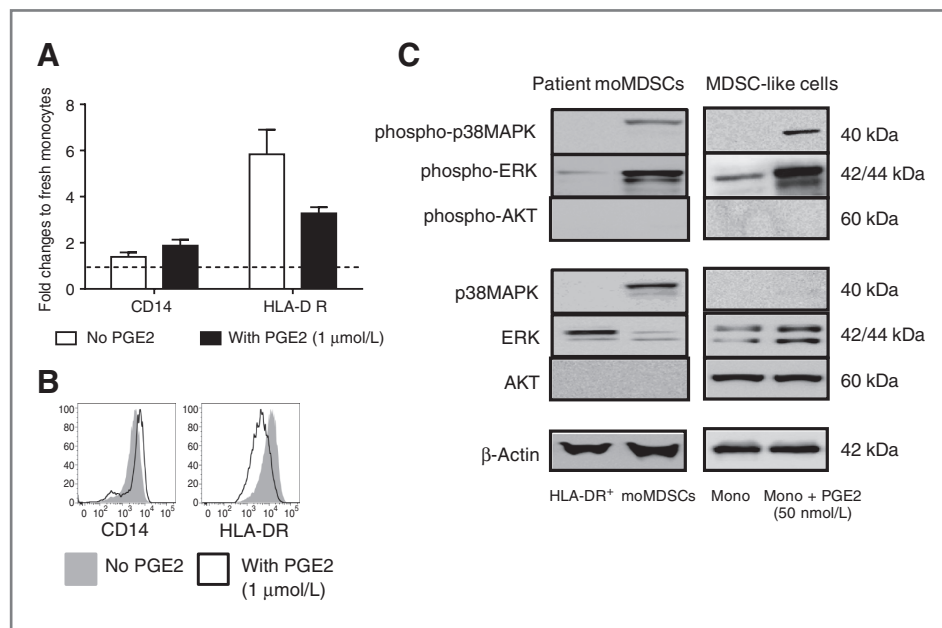


Figure 3. PGE2-treated monocytes resemble patient-derived moMDSCs. A, surface expression of CD14 and HLA-DR on monocytes cultured in the presence or absence of PGE2 (1 μmol/L, 48 hours). Data, mean ± SD from three individual experiments. B, representative histograms of CD14 and HLA-DR staining. C, representative micrograph of expression and phosphorylation of p38MAPK, ERK, and AKT in monocytes from healthy donors cultured in the presence or absence of PGE2 (50 nmol/L, 30 minutes) or freshly isolated patient-derived HLA-DR⁺ cells or moMDSCs (*n* = 3).

in vitro culture (Fig. 3C). Because of 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 cocultured with MDSC-like cells or control monocytes. Because PGE2 directly suppressed proliferation of IL2-activated NK cells in a dose-dependent manner (Supplementary Fig. S2B), a noninhibitory level of PGE2 (50 nmol/L) 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 (Fig. 4A and Supplementary Fig. S2C). Moreover, MDSC-like cells significantly inhibited the proliferation of NK cells ($P < 0.01$; Fig. 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, coculture with monocytes downregulated the expression of these molecules (Fig. 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 β significantly restored NK-cell-mediated cytotoxicity ($P < 0.01$) and proliferation ($P < 0.05$) to similar levels to that of NK cells cultured alone (Fig. 4D and E). Importantly, neutralization of TGF β or PGE2 had no direct impact on the cytotoxicity of NK cells (Supplementary Fig. 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 cocultures. Interrupting the activity of EP2 and EP4, but not of EP3, significantly blocked the suppressive capacity of the MDSC-like cells on NK cells ($P < 0.01$; Fig. 4D). In addition, significant recovery of NK-cell proliferative ability was observed by antagonizing the EP2 receptor ($P < 0.05$; Fig. 4E).

PGE2 enhances the production of TGF β by monocytes

Because TGF β plays a major role in suppression of NK-cell responses by MDSC-like cells and moMDSCs,

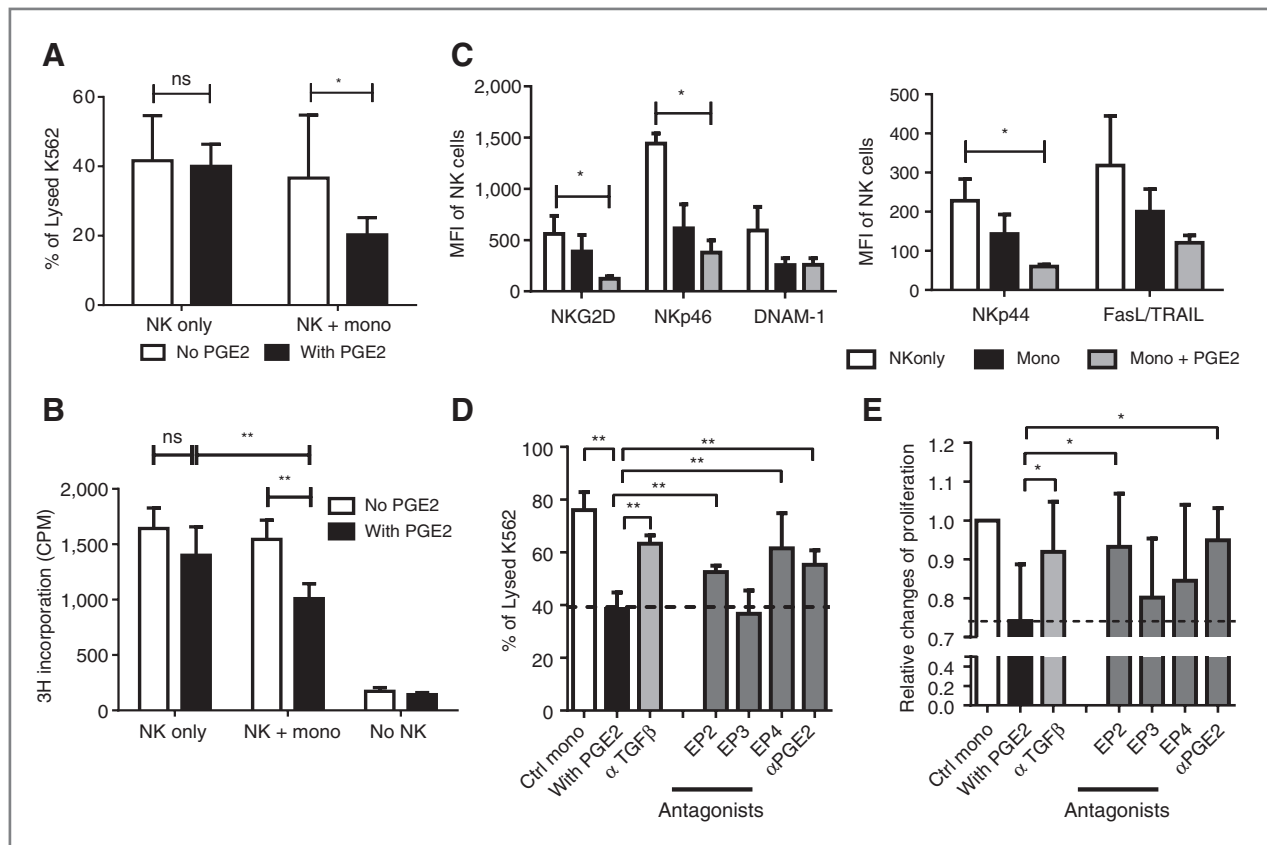


Figure 4. MDSC-like cells suppress NK cells through TGF β . NK cells isolated from healthy individuals were activated with IL2 (200 U/mL, 48 hours) alone or together with monocytes in the presence or absence of PGE2 (50 nmol/L) and their cytotoxicity against K562 ($n = 8$; A), proliferation ($n = 5$; B), and phenotype ($n = 4$; C) were analyzed. Furthermore, ability of IL2-activated NK cells to lyse K562 cells ($n = 6$; D) and proliferate, when cocultured with monocytes in the presence or absence of PGE2, neutralizing antibodies against TGF β , PGE2, or antagonists of EP2 (AH6809), EP3 (L798106), or EP4 (AH23848) (all at 20 μ mol/L, $n = 5$; E) were demonstrated. Data, mean \pm SD; Mann-Whitney nonparametric U test, *, $P < 0.05$; **, $P < 0.01$; n.s., not significant.

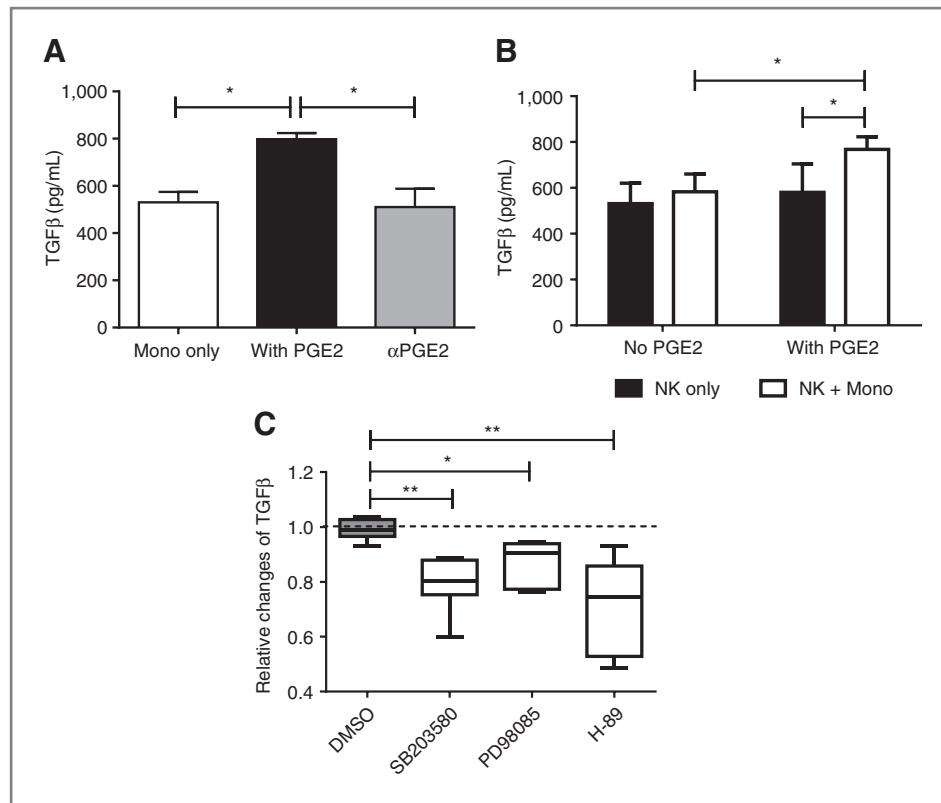


Figure 5. PGE2 enhances the production of TGF β on monocytes. A, monocytes isolated from healthy donors were cultured alone or in the presence of PGE2 (50 nmol/L, 48 hours) or together with a neutralizing antibody against PGE2 and analyzed for production of TGF β ($n = 3$). B, production of TGF β in cultures of NK cells alone or when cocultured with monocytes, in the presence or absence of PGE2 (50 nmol/L, $n = 4$). 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 μ mol/L) and analyzed for production of TGF β ($n = 5$) relative to nontreated moMDSCs. Data, mean \pm SD; Student t test; *, $P < 0.05$; **, $P < 0.01$.

we next evaluated if monocytes differ in their ability to produce TGF β in the presence or absence of PGE2. Compared with untreated monocytes, MDSC-like cells produced significantly higher levels of soluble TGF β ($P < 0.05$; Fig. 5A), which was abolished when cultured in the presence of neutralizing antibodies to PGE2 ($P < 0.05$; Fig. 5A). These observations were confirmed in the NK-MDSC-like cocultures, excluding the possibility of TGF β -driven auto-regulation by PGE2-treated NK cells (Fig. 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 the 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 (Fig. 5C), confirming the functional contribution of these pathways to MDSC-mediated suppression of NK cells in humans.

Overexpression 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 carcino-

ma cells were inoculated subcutaneously (Supplementary Fig. S3A).

Inoculation of control 4T1 cells resulted in a significant accumulation of CD11b⁺Gr1⁺ MDSCs ($P < 0.001$; Fig. 6A), but a decrease in the percentage of NK cells in the spleen of BALB/c mice ($P < 0.05$; Fig. 6B). Notably, significantly fewer CD11b⁺Gr1⁺ MDSCs ($P < 0.01$; Fig. 6A) and restored numbers of NK cells ($P < 0.01$; Fig. 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⁺, $P < 0.01$; Fig. 6C and Supplementary Fig. S3B) and lower percentages of CD3⁺ T cells ($P < 0.001$; Supplementary Fig. S3C) were observed in mice inoculated with control 4T1 tumor cells, these cell populations were not regulated by tumor-derived COX-2 production (Fig. 6C and Supplementary Fig. S3C). Moreover, the percentage of CD4⁺ T cells was comparable in all groups (Supplementary Fig. S3D).

To evaluate the cytolytic capacity of NK cells *in vivo*, naïve 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 ($P < 0.05$; Fig. 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 (Fig. 6E). No difference in the

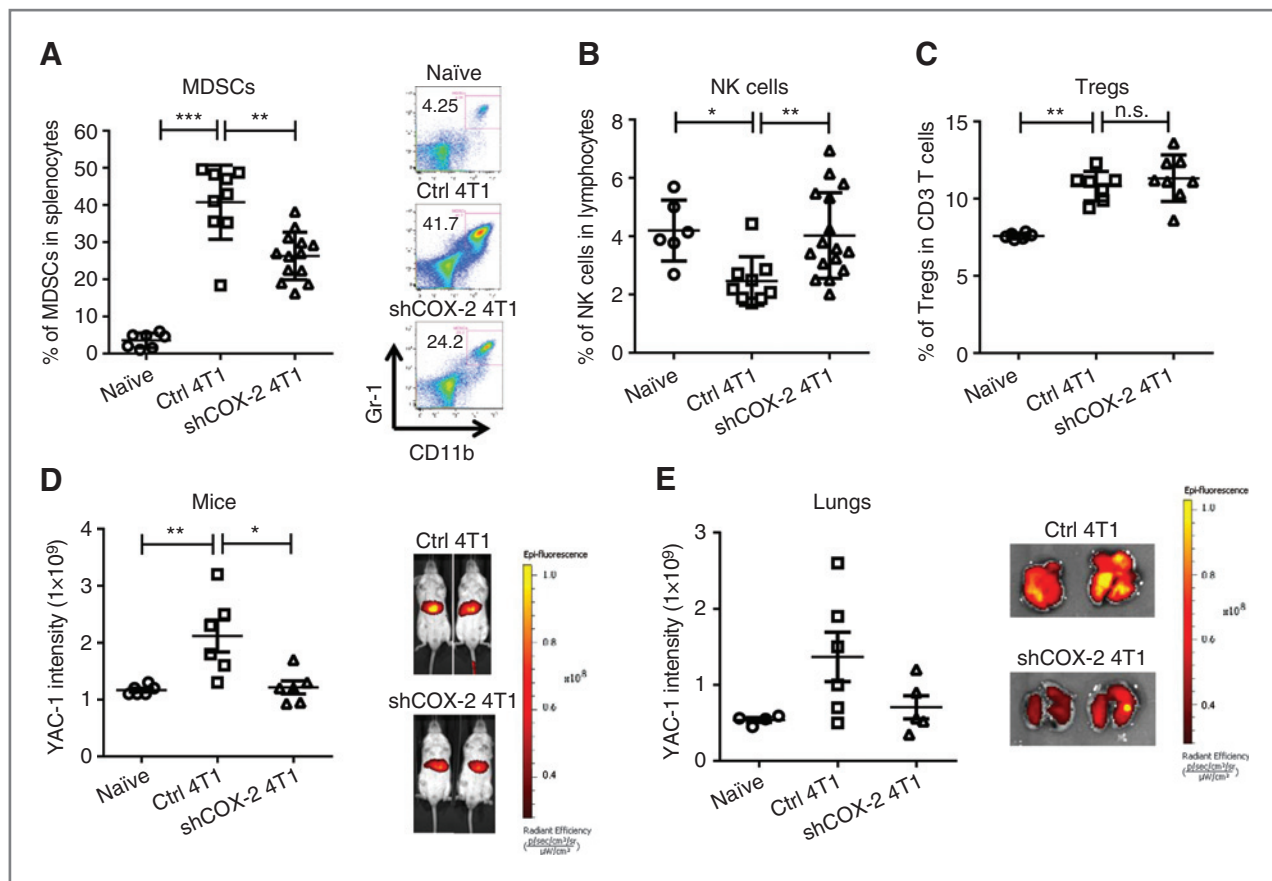


Figure 6. Overexpression of COX-2 in tumor cells regulates MDSC accumulation and NK-cell cytotoxicity *in vivo*. Splenocytes were isolated from naïve BALB/c or mice bearing similar tumor burden (control or COX-2 silenced 4T1 cells). Percentages of MDSCs (CD11b⁺Gr1⁺; A), NK cells (CD3⁺CD49b⁺; B), or regulatory T cells (CD3⁺CD4⁺CD25⁺FoxP3⁺; C) 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, mean \pm SD; Mann-Whitney nonparametric *U* test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

fluorescence intensity of inoculated YAC-1 cells was observed within 1 hour after inoculation, showing that the fluorescence labeling intensity was similar among the groups (Supplementary Fig. 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 used by tumor cells (32), regulatory T cells (33, 34), and MDSCs (23, 25). Although 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.

Coculture of moMDSCs isolated from patients with melanoma with NK cells resulted in reduced cytolytic function and IFN γ release of resting and IL2-activated NK cells.

Because 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 the presence of moMDSCs. In contrast, production of IFN γ from resting NK cells was not improved in the presence of catalase. This implies that although 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^{dim} NK cells are more susceptible to ROS-induced apoptosis, compared with cytokine-producing CD56^{bright} NK cells (35, 36). The distinction in sensitivity to ROS between the 2 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 2 well-documented mechanisms that MDSCs use 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, that is TGF β (5) or ROS (38). Thus, because of the multifaceted 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 and colleagues, we have previously shown that coculture 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 coculture. 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 the 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⁺ cells isolated from patients with advanced stage melanoma (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 patients with cancer (41). However, the functional implications and molecular basis of these pathways remain unknown. Here, we provide a novel perspective, where elevated PGE2 levels in patients with cancer 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⁺ cells. Thus, targeting similar pathways in MDSCs with BRAF or MEK1/2 inhibitors may contribute to the clinical responses in patients with cancer (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 accumu-

lation 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 tumors. Notably, COX-2 silencing did not 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 overexpression 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 the EP2/4 receptor and the p38MAPK/ERK pathway. Given the prevalence of COX-2 overexpression 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 patients with cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Mao, D. Sarhan, B. Seliger, R. Kiessling, A. Lundqvist

Development of methodology: Y. Mao, D. Sarhan

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Mao, D. Sarhan, A. Steven, R. Kiessling, A. Lundqvist

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Mao, D. Sarhan, A. Steven, B. Seliger,

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Writing, review, and/or revision of the manuscript: Y. Mao, D. Sarhan,

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Sarhan

Study supervision: Y. Mao, D. Sarhan, R. Kiessling, A. Lundqvist

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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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