Cancer Research

Melanoma-Educated CD14$^+$ Cells Acquire a Myeloid-Derived Suppressor Cell Phenotype through COX-2–Dependent Mechanisms

Yumeng Mao[1], Isabel Poschke[1,3], Erik Wennerberg[1], Yago Pico de Coaña[1], Suzanne Egyhazi Brage[1], Inkeri Schultz[2], Johan Hansson[1], Giuseppe Masucci[1], Andreas Lundqvist[1], and Rolf Kiessling[1]

Abstract

Tumors can suppress the host immune system by employing a variety of cellular immune modulators, such as regulatory T cells, tumor-associated macrophages, and myeloid-derived suppressor cells (MDSC). In the peripheral blood of patients with advanced stage melanoma, there is an accumulation of CD14$^+$HLA-DR$^{lo/-}$ MDSC that suppress autologous T cells *ex vivo* in a STAT-3–dependent manner. However, a precise mechanistic basis underlying this effect is unclear, particularly with regard to whether the MDSC induction mechanism relies on cell–cell contact of melanoma cells with CD14$^+$ cells. Here, we show that early-passage human melanoma cells induce phenotypic changes in CD14$^+$ monocytes, leading them to resemble MDSCs characterized in patients with advanced stage melanoma. These MDSC-like cells potently suppress autologous T-cell proliferation and IFN-γ production. Notably, induction of myeloid-suppressive functions requires contact or close proximity between monocytes and tumor cells. Further, this induction is largely dependent on production of cyclooxygenase-2 (COX-2) because its inhibition in these MDSC-like cells limits their ability to suppress T-cell function. We confirmed our findings with CD14$^+$ cells isolated from patients with advanced stage melanoma, which inhibited autologous T cells in a manner relying up prostaglandin E2 (PGE_2), STAT-3, and superoxide. Indeed, PGE_2 was sufficient to confer to monocytes the ability to suppress proliferation and IFN-γ production by autologous T cells *ex vivo*. In summary, our results reveal how immune suppression by MDSC can be initiated in the tumor microenvironment of human melanoma. *Cancer Res; 73(13); 3877–87. ©2013 AACR.*

Introduction

Myeloid-derived suppressor cells (MDSC) are present in a low frequency in healthy individuals, but are increased in sepsis and trauma to facilitate wound healing (1, 2). Recently, they have been recognized as cellular immune modulators that tumors employ to suppress both innate and adaptive immune responses in a variety of human solid and hematologic malignancies (1, 3, 4). MDSCs consist of immature myeloid cells and contain progenitors of granulocytes, dendritic cells, and macrophages. In mice, coexpression of CD11b and Gr1 was used to define MDSCs, which can be subdivided into CD11b$^+$ Ly6-G$^-$Ly6-C^{high} monocytic and CD11b$^+$Ly6-G$^+$Ly6-C^{lo} granulocytic MDSCs (5, 6). In humans, different MDSC phenotypes have been reported to be associated with different cancer types. In patients with melanoma, we and others have reported that CD14$^+$HLA-DR$^{lo/-}$ monocytic MDSCs accumulate in the peripheral blood (7, 8). MDSCs can directly suppress T cells via a variety of factors, including reactive oxygen species (ROS), arginase activity, TGFβ, and indoleamine 2,3-dioxygenase (IDO; refs. 5, 9, 10).

Tumor cells, together with tumor-associated fibroblast and other stromal cells, release a number of factors, such as TGF-β, interleukin (IL)-10, PGE_2, GM-CSF, VEGF, and IL-6, to facilitate immune escape and tumor progression (11, 12). Recent studies have shown that a combination of different cytokines, that is, GM-CSF and IL-6 (13, 14), or coculture with tumor cell lines (15, 16), can induce human MDSC-like cells *in vitro* from healthy monocytes or bone marrow aspirates to conduct potent immunosuppression.

Notably, Obermajer and colleagues have elegantly shown the importance of PGE_2 in the induction of MDSCs (17). This is in accordance with previous observations that COX-2 and its direct product PGE_2 are expressed at high levels in malignant melanomas and often correlate with poor prognosis (18, 19). Interruption of the COX-2/PGE_2 pathway has been shown to be effective in blocking MDSCs induced by a mixture of cytokines *in vitro* (17, 20). Similar effects were also observed in a mouse model (21).

Yet, the importance of these factors versus that of a direct interplay between tumor cells and MDSCs in the tumor

Authors' Affiliations: [1]Cancer Center Karolinska; [2]Section of Reconstructive Plastic Surgery, Institution of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden; and [3]German Cancer Research Center, DKFZ, Heidelberg, Germany

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Rolf Kiessling, Cancer Center Karolinska, Karolinska Institutet, Stockholm, Sweden. Phone: 468-517-76857; Fax: 468-30-9195; E-mail: rolf.kiessling@ki.se

doi: 10.1158/0008-5472.CAN-12-4115

microenvironment remains to be elucidated. MDSCs in multiple myeloma microenvironment potently promote tumor growth and suppress T-cell functions (22). In contrast, Gros and colleagues reported the absence of T-cell suppressing capacity of those MDSCs that are retrieved from melanoma tumor tissues (23). In this study, we show that human melanoma cells can induce $CD14^+$ monocytes from healthy individuals to acquire an MDSC-like phenotype and suppressive properties mediated by COX-2/PGE_2 and STAT-3 signaling. We also present data supporting PGE_2 as a T-cell–suppressive mediator employed by monocytes from patients with advanced stage melanoma.

Materials and Methods

Cell isolation and patient materials

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donor buffy coats (Karolinska University Hospital, ethical permit: #20010305,01-50) by gradient centrifugation (Ficoll-Paque Plus, GE Healthcare), followed by monocyte isolation using a $CD14^+$ monocyte isolation kit and MACS LS column (Miltenyi Biotech). Autologous T cells were purified from the negative fraction of the monocyte isolation by a Pan T-cell Isolation Kit (Miltenyi Biotech), according to the manufacturer's instructions.

Five patients with stage IV melanoma were accrued with informed donor consent according to the Declaration of Helsinki and approved by the Karolinska Institutet review board (ethical permit: #2011/143-32/1). PBMCs were isolated from patient peripheral blood and $CD14^+$ cells were purified as stated above. Monocyte-depleted autologous lymphocytes were collected for functional studies.

Tumor coculture and MDSC isolation

The EST human melanoma cell lines were from the European Searchable Tumor Line Database (ESTDAB, http://www.ebi.ac.uk/ipd/estdab) and maintained as described (24). Cell line authentication was carried out by an STR identifier kit (Applied Biosystems) and both cell lines used in the study showed at least a 90% match to the STR profiles of ESTDAB (Supplementary Table S1). The early-passage GEWA and KADA melanoma cell lines were established from patients at the oncology clinic at Karolinska University Hospital (ethical permit: #2011/143-32/1) based on the published protocol (25). Mycoplasma-free (MycoAlert kit) GEWA and KADA cell lines were in their 7^{th} and 6^{th} passage when used for tumor–monocyte coculture, respectively.

One million isolated $CD14^+$ cells were cultured in 3.5 mL IMDM medium supplemented with 10% human AB serum (Karolinska University Hospital) per well in a 6-well plate. For the tumor–monocyte cocultures, 400,000 tumor cells were added to the monocytes. In the transwell experiments, $CD14^+$ cells were separated from the melanoma cells by 0.4 μm inserts (Corning) during coculture. Tumor cocultured and control monocytes cultured without tumor cells were harvested by gently scraping the bottom of the wells after 64 hours of coculture and $CD11b^+$ cells were purified by anti-$CD11b^+$ microbeads and MS columns (Miltenyi Biotec). To investigate the induction of suppressive MDSC-like cells, tumor–monocyte cocultures were treated with nontoxic levels of 20 μmol/L AG490 (STAT-3 inhibitor, Sigma-Aldrich) and 40 μmol/L celecoxib (COX-2 inhibitor, Biovision). PGE_2 concentrations in the melanoma–monocyte coculture were measured by a PGE_2 ELISA kit according to the manufacturer's instructions (Enzo Life Sciences). To study which PGE2 receptor is responsible for the phosphorylation of STAT-3 protein on the tumor-educated monocytes, EP receptor-selective antagonists AH6809 (EP1/EP2), L798106 (EP3) or AH23848 (EP4; all purchased from Sigma-Aldrich) were added at 20 μmol/L during the tumor–monocyte coculture and the phosphorylation of STAT-3 protein was evaluated by flow cytometry.

Monoclonal antibodies and flow cytometry

Information on the antibodies used in this study is provided in Supplementary Table S2. A panel of myeloid cell surface markers was measured on monocytes when they were freshly isolated (0 hours), and/or after 14, 40, and 64 hours of coculture with EST cell lines. For the monocytes cocultured with early-passage melanoma cell lines, the myeloid markers were evaluated at 0 hours and 64 hours. In brief, cells were harvested by gentle scraping and stained in PBS supplemented with 1% human serum albumin (HSA) at 4°C for 30 minutes. For the pSTAT-3 staining, cells were fixed with BD Phosflow Lyse/Fix Buffer (BD Biosciences), followed by permeabilization with BD Phosflow Perm Wash III (BD Biosciences). Cells were stained with antibodies for detecting phosphorylation of Serine 727 (Ser727) or Tyrosine 705 (Tyr705) on STAT-3 protein as mentioned above. All data were acquired on an LSRII flow cytometer (BD Biosciences) using FACS Diva software and were analyzed using FlowJo software (Treestar).

T-cell proliferation and cytokine production assays

To evaluate the suppressive functions of melanoma-educated monocytes, 5 million T cells were labeled in 1 mL PBS containing 1.4 μmol/L Carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) for 5 minutes at room temperature and seeded at 100,000 cells per well in a 96-well U bottom plate in 100 μL IMDM medium containing 10% human AB serum. $CD11b^+$ monocytes were purified from cocultures with or without tumor cells or from transwells at 64 hours and added to T cells at ratios of 1:2 (50,000 cells) or 1:4 (25,000 cells). Next, T cells were activated by addition of 1.5 μL anti-CD3/CD28 mAb-coated beads per well for 4 days. Subsequently, T-cell proliferation and CD62L expression were measured by flow cytometry and IFNγ concentrations secreted to the supernatants were determined by ELISA (MabTech). Nonactivated T cells or activated T cells without monocyte coculture were used as controls. For the patient samples, monocyte-depleted lymphocytes were labeled with CFSE and seeded at 100,000 cells per well. Purified $CD14^+$ monocytes were added at ratios of 2:1, 1:1, and 1:2 to each well and the parameters described above were measured.

Blocking assays for monocyte-suppressive functions were conducted at 1:2 monocyte:T-cell ratio for melanoma-educated monocytes and 1:1 ratio for *ex vivo* patient-derived monocytes. Briefly, monocytes were treated for 2 hours with 20 μmol/L AG490 (Sigma-Aldrich), 40 μmol/L celecoxib

(Biovision), 400 IU/mL superoxide dismutase (Sigma-Aldrich), 200 IU/mL catalase (Sigma-Aldrich), 1 mmol/L *N*(ω)-hydroxy-nor-L-arginine (nor-NOHA, Calbiochem), 20 μg/mL anti-TGFβ mAb (R&D Systems), and 20 μg/mL anti-CD80 mAb (Biolegend) in 100 μL medium. To minimize the effects on T cells, AG490 and celecoxib were removed from the wells by replacing with fresh medium, followed by adding T cells in 100 μL medium to all wells.

In addition, 50 μmol/L α,β-methylene ADP (Sigma-Aldrich), 20 nmol/L SCH 58261 (Sigma-Aldrich), 20 μg/mL anti-PGE_2 mAb (Cayman Chemical), or 10 μmol/L PGE_2 (Sigma-Aldrich) were added to the *ex vivo* isolated patient monocytes in the T-cell proliferation assay.

Statistical analysis

All data were summarized by Prism Version 6 software (GraphPad) and differences were analyzed by nonparametric, Mann–Whitney *U* test. Unless otherwise stated, the data from multiple experimental and control groups are presented as means and SD from the number of donors that are indicated in the figure legends. All results were confirmed in at least 3 individual experiments and the representative figures were chosen according to the average values.

Results

Melanoma-educated monocytes phenotypically resemble myeloid-derived suppressor cells

We previously described that $CD14^{+}HLA\text{-}DR^{lo/-}$ MDSCs overexpress CD80, CD83, and DC-Sign in patients with advanced stage melanoma (7). To study if a similar MDSC population could be induced *in vitro*, we established an MDSC induction model by coculturing monocytes enriched from healthy individuals with melanoma cell lines.

When cocultured with established melanoma cell lines (EST025 and EST094), the upregulation of HLA-DR was markedly blocked compared with control monocytes

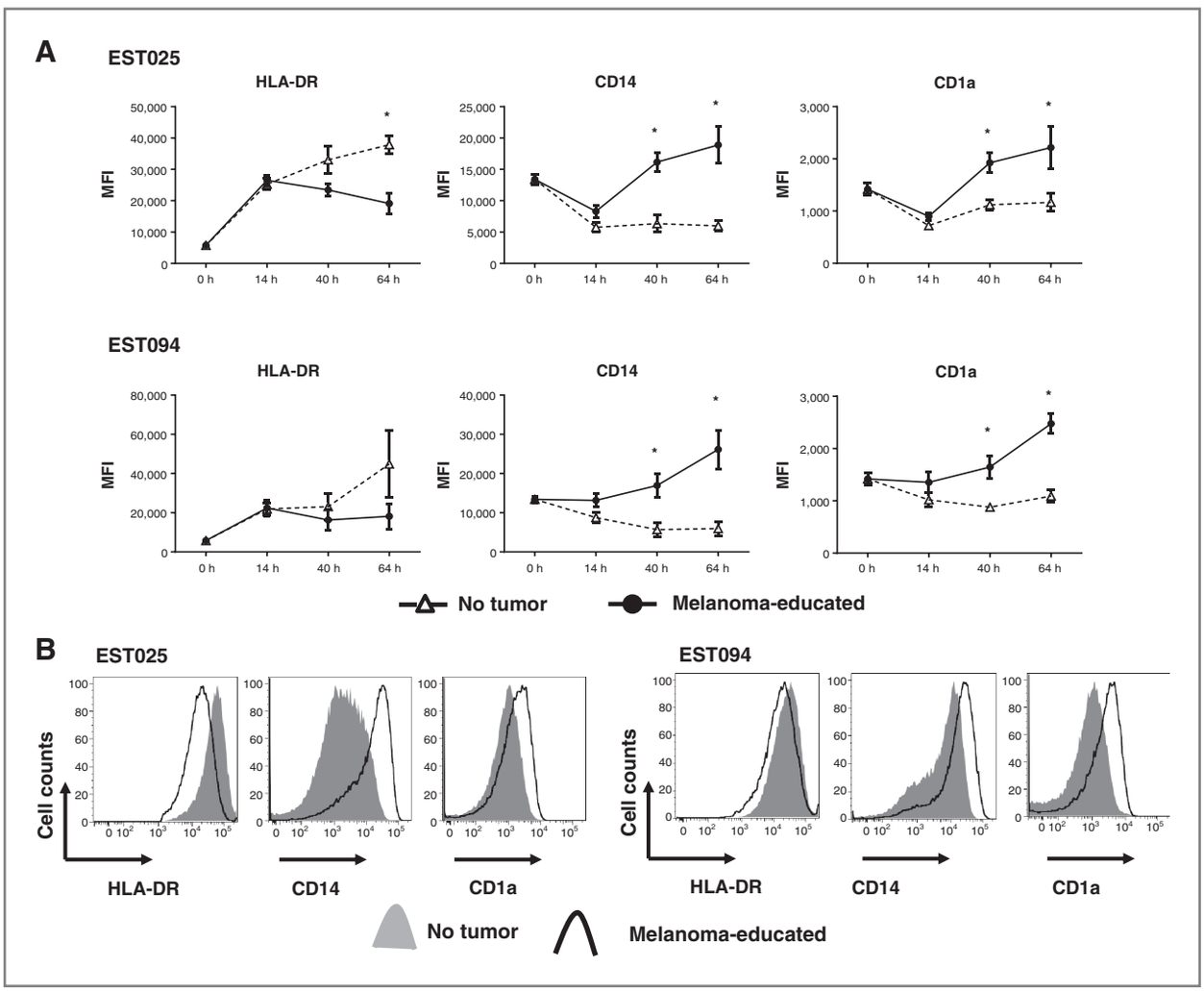

Figure 1. Melanoma-educated monocytes phenotypically resemble MDSCs. Mean fluorescence intensity (MFI; A) and representative histograms (B) depicting HLA-DR, CD14, and CD1a expression kinetics at 0, 14, 40, and 64 hours on healthy donor monocytes ($n = 4$) cultured with or without EST025 (top) or EST094 (bottom) melanoma cell lines. Data are presented as means ± SD. *, $P < 0.05$.

(Fig. 1A and B). Conversely, expression of CD14 and CD1a significantly increased over time on tumor cocultured monocytes compared with control monocytes ($P < 0.05$, Fig. 1A and B). Although not significant, expression of CD80 and DC-Sign were upregulated in monocyte–tumor cell cocultures compared with controls (Supplementary Fig. S1). Neither of the tumor cell lines induced significant changes of other myeloid and/or immunosuppressive surface molecules, such as, CD33, CD11b, CD11c, CD83, CD137, CD137L, PD-L1, and CD86 (data not shown).

Early-passage melanoma cells can induce MDSC-like cells in a proximity-dependent fashion

To exclude that alterations induced by long-term *in vitro* culture of the established melanoma lines could account for the observed effects, short-term ($\leq$7 passages) cultured melanoma cells were cocultured with freshly isolated healthy donor monocytes.

In line with our observations from long-term established melanoma cell lines, the short-term cultured cell line also impaired the expression of HLA-DR and CD86 on monocytes, and induced significant upregulation of CD14 and CD1a (Fig. 2A).

Because separation of melanoma cells and monocytes in a transwell system did not result in the upregulated expression of CD14 and CD1a, as compared with levels observed on control cultured monocytes (Fig. 2A), we conclude that the effect was dependent on cell–cell contact or mechanisms that require close proximity to tumor cells. This argued against the possibility that the observed phenotypic changes were induced by nonspecific factors, that is, nutrient deprivation or pH value fluctuation due to the fast-growing tumor cells in the coculture.

In contrast, expression levels of HLA-DR and CD86 decreased even when cultured in the transwell system, suggesting that contact/proximity independent mechanisms may

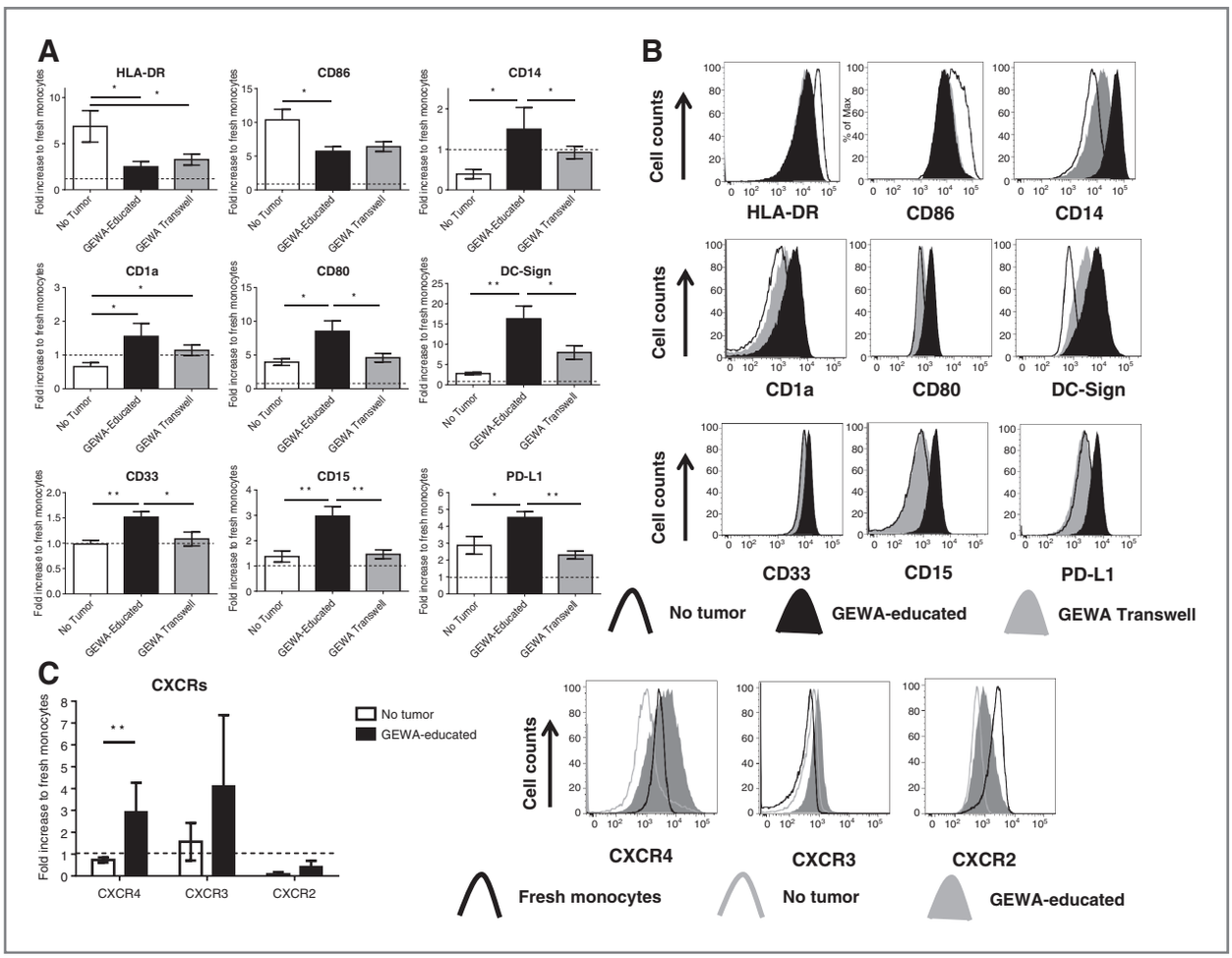

Figure 2. Early-passage melanoma cells can induce MDSC-like cells in a proximity-dependent fashion. Expression levels (A) and representative histograms (B) of HLA-DR, CD86, CD14, CD1a, CD80, DC-Sign, CD33, CD15, and PD-L1 on healthy donor monocytes ($n = 6$) cultured for 64 hours without tumor cells (empty columns), directly cocultured (black columns) with, or separated by a transwell setup (gray columns) from the early-passage GEWA melanoma tumor cell line. C, expression levels of CXCR2, 3, and 4, as well as the representative histograms, of healthy monocytes ($n = 5$) cultured for 64 hours without (empty columns) or cocultured with (black columns) early-passage GEWA tumor cell line. Data are presented as relative fold changes in MFI to freshly isolated $CD14^+$ cells (dashed horizontal line), means $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$.

be involved in regulating tumor-induced changes for these markers (Fig. 2A and B).

Notably, the early-passage cell line was capable of inducing phenotypic changes that we did not observe by using long-term cultured melanoma cell lines but that were similar to those observed in patient-isolated MDSCs (7). Expression of CD80 ($P < 0.05$) and DC-Sign ($P < 0.01$) were significantly increased in melanoma-educated monocytes, but normalized by using transwells (Fig. 2A and B). Melanoma coculture also increased the expression of the myeloid/granulocytic cell markers CD33 and CD15 ($P < 0.01$), as well as PD-L1 ($P < 0.05$), all of which were normalized by using the transwell system. Moreover, expression of chemokine receptor CXCR4 on these monocytes were significantly increased when cocultured with tumor cells ($P < 0.01$). Due to the phenotypic similarities of these melanoma-educated monocytes to the MDSCs in patients with melanoma, we hereafter refer them as "MDSC-like" cells.

Tumor-educated MDSC-like cells are potent suppressors of autologous T cells

Since CD11b expression was unaffected by the presence of tumor cells (data not shown), this marker was used to purify $CD14^+$ monocytes after 64 hours of coculture with the early-passage melanoma cell line. $CD11b^+$ cells were then mixed with CFSE-labeled autologous T cells at monocyte:T-cell ratios of 1:2 and 1:4, followed by T-cell activation using anti-CD3/CD28 mAb-coated beads. T-cell proliferation and IFNγ production were evaluated after 4 days.

Of note, tumor education not only induced MDSC-like phenotype, but also resulted in potent suppressive activity of these healthy donor-derived monocytes. At a 1:2 MDSC-like cell:T-cell ratio, proliferation of T cells was significantly inhibited (73.6 ± 13.4% with MDSC-like cells versus 20.5 ± 26.1% with controls, $P < 0.05$) and the IFNγ production (2351.8 ± 934.1 pg/mL with MDSC-like cells versus 139.1 ± 136.8 pg/mL with controls, $P < 0.05$) was completely abrogated. T-cell suppression mediated by these cells was highly dose dependent and clearly alleviated by reducing the number of MDSC-like cells (Fig. 3A). In accordance with the functional assay, CD62L, a surface molecule that is downregulated upon T-cell activation, was maintained on the surface at higher levels if T cells were stimulated in presence of MDSC-like cells (Supplementary Fig. S2). Remarkably, monocytes cultured in transwells during the tumor education phase failed to suppress T-cell proliferation and IFNγ production (Fig. 3A) and did not prevent CD62L downregulation (Supplementary Fig. S2).

To ensure that these results were not dependent on the allogenicity between healthy donor monocytes and patient tumor cells, $CD14^+$ cells were isolated from the patient, from whom the melanoma cell line was established. Identical experiments were conducted in this completely HLA-matched setting. Indeed, melanoma cells induced autologous $CD14^+$ cells to potently suppress both autologous T-cell proliferation and IFNγ production, whereas transwell separation abolished the MDSC induction (Fig. 3B).

MDSC-like cells suppress T-cell function through COX-2 production

To explore the mechanisms of T-cell suppression, MDSC-like cells induced by the early-passage human melanoma cell line were used in the T-cell suppression assays. Cells were treated with a panel of inhibitors, including the ROS-inactivating enzymes superoxide dismutase and catalase, iNOS and arginase inhibitors NG-monomethyl-L- arginine-acetate (L-NMMA), and *N*(ω)-hydroxy-nor-L-arginine (nor-NOHA), as well as a TGFβ neutralizing antibody. These reagents only showed inconsistent or marginal improvements of T-cell proliferation (data not shown).

Obermajer and colleagues have recently shown that a PGE_2-COX-2–mediated positive feedback loop is essential for the induction of suppressive factors by human MDSCs (17). Thus, we pretreated the MDSC-like cells with the COX-2 inhibitor celecoxib. Strikingly, COX-2 blocking significantly reduced their ability to inhibit T-cell proliferation ($P < 0.05$, Fig. 4A and C), indicating the importance of COX-2 enzymatic activity in MDSC-mediated T-cell suppression. PGE_2, a direct product of the COX-2 enzyme, has been reported to contribute to the activation of STAT-3 signaling (26). Therefore, MDSC-like cells were pretreated with the STAT-3 signaling inhibitor AG490. This resulted in reduced ability of these cells to inhibit T-cell proliferation and IFNγ production, although it did not reach statistical significance (Fig. 4).

PGE_2 production and STAT-3 signaling are involved in the T-cell suppression mediated by $CD14^+$ monocytes from patients with advanced stage melanoma

We next considered it of importance to confirm these observations in MDSCs derived from patients with melanoma. Similar to the *in vitro*-induced MDSC-like cells, $CD14^+$ monocytes isolated from patients with melanoma were found to inhibit autologous T-cell proliferation and IFNγ production in a dose-dependent fashion (Fig. 5A). A similar trend was also observed for the induction of a decreased CD62L expression on $CD3^+$ T cells (Fig. 5B). In accordance with previously published data (7), inhibition of STAT-3 signaling in patient-derived $CD14^+$ cells significantly decreased their T-cell inhibitory functions ($P < 0.05$), resulting in improvements of T-cell proliferation (Fig. 5C). Blockade of PGE_2 and superoxide production resulted in consistent improvements of T-cell proliferation in the cocultures with the $CD14^+$ patient-derived monocytes ($P < 0.05$, Fig. 5C), confirming the important role of PGE_2 and ROS in mediating T-cell suppression. Blocking COX-2, CD80, neutralizing TGFβ and arginase production, or interrupting adenosine-related pathways showed a nonsignificant trend toward alleviating T-cell suppression (Fig. 5C).

To further investigate its T-cell–suppressive effect, synthetic human PGE_2 was added to the activated lymphocytes, with or without patient-derived monocytes. Strikingly, in the presence of PGE_2, $CD14^+$ monocytes almost completely abolished T-cell proliferation and IFNγ production (Fig. 5D). This suppression was predominantly mediated by the presence of $CD14^+$ cells, because adding PGE_2 without monocytes did not affect T-cell proliferation. However, a direct suppression of T-cell IFNγ production by PGE_2 was observed (Fig. 5D).

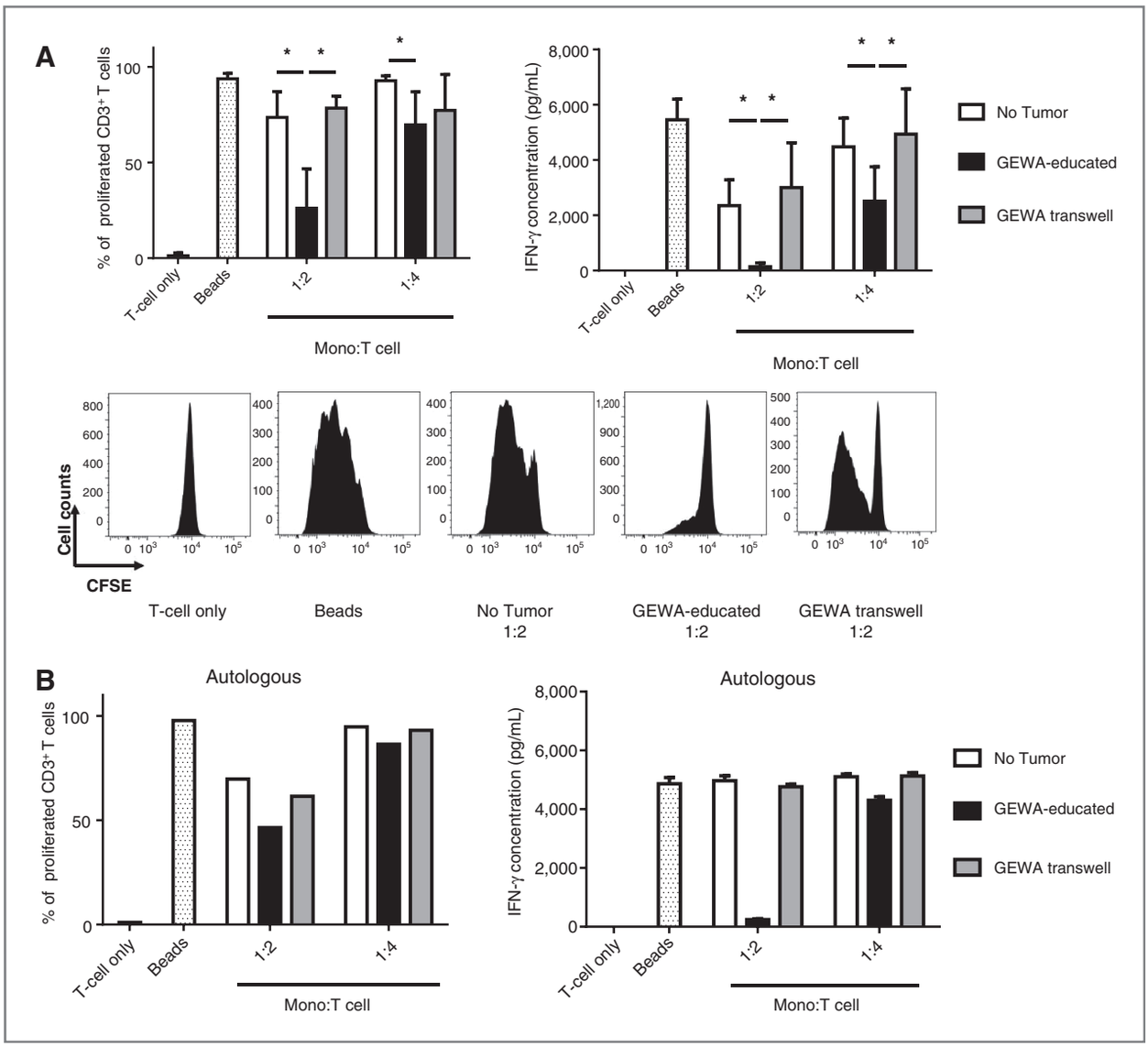

Figure 3. Tumor-educated MDSC-like cells are potent suppressors of autologous T cells. A, percentage of proliferated $CD3^+$ T cells (top left), IFNγ production (top right), and representative histograms of T-cell proliferation (bottom) with the presence of control (empty columns), GEWA-educated (black columns), and GEWA transwell cocultured (gray columns) healthy donor monocytes ($n = 4$) after 4 days. Data are presented as means ± SD. *, $P < 0.05$. B, T-cell proliferation (top left) and IFNγ production (triplicates, top right) were investigated in setup as described in A, but with the autologous $CD14^+$ monocytes from the GEWA melanoma patient.

Melanoma cells induce MDSC-like cells through COX-2/PGE_2 production

Given that COX-2/PGE_2 and STAT-3 were essential for mediating T-cell suppression by MDSCs, we investigated whether these 2 mechanisms also contributed to the induction of MDSC-like cells by melanoma cells.

Both celecoxib and AG490 markedly abolished the induction of MDSC-like cells from monocytes by early-passage melanoma tumor cells. Thus, T-cell proliferation and IFNγ production in cocultures of MDSC-like cells pre-treated with these reagents were comparable with the levels observed in control monocytes cultured without tumor cells (Fig. 6A–C). A similar trend was observed when measuring their ability to decrease CD62L expression on activated $CD3^+$ T cells, although not as pronounced as in the functional assay (Supplementary Fig. S3).

Next, we tested the PGE_2 concentrations in the culture medium collected from the *in vitro* MDSC induction model by ELISA. As shown in Fig. 6D, PGE_2 was produced only at low levels by control monocytes. In contrast, high amount of PGE_2 was secreted by early-passage melanoma tumor cells, similar to the levels observed in the tumor–monocyte coculture. Celecoxib, but not AG490, was found to efficiently reduce the PGE_2 production to a nondetectable level in the tumor–monocyte coculture. We also observed that phosphorylation of STAT-3 protein was markedly increased on monocytes after being cocultured with tumor cells. Interrupting binding

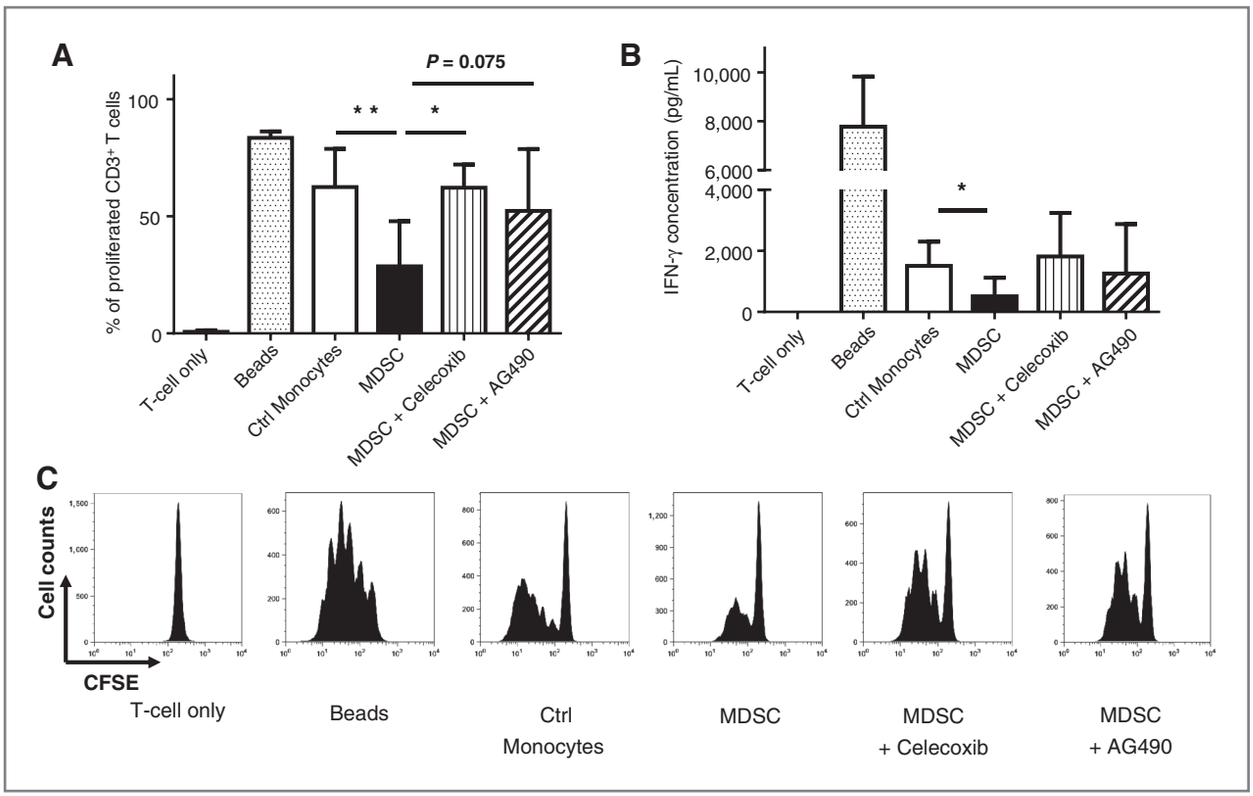

Figure 4. MDSC-like cells suppress T-cell function through COX-2 production. A, percentage of proliferated $CD3^+$ T cells ($n = 4$) in the presence of control monocytes, MDSC-like cells, MDSC-like cells pretreated by COX-2 inhibitor (celecoxib) or STAT-3 inhibitor (AG490) at a ratio of 1:2 (monocyte:T cells). Data are presented as means ± SD. *, $P < 0.05$; **, $P < 0.01$. B, IFNγ produced by the T cells from the same experiments. C, representative histograms of T-cell proliferation after 4 days.

between PGE_2 to its EP3 receptor resulted in a marginal inhibition of this phosphorylation (Supplementary Fig. S4).

Collectively, our data provide strong evidence that COX-2/PGE_2 production and STAT-3 activation in melanoma tumor microenvironment play an essential role in converting normal $CD14^+$ cells to display MDSC-like properties.

Discussion

We have developed an *in vitro* model to study how melanoma cells can induce monocytic MDSCs by coculturing monocytes from healthy donors with long- or short-term established melanoma tumor cell lines. The resulting MDSCs bear a striking resemblance to those isolated from patients with advanced stage melanoma, with significant upregulation of CD14 and downregulation of HLA-DR and CD86. Our data suggest that melanoma tumor cells can arrest monocytes from healthy individuals in an immature stage associated with a capacity to impair T-cell functions through novel mechanisms involving COX-2/PGE_2 production and STAT-3 signaling.

A variety of dendritic cell and/or macrophage markers, such as CD80, DC-Sign, PD-L1, and CD1a, were significantly upregulated on monocytes after being cocultured with early-passage melanoma tumor cells. This phenotype is similar to the overexpression of CD80 and DC-Sign that we described in MDSCs isolated from patients with advanced stage melanoma (7), even though the expression of PD-L1 was not evaluated in that study. The presence of these molecules may have functional implications for MDSCs via contact-dependent mechanisms, since CD80 has been proposed to play an immune regulatory role (27–29) and PD-L1 (B7-H1) is a B7 family member that transmits a negative signal to limit T-cell functions via PD1 (30, 31). This raises the possibility that the clinical effect of interfering with the PD-L1–PD1 interaction noted in a number of studies (32, 33) may involve blocking the negative influence of MDSCs on T cells.

Similar to what has been reported in patients with ovarian cancer (34), we observed that the presence of melanoma tumor cells is able to induce the upregulation of CXCR4 on monocytes, which may result in the modulation of homing capacity of these MDSC-like cells.

The induction of MDSCs by early-passage melanoma tumor cells was found to be dependent on COX-2 production and STAT-3 signaling, although inhibition of the latter did not reach statistical significance. This has also been confirmed by using tumor cells directly expanded from melanoma lymph node metastasis (data not shown). As expected (35), the early-passage melanoma lines utilized in this study produce significant amounts of PGE_2, which induced increased

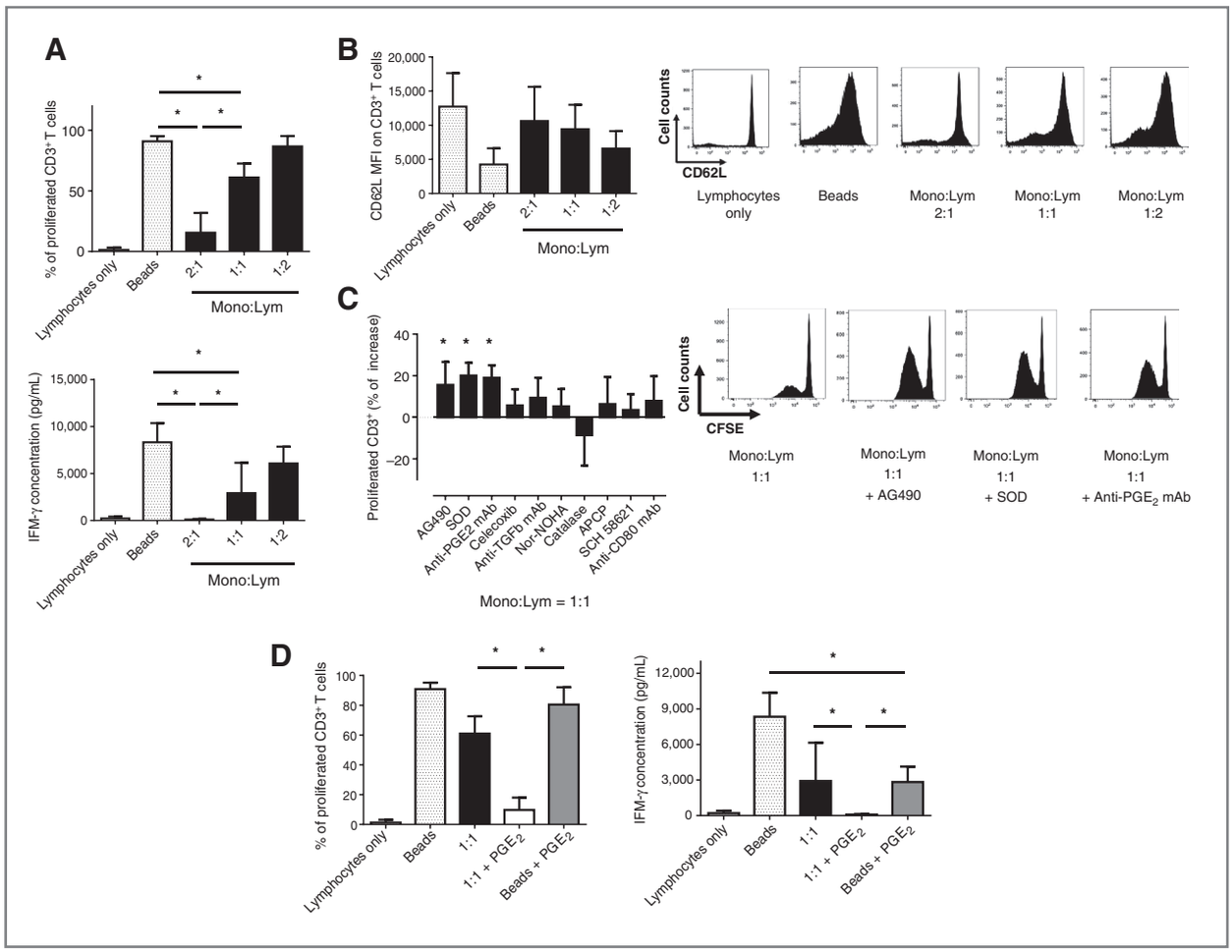

Figure 5. PGE_2 production and STAT-3 signaling are involved in the T-cell suppression mediated by $CD14^+$ monocytes from patients with advanced stage melanoma. A, percentage of proliferated $CD3^+$ T cells (top left) and IFNγ production (top right) when cocultured with $CD14^+$ monocytes isolated from patients with advanced stage melanoma at monocyte:lymphocyte ratios of 2:1 ($n = 3$), 1:1 ($n = 5$), and 1:2 ($n = 5$). Data are presented as means ± SD. *, $P < 0.05$. B, MFI (top) and representative histograms (bottom) of CD62L on $CD3^+$ T cells from the same T-cell proliferation assay. C, increased percentage of proliferated $CD3^+$ T cells ($n = 4$) in the presence of patient-derived monocytes (monocyte:T cells = 1:1) with a panel of inhibitors, including AG490 (STAT-3), SOD (superoxide), anti-PGE_2 mAb, celecoxib (COX-2), anti-TGFβ mAb, nor-NOHA (arginase), catalase (hydrogen peroxide), APCP (CD73), SCH 58621 (adenosine receptor), and anti-CD80 mAb (*top*). Representative histograms with AG490, SOD, and anti-PGE_2 mAb are shown in the right. D, percentage of proliferated $CD3^+$ T cells (top left) and IFNγ production (top right) when cocultured at a ratio of 1:1 with $CD14^+$ monocytes isolated from patients with melanoma. Human synthetic PGE_2 was added to monocyte-activated T-cell coculture (empty column) or activated T cells without monocytes (gray column), $n = 4$. Data are presented as means ± SD. *, $P < 0.05$.

phosphorylation of STAT-3 on monocytes primarily via binding to the EP3 receptor. Although MDSC induction was completely abolished by the COX-2 inhibitor celecoxib, a direct involvement of tumor cell-derived COX-2 in the MDSC induction remains to be established.

In addition, we find that COX-2 production and STAT-3 signaling are also involved in T-cell suppression mediated by tumor-educated, MDSC-like cells. By blocking either COX-2 or STAT-3 activity, their T-cell–suppressive function was impaired. This is in accordance with the observation that activation of STAT-3 signaling was shown to be related to MDSC infiltration (36, 37), possibly in response to tumor-derived inflammatory factors, such as PGE_2, GM-CSF, and IL6 (26, 38, 39), all of which have been implicated as MDSCs inducers (13, 17, 40–42).

We have recently shown that MDSCs can induce a dose-dependent decrease in the maturation, ability to take up antigen, migrate, and induce T-cell IFNγ production of DC (43). Changes in DC characteristics were most notable when "pathologic" frequencies of more than 50% $CD14^+HLA\text{-}DR^{lo/-}$ cells were present in the starting culture. In light of the findings reported here and by Obermajer and colleagues (17), it is likely that this negative effect on DC maturation by MDSCs may be mediated via a COX-2/PGE_2–dependent effect, where the presence of PGE_2 at early stages of DC development suppresses the differentiation of human monocytes into functional DCs.

Our group has previously proposed STAT-3 signaling as a novel T-cell–suppressive mechanism employed by human MDSCs in patients with melanoma (7). By using $CD14^+$ monocytes isolated from patients with advanced stage melanoma,

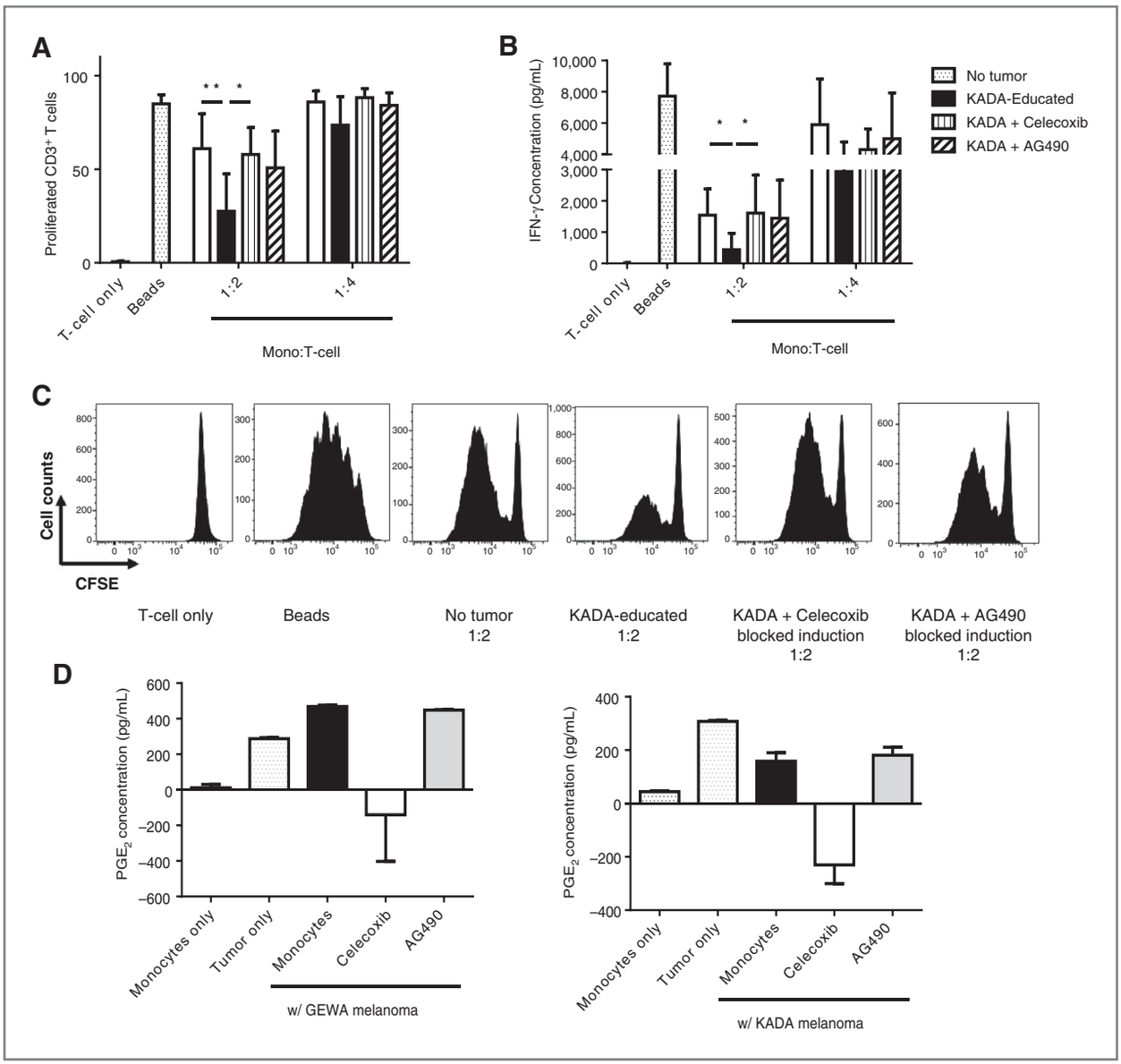

Figure 6. Melanoma cells induce MDSC-like cells through COX-2/PGE_2 production. A, percentage of proliferated $CD3^+$ T cells ($n = 5$) when control monocytes, MDSC-like cells, monocytes educated by COX-2 or STAT-3 blocked KADA melanoma were added at monocyte–T-cell ratios of 1:2 and 1:4. Data are presented as means ± SD. *, $P < 0.05$; **, $P < 0.01$. B, IFNγ produced by T cells from the same experiments. C, representative histograms from the T-cell proliferation assay at monocyte–T-cell ratio of 1:2. D, PGE_2 produced by control monocytes, melanoma tumor cells alone, melanoma–monocyte coculture, or coculture in the presence of COX-2 or STAT-3 inhibitor was measured in triplicate by ELISA. Data are presented as means ± SD.

we here confirm that MDSCs mediate T-cell suppression in a STAT-3–dependent fashion *ex vivo*. We also identified a novel mechanism showing that neutralization of PGE_2 secreted from these monocytes resulted in consistent and significant improvement of T-cell proliferation. Moreover, we confirmed the colocalization of $COX\text{-}2^+$ tumor cells and $COX\text{-}2^+$ infiltrating $CD14^+$ monocytes by evaluating the immunohistochemistry (IHC) staining on paraffin-embedded tumor tissues from patients with stage III melanoma (data not shown). However, blockade of COX-2 activity in the patient-derived monocytes did not yield consistent T-cell rescue, which suggests that PGE_2 from patient MDSCs are synthesized via an alternative pathway, that is, COX-1 activity (44).

Strikingly, we found that treatment of melanoma patient-derived monocytes with PGE_2 provided them with capacity to induce complete abrogation of T-cell proliferation and IFNγ production. This effect of PGE_2-treated monocytes was significantly more pronounced than the suppressive effects exerted by PGE_2 alone on the activated T cells. Similar results were also observed by treating monocytes isolated from healthy donors (data not shown). In contrast to other MDSC induction models, in which healthy donor monocytes are treated by a

combination of cytokines for as long as 7 days (13, 17), we here show PGE_2 as a single reagent to induce potent immunosuppressive MDSCs within a short period of time. This model may offer a new perspective of the biologic characteristics of MDSC induction, which could facilitate the usage of MDSCs as a therapeutic approach in autoimmune diseases or transplantation (45).

Collectively, our findings show that once monocytes are exposed to the tumor microenvironment containing high concentration of cytokines and other inflammatory mediators, COX-2 production and STAT-3 signaling will be elevated and immune-suppressive MDSCs will be induced. During tumor progression, MDSCs may also migrate from the tumor microenvironment, explaining the enhanced levels of $CD14^+HLA\text{-}DR^{lo/-}$ cells in the blood of patients with melanoma. Alternatively, MDSCs could be induced in the periphery by systemic release of inflammatory mediators, although our finding that direct tumor cell contact or a close proximity to the tumor is required for MDSCs is less compatible with this view.

A better understanding of how the interaction between tumor cells and the immune system can lead to immune suppression seems crucial for the development of strategies aimed at breaking tumor-induced immune tolerance. Our results may help to explain the mechanisms behind melanoma-induced impairment of T-cell functions, and suggest an important role for $COX\text{-}2/PGE_2$ and STAT-3 activation in MDSC-mediated immune suppression. Consequently, methods where immunotherapy is combined with inhibiting COX-2 or STAT-3, might offer a better option to eliminate MDSCs in patients with advanced stage melanoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Mao, I. Poschke, J. Hansson, A. Lundqvist, R. Kiessling
Development of methodology: Y. Mao, I. Poschke, E. Wennerberg, R. Kiessling
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Mao, E. Wennerberg, Y. Pico de Coaña, S.E. Brage, I. Schultz, J. Hansson, G. Masucci, R. Kiessling
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Mao, Y. Pico de Coaña, S.E. Brage, G. Masucci, A. Lundqvist, R. Kiessling
Writing, review, and/or revision of the manuscript: Y. Mao, I. Poschke, Y. Pico de Coaña, I. Schultz, J. Hansson, G. Masucci, A. Lundqvist, R. Kiessling
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Mao, S.E. Brage, R. Kiessling
Study supervision: A. Lundqvist, R. Kiessling

Acknowledgments

We thank Giusy Gentilcore and Maria Nyström for help in collecting the patient blood and Juan Castro for help with the cell line authentication. We also appreciate the help from Inger Bodin for establishing the immunohistochemistry staining.

Grant Support

This study was supported by Swedish Cancer Society, the Cancer Society of Stockholm, the Swedish Medical Research Council, and an ALF-Project grant from Stockholm City Council.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 6, 2012; revised March 5, 2013; accepted March 25, 2013; published OnlineFirst April 30, 2013.

References

1. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009;9:162–74.
2. Munera V, Popovic PJ, Bryk J, Pribis J, Caba D, Matta BM, et al. Stat 6-dependent induction of myeloid-derived suppressor cells after physical injury regulates nitric oxide response to endotoxin. Ann Surg 2010;251:120–6.
3. Movahedi K, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschin A, et al. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. Blood 2008;111:4233–44.
4. Poschke I, Kiessling R. On the armament and appearances of human myeloid-derived suppressor cells. Clin Immunol 2012;144:250–68.
5. Condamine T, Gabrilovich DI. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. Trends Immunol 2011;32:19–25.
6. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J Immunol 2008;181:5791–802.
7. Poschke I, Mougiakakos D, Hansson J, Masucci GV, Kiessling R. Immature immunosuppressive CD14+HLA-DR-/low cells in melanoma patients are Stat3hi and overexpress CD80, CD83, and DC-sign. Cancer Res 2010;70:4335–45.
8. Filipazzi P, Valenti R, Huber V, Pilla L, Canese P, Iero M, et al. Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. J Clin Oncol 2007;25:2546–53.
9. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol 2009;182:4499–506.
10. Marigo I, Dolcetti L, Serafini P, Zanovello P, Bronte V. Tumor-induced tolerance and immune suppression by myeloid-derived suppressor cells. Immunol Rev 2008;222:162–79.
11. Kusmartsev S, Gabrilovich DI. Effect of tumor-derived cytokines and growth factors on differentiation and immune suppressive features of myeloid cells in cancer. Cancer Metastasis Rev 2006;25:323–31.
12. Lin WW, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest 2007;117:1175–83.
13. Lechner MG, Liebertz DJ, Epstein AL. Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. J Immunol 2010;185:2273–84.
14. Solito S, Falisi E, Diaz-Montero CM, Doni A, Pinton L, Rosato A, et al. A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. Blood 2011;118:2254–65.
15. Lechner MG, Megiel C, Russell SM, Bingham B, Arger N, Woo T, et al. Functional characterization of human Cd33+ and Cd11b+ myeloid-derived suppressor cell subsets induced from peripheral blood mononuclear cells co-cultured with a diverse set of human tumor cell lines. J Transl Med 2011;9:90.
16. Rodrigues JC, Gonzalez GC, Zhang L, Ibrahim G, Kelly JJ, Gustafson MP, et al. Normal human monocytes exposed to glioma cells acquire myeloid-derived suppressor cell-like properties. Neuro Oncol 2010;12:351–65.
17. Obermajer N, Muthuswamy R, Lesnock J, Edwards RP, Kalinski P. Positive feedback between PGE2 and COX2 redirects the differentiation of human dendritic cells toward stable myeloid-derived suppressor cells. Blood 2011;118:5498–505.

18. Cryan LM, Paraoan L, Hiscott P, Damato BE, Grierson I, Gray D, et al. Expression of COX-2 and prognostic outcome in uveal melanoma. Curr Eye Res 2008;33:177–84.
19. Johansson CC, Egyhazi S, Masucci G, Harlin H, Mougiakakos D, Poschke I, et al. Prognostic significance of tumor iNOS and COX-2 in stage III malignant cutaneous melanoma. Cancer Immunol Immunother 2009;58:1085–94.
20. Obermajer N, Wong JL, Edwards RP, Odunsi K, Moysich K, Kalinski P. PGE(2)-driven induction and maintenance of cancer-associated myeloid-derived suppressor cells. Immunol Invest 2012;41:635–57.
21. Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. Cancer Res 2007;67:4507–13.
22. Gorgun GT, Whitehill G, Anderson JL, Hideshima T, Maguire C, Laubach J, et al. Tumor promoting immune-suppressive myeloid-derived suppressor cells in the multiple myeloma microenvironment in humans. Blood 2013;121:2975–87.
23. Gros A, Turcotte S, Wunderlich JR, Ahmadzadeh M, Dudley ME, Rosenberg SA. Myeloid cells obtained from the blood but not from the tumor can suppress T-cell proliferation in patients with melanoma. Clin Cancer Res 2012;18:5212–23.
24. Hallermalm K, De Geer A, Kiessling R, Levitsky V, Levitskaya J. Autocrine secretion of Fas ligand shields tumor cells from Fas-mediated killing by cytotoxic lymphocytes. Cancer Res 2004;64: 6775–82.
25. Selvan SR, Carbonell DJ, Fowler AW, Beatty AR, Ravindranath MH, Dillman RO. Establishment of stable cell lines for personalized melanoma cell vaccine. Melanoma Res 2010;20:280–92.
26. Han C, Demetris AJ, Stolz DB, Xu L, Lim K, Wu T. Modulation of Stat3 activation by the cytosolic phospholipase A2alpha and cyclooxygenase-2-controlled prostaglandin E2 signaling pathway. J Biol Chem 2006;281:24831–46.
27. Yang R, Cai Z, Zhang Y, Yutzy WHt, Roby KF, Roden RB. CD80 in immune suppression by mouse ovarian carcinoma-associated Gr-1+CD11b+ myeloid cells. Cancer Res 2006;66:6807–15.
28. Mencacci A, Montagnoli C, Bacci A, Cenci E, Pitzurra L, Spreca A, et al. CD80+Gr-1+ myeloid cells inhibit development of antifungal Th1 immunity in mice with candidiasis. J Immunol 2002;169:3180–90.
29. Liu Y, Yu Y, Yang S, Zeng B, Zhang Z, Jiao G, et al. Regulation of arginase I activity and expression by both PD-1 and CTLA-4 on the myeloid-derived suppressor cells. Cancer Immunol Immunother 2009;58:687–97.
30. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. Nat Med 1999;5:1365–9.
31. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000;192:1027–34.
32. Ascierto PA, Simeone E, Sznol M, Fu YX, Melero I. Clinical experiences with anti-CD137 and anti-PD1 therapeutic antibodies. Semin Oncol 2010;37:508–16.
33. Topalian SL, Drake CG, Pardoll DM. Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. Curr Opin Immunol 2012;24:207–12.
34. Obermajer N, Muthuswamy R, Odunsi K, Edwards RP, Kalinski P. PGE (2)-induced CXCL12 production and CXCR4 expression controls the accumulation of human MDSCs in ovarian cancer environment. Cancer Res 2011;71:7463–70.
35. Denkert C, Kobel M, Berger S, Siegert A, Leclere A, Trefzer U, et al. Expression of cyclooxygenase 2 in human malignant melanoma. Cancer Res 2001;61:303–8.
36. Corzo CA, Cotter MJ, Cheng P, Cheng F, Kusmartsev S, Sotomayor E, et al. Mechanism regulating reactive oxygen species in tumor-induced myeloid-derived suppressor cells. J Immunol 2009;182:5693–701.
37. Nefedova Y, Cheng P, Gilkes D, Blaskovich M, Beg AA, Sebti SM, et al. Activation of dendritic cells via inhibition of Jak2/STAT3 signaling. J Immunol 2005;175:4338–46.
38. Fortin CF, Larbi A, Dupuis G, Lesur O, Fulop T Jr. GM-CSF activates the Jak/STAT pathway to rescue polymorphonuclear neutrophils from spontaneous apoptosis in young but not elderly individuals. Biogerontology 2007;8:173–87.
39. Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. Eur J Cancer 2005;41:2502–12.
40. Serafini P, Carbley R, Noonan KA, Tan G, Bronte V, Borrello I. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. Cancer Res 2004;64:6337–43.
41. Bayne LJ, Beatty GL, Jhala N, Clark CE, Rhim AD, Stanger BZ, et al. Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer. Cancer Cell 2012;21:822–35.
42. Pylayeva-Gupta Y, Lee KE, Hajdu CH, Miller G, Bar-Sagi D. Oncogenic Kras-induced GM-CSF production promotes the development of pancreatic neoplasia. Cancer Cell 2012;21:836–47.
43. Poschke I, Mao Y, Adamson L, Salazar-Onfray F, Masucci G, Kiessling R. Myeloid-derived suppressor cells impair the quality of dendritic cell vaccines. Cancer Immunol Immunother 2012;61:827–38.
44. Caughey GE, Cleland LG, Penglis PS, Gamble JR, James MJ. Roles of cyclooxygenase (COX)-1 and COX-2 in prostanoid production by human endothelial cells: selective up-regulation of prostacyclin synthesis by COX-2. J Immunol 2001;167:2831–8.
45. Obermajer N, Kalinski P. Generation of myeloid-derived suppressor cells using prostaglandin E2. Transplant Res 2012;1:15.