COX-2 promotes metastasis in nasopharyngeal carcinoma by mediating interactions between cancer cells and myeloid-derived suppressor cells

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Keywords: COX-2, myeloid-derived suppressor cells, nasopharyngeal carcinoma

Abbreviations: ARG-1, arginase 1; COX-2, cyclooxygenase-2; DFS, disease-free survival; EMT, epithelial-mesenchymal transition; GM-CSF, granulocyte-monocyte-colony stimulating factor; iNOS, inducible nitric oxide synthase; LNMA, L-NG-monomethylarginine; MDSCs, myeloid-derived suppressor cells; NAC, N-acetylcysteine; NOHA, N-hydroxy-nor-L-arginine; NOX2, NADPH oxidase; NPC, nasopharyngeal carcinoma; ROS, reactive oxygen species; siRNA, small interfering RNA; TCF4, T-cell factor 4; TGF, transforming growth factor β; T-MDSCs, tumor-induced MDSCs; VEGF, vascular endothelial growth factor

The expansion of myeloid-derived suppressor cells (MDSCs) is a common feature of cancer, but its biological roles and molecular mechanism remain unclear. Here, we investigated a molecular link between MDSC expansion and tumor cell metastasis in nasopharyngeal carcinoma (NPC). We demonstrated that MDSCs expanded and were positively correlated with the elevated tumor COX-2 expression and serum IL-6 levels in NPC patients. Importantly, COX-2 and MDSCs were poor predictors of patient disease-free survival (DFS). Knocking down tumor COX-2 expression hampered functional TW03-mediated-MDSC cell (T-MDSC) induction with IL-6 blocking. We identified that T-MDSCs promoted NPC cell migration and invasion by triggering the epithelial-mesenchymal transition (EMT) on cell-to-cell contact, and T-MDSCs enhanced tumor experimental lung metastasis in vivo. Interestingly, the contact between T-MDSCs and NPC cells enhanced tumor COX-2 expression, which subsequently activated the β-catenin/TCF4 pathway, resulting in EMT of the cancer cells. Blocking transforming growth factor β (TGFβ) or inducible nitric oxide synthase (iNOS) significantly abolished the T-MDSC-induced upregulation of COX-2 and EMT scores in NPC cells, whereas the administration of TGFβ or L-arginine supplements upregulated COX-2 expression and EMT scores in NPC cells. These findings reveal that COX-2 is a key factor mediating the interaction between MDSCs and tumor cells, suggesting that the inhibition of COX-2 or MDSCs has the potential to suppress NPC metastasis.

Introduction

NPC has a high incidence in southern China and Southeast Asia.1 NPC has the highest rate of metastasis among head and neck cancers, and distant metastasis is the main cause of treatment failure.2 NPC is consistently correlated with EBV infection; the expression of neo-viral antigens in tumor cells induces numerous immune cells, referred to as tumor-infiltrating leukocytes, which are attracted to tumor tissues.3,4 Most studies of the mechanisms of cancer-related inflammation have focused on cancer initiation; in fact, inflammatory mediators and cells are also involved in the migration, invasion and metastasis of malignant cells.5-7 Metastatic cancer cells typically undergo morphological changes that result in decreased intercellular adhesion and increased cell motility, a process defined as the EMT.8-10 A variety of cytokines, including chemokines, inflammatory factors, and growth factors, and tumor-infiltrating bone marrow-derived components, including macrophages and related cell types, are involved in tumor invasion and metastasis.11,12 However, the involvement of inflammatory cells in cancer cell metastasis remains controversial.

Myeloid cells in cancer are represented by populations of mature cells, including macrophages, granulocytes, and dendritic cells (DC), and pathologically activated immature myeloid cells termed MDSCs, which are not present under normal conditions.7,13 MDSCs are a heterogeneous population of bone
marrow-derived myeloid progenitors and immature myeloid cells that expand dramatically during tumor growth, leading to an increased proportion of MDSCs in the peripheral blood and tumor microenvironments of cancer patients. The MDSC phenotype varies by differentiation status and function in response to the environmental conditions of different cancers and generally has been termed as the HLA-DR<sup>+</sup>CD33<sup>+</sup>CD11b<sup>+</sup> cell population, including PMN- and MO-MDSCs, in many human cancers. In humans, these tumor-associated MDSCs have drawn attention due to their role in tumor progression. MDSCs enhance tumor growth by negatively regulating immune responses and facilitating tumor metastasis and angiogenesis. The reported mechanisms of MDSC-mediated T-cell inhibition include arginase 1 (ARG-1)-mediated depletion of L-arginine, iNOS-mediated and NADPH oxidase (NOX2)-mediated production of reactive nitrogen species and reactive oxygen species (ROS), increased vascular endothelial growth factor (VEGF) expression, cysteine depletion, and T-regulatory (Treg) cell expansion. However, a direct link between the non-immune function of tumor-associated MDSC populations and tumor growth and metastasis has yet to be established.

Cyclooxygenase (COX), also known as prostaglandin (PG) H2 synthase, is the rate-limiting enzyme in the conversion of arachidonic acid to PGs. Two isoforms of COX are referred to as COX-1 and COX-2. COX-1 is a constitutively expressed housekeeping gene, whereas COX-2 expression is normally restricted to a few organs but can be induced by a variety of stimuli, including cytokines, oncopgenes, growth factors, and hormones. Increased expression of COX-2 is frequently detected in many cancers, including NPC. COX-2 is an inducible enzyme that produces PGs in inflammatory and tumorigenic settings. This function of the COX-2/PGE2 pathway affects multiple aspects of cell physiology required for tumor development. The involvement of COX-2 in the induction of MDSCs in tumor hosts and tumor metastasis has been documented in recent years, however, the link between COX-2, MDSCs and tumor metastasis and its mechanism of regulation in tumors is unknown.

In the present study, we detected the upregulation of COX-2 in NPC cells and the expansion of circulating MDSCs in NPC patients as well as the expansion of tumor-infiltrating MDSC populations in NPC tissues. Moreover, COX-2 and MDSCs were found to be predictors of poor DFS of the patients, and a positive correlation was observed between COX-2 levels and the number of circulating and tumor-infiltrating MDSCs. We further found that COX-2 promotes the induction of NPC-activated MDSCs by increasing IL-6 secretion in vitro, and serum IL-6 levels were positively associated with circulating HLA-DR<sup>+</sup>CD33<sup>+</sup>CD11b<sup>+</sup> MDSCs in NPC patients. Furthermore, NPC-activated MDSCs promoted EMT and tumor metastasis in vitro and in vivo. This MDSC-mediated EMT was based on cell-to-cell contact; in turn, NPC-activated MDSC contact with NPC cells promoted the production of TGFβ and nitric oxide (NO), which upregulated COX-2 expression and subsequently activated the β-catenin/TCF4 pathway, leading to EMT in NPC cells. Here, we delineate how COX-2 drives the interaction between MDSCs and tumor cells to promote tumor progression and metastasis in NPC patients.

**Results**

**Clinical impact of COX-2 and MDSCs in patients with NPC**

Recent studies have reported that upregulation of COX2 in different malignances is associated with advanced disease stage and reduced survival. Here, the expression level of COX-2 was significantly higher in tumor biopsies compared with tumor-adjacent tissues from 26 paired NPC patients (P < 0.05, n = 26), as shown in Fig. 1A. In addition, the COX-2 protein is highly expressed in several NPC cell lines, including TW03, CNE1 and CNE2, compared with the normal NP cell line NP69 (Fig. 1B).

Next, we measured the percentages of HLA-DR<sup>+</sup>CD33<sup>+</sup>, HLA-DR<sup>+</sup>CD33<sup>+</sup>CD11b<sup>+</sup>, HLA-DR<sup>+</sup>CD33<sup>+</sup>CD11b<sup>−</sup> and HLA-DR<sup>+</sup>CD33<sup>+</sup>CD11b<sup>+</sup> MDSC subsets in peripheral blood from 49 NPC patients and 32 age-matched healthy donors. The percentages of HLA-DR<sup>+</sup>CD33<sup>+</sup>, HLA-DR<sup>+</sup>CD33<sup>+</sup>CD11b<sup>+</sup> and HLA-DR<sup>+</sup>CD33<sup>+</sup>CD11b<sup>−</sup> MDSCs were significantly higher in peripheral blood from NPC patients compared with healthy controls (P < 0.05), whereas the percentage of HLA-DR<sup>+</sup>CD33<sup>+</sup>CD11b<sup>+</sup> MDSCs was only slightly increased in peripheral blood from NPC patients compared with healthy controls (P > 0.05), as shown in Fig. 1C and Fig. S1. Moreover, we discovered that the number of CD33<sup>+</sup> cells was significantly increased in tumor tissue compared with paired adjacent tissues from NPC patients (P < 0.05, n = 26), as shown in Fig. 1D. These HLA-DR<sup>+</sup>CD33<sup>+</sup>CD11b<sup>−</sup> and HLA-DR<sup>+</sup>CD33<sup>+</sup>CD11b<sup>+</sup> cells in peripheral blood mononuclear cells (PBMCs) and CD33<sup>+</sup> cells in tumor tissues expressed myeloid cell markers, including CD45, CD34, CD66b, ARG-1, iNOS and ROS (data not shown). Therefore, we refer to circulating HLA-DR<sup>+</sup>CD33<sup>+</sup> cells, including CD11b<sup>+</sup> and CD11b<sup>−</sup> cells, and CD33<sup>+</sup> cells in tumor tissues as MDSCs in this study.

More importantly, the expression of COX-2 in NPC tissues was positively correlated with the number of circulating HLA-DR<sup>+</sup>CD33<sup>+</sup>CD11b<sup>−</sup> cells (n = 45, P = 0.001, R = 0.476) and tumor-infiltrating CD33<sup>+</sup> cells (n = 112, P < 0.0001, R = 0.552) in NPC patients (Fig. 1E).

As shown in Table 1, we found that the proportion of circulating HLA-DR<sup>+</sup>CD33<sup>+</sup> MDSCs was increased in late tumor stage NPC patients compared with early tumor stage NPC patients (P < 0.05), and the high levels of COX-2 in NPC tissues were associated with advanced clinical and tumor stage (P < 0.05). Furthermore, based on an optimal cut-off determined by circulating or tumor-infiltrating MDSC frequencies or COX-2 levels, we divided the patients into “low” and “high” MDSC groups. The patients with higher numbers of circulating HLA-DR<sup>+</sup>CD33<sup>+</sup> MDSCs and tumor-infiltrating CD33<sup>+</sup> MDSCs was significantly associated with shortened DFS (P = 0.04, n = 49; P = 0.049, n = 112, respectively) based on Kaplan-Meier analysis, as shown in Figs. 2A, B. Moreover, high levels of
Figure 1. The expression of COX-2 is correlated with the expansion of MDSC populations in NPC. (A) Two representative cases of staining for COX-2 and statistical analysis of the levels of COX-2 in tumor and adjacent tissues from 26 paired NPC patients are shown. (B) Western blot for the expression of COX-2 in NPC cell lines, including TW03, CNE1 and CNE2, or the normal NP cell line NP69. (C) The percentages of CD33^+HLA-DR^+CD11b^+HLA-DR^- and CD33^+CD11b^-HLA-DR^- MDSC populations in blood from 45 NPC patients were compared with those from 32 healthy donors. (D) Paraffin-embedded NPC tissues and adjacent tissues were collected from 26 paired patients to detect CD33^+ cells or the expression of COX-2 in situ by IHC staining. Some CD33^+ cells (brown staining on the cell membrane) were detected in the stroma of the NPC tissues, but very few of these cells were detected in the adjacent tissues (original magnification 400x); two representative cases of staining for CD33 or CD11b and statistical analysis of the frequency of CD33^+ and CD11b^+ cells in tumor and adjacent tissues from 26 paired NPC patients are shown. *P < 0.05; **P < 0.01. (E) Correlation analysis between circulating MDSC subsets or tumor-infiltrating CD33^+ cells and the score of tumor COX-2 or serum levels of IL-6. The statistical analysis was performed using Pearson’s correlation coefficient and linear regression. R = Spearman’s correlation; P = significance of correlation.
| Clinical parameter | Total cases | Peripheral blood | Tumor tissue |
|--------------------|-------------|------------------|--------------|
|                    |             | High level of CD33<sup>+</sup> | High level of CD11b<sup>+</sup> | High level of CD33<sup>+</sup> | High level of CD11b<sup>+</sup> | Total cases | High density of CD33<sup>+</sup> cells | High expression of COX-2 |
|                    |             | HLA-DR<sup>-</sup> | HLA-DR<sup>-</sup> | HLA-DR<sup>-</sup> | HLA-DR<sup>-</sup> |          |          |          |
| Total               | 49          |                  |                |                  |                | 112      |                  |            |
| Age                 |             |                  |                |                  |                |          |                  |            |
| < 46 y              | 24          | 17 (70.8%)       | 9 (37.5%)      | 14 (58.3%)       | 15 (62.5%)     | 24       | 17 (68.0%)       | 14 (58.5%) |
| ≥ 46 y              | 25          | 17 (68.0%)       | 5 (20.0%)      | 13 (52.0%)       | 11 (44.0%)     | 25       | 17 (68.0%)       | 13 (52.0%) |
| Gender              |             |                  |                |                  |                |          |                  |            |
| Male                | 43          | 30 (69.8%)       | 12 (27.9%)     | 24 (55.8%)       | 25 (58.1%)     | 43       | 43 (46.7%)       | 22 (43.0%) |
| Female              | 6           | 4 (66.7%)        | 2 (33.3%)      | 3 (50.0%)        | 1 (16.7%)      | 20       | 14 (70.0%)       | 10 (50.0%) |
| Clinical Stage      |             |                  |                |                  |                |          |                  |            |
| I–II                | 5           | 3 (60.0%)        | 1 (20.0%)      | 3 (60.0%)        | 1 (20.0%)      | 23       | 13 (46.7%)       | 6 (26.1%)  |
| III–IV              | 44          | 31 (70.5%)       | 13 (29.5%)     | 24 (54.5%)       | 25 (56.8%)     | 44       | 44 (49.4%)       | 48 (53.9%) |
| T Stage             |             |                  |                |                  |                |          |                  |            |
| T1–T2               | 11          | 4 (36.4%)        | 1 (9.1%)       | 4 (36.4%)        | 4 (36.4%)      | 11       | 14 (50.0%)       | 7 (25.0%)  |
| T3–T4               | 38          | 30 (78.9%)       | 13 (34.2%)     | 23 (60.5%)       | 22 (57.9%)     | 38       | 43 (51.2%)       | 47 (56.0%) |
| N Stage             |             |                  |                |                  |                |          |                  |            |
| N0–N1               | 32          | 22 (68.8%)       | 9 (28.1%)      | 16 (50.0%)       | 17 (53.1%)     | 32       | 35 (53.0%)       | 32 (48.5%) |
| N2–N3               | 17          | 12 (70.6%)       | 5 (29.4%)      | 11 (64.7%)       | 9 (52.9%)      | 17       | 22 (47.8%)       | 22 (47.8%) |

*p < 0.05, as determined by Pearson’s χ<sup>2</sup> test.
**COX-2 enhances the induction of T-MDSCs by upregulating IL-6 in vitro**

First, we developed an *in vitro* short-term co-culture model according to a previous report, in which CD33^+ myeloid progenitors from healthy donors were co-cultured with TW03 using a transwell system (pore diameter 0.4 μM) for 48 h to effectively induce the rapid generation of functional MDSC-like cells by tumor cells, defined as tumor (TW03)-induced MDSCs (T-MDSCs). More than 80% of the CD33^+ PBMCs became HLA-DR^+CD33^+CD11b^+ cells (T-MDSCs) after co-culture with TW03 cells for 48 h. In addition, several different NPC cell lines induced the rapid generation of functional T-MDSCs in this transwell system (Figs. S2A, B and C). Next, we suppressed the expression of COX-2 in TW03 and CEN1 cells by targeting COX-2 with small interfering RNAs (siRNAs) (Fig. 3A); consequently, the induction of T-MDSC was significantly hampered in siCOX-2-TW03-mediated co-culture, but the induction of T-MDSC was reversed when IL-6 was added to siCOX-2-TW03-mediated co-culture (*P < 0.05, Fig. 3B*). Moreover, the T-MDSCs induced by TW03 cells suppressed both CD4^+ and CD8^+ T-cell activity, whereas those induced by siCOX-2-TW03 demonstrated weak suppression (*P < 0.05, Figs. 3C and D*).

In addition, expansion and infiltration of MDSCs are regulated by cytokines and chemokines from tumor cells, including IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF). The levels of IL-6 and G-CSF were high in NPC patients (*P < 0.016, n = 112, Fig. 2C*). COX-2 (> 2.0 score), as defined elsewhere, in NPC tissues were significantly associated with shortened DFS (*P = 0.016, n = 112, Fig. 2C).

**T-MDSCs induce cancer cell EMT by cell-to-cell contact in vitro**

Several recent studies have reported that MDSCs promote tumor progression and metastasis by provoking EMT and tumor cell stemness in some solid tumors. Here, we hypothesized that T-MDSCs directly promote NPC cell growth, migration and invasion. First, a spindle-shaped appearance among CNE2 cells was observed when these cells were co-cultured with T-MDSCs for 48 h (Fig. 4A). We performed Boyden chamber and wound healing assays to determine the potential for MDSCs to stimulate the invasiveness NPC cell lines, including TW03 and CNE2, in an *in vitro* co-culture system. A significant increase in the number of TW03 or CNE2 cells that invaded through basement membrane-like Matrigel barriers was detected when the NPC cells were co-cultured with T-MDSCs. T-MDSCs also accelerated the wound healing process based on the more rapid healing of a “wound” scratched into an epithelial monolayer (Figs. 4B–E).

**EMT is an indispensable step for cancer cells to acquire migratory and invasive capacities.** After TW03 or CNE2 cells were co-cultured with T-MDSCs for 48 h, the expression of EMT-like cellular markers was altered in TW03 and CNE2 cells, including a dramatic upregulation of COX-2, the mesenchymal markers vimentin and fibronectin and the EMT-associated transcription factor snail along with the downregulation of the epithelial marker E-cadherin, as shown in Fig. 4F. These findings indicate that T-MDSCs induce EMT in NPC cells. Surprisingly, the alteration of EMT-like cellular markers, including...
E-cadherin, vimentin, fibronectin and snail, as well as COX-2 was reversed when TW03 or CNE2 cells were co-cultured with T-MDSCs using a transwell insert (0.4 μM, Fig. 4F). This result indicates that T-MDSCs promote EMT induction in a cell-to-cell contact-dependent manner. In addition, studies have shown that TGFβ plays an important role in the biological function of MDSCs.39,40 Here, we found that TGFβ levels were increased in the cell culture supernatant when TW03 and T-MDSCs were co-cultured but were significantly decreased in the supernatant when TW03 and T-MDSCs were co-cultured with a transwell insert, as shown in Fig. S3. These observations provide evidence that contact between T-MDSCs and NPC cells stimulates the production of TGFβ, which may correlate with the promotion of EMT mediated by T-MDSCs.

T-MDSCs promote NPC metastasis in vivo

We next evaluated the in vivo effects of T-MDSCs on tumor metastasis with an experimental metastasis model. CNE2 cells and T-MDSCs were injected into the tail veins of nude mice after co-culture for one week in vitro; the control group received CNE2 cells only (P < 0.05, n = 13). Histological analyses further revealed that co-injection of T-MDSCs with cancer cells promoted NPC metastases in the lung (Figs. 5B–D).

Alternatively, CNE2 cells were subcutaneously injected into the flanks of nude mice with or without T-MDSCs. The volume and weight of the tumors resulting from the injection containing both CNE2 and T-MDSC cells were not significantly different from those resulting from the inoculation of CNE2 cells alone (n = 10) (Figs. S4A and B), suggesting that T-MDSCs promote NPC metastasis without influencing NPC growth.

Upregulation of COX-2 and the β-catenin/TCF4 pathway

Recently, accumulating evidence has shown that a PGE2-COX-2-mediated positive feedback loop is essential for the induction of immunosuppressive factors secreted by human MDSCs31 and that COX-2 is implicated in tumor angiogenesis, evasion of apoptosis, and induction of EMT.42,43 Therefore, we suppressed the expression of COX-2 in TW03 and CNE1 cells using siRNAs targeting COX-2 (siCOX-2). The invasive ability of TW03 and CNE1 cells was significantly decreased when the expression of COX-2 was knocked down using siCOX-2 even when these NPC cells were co-cultured with T-MDSCs (P <
Immunoblotting revealed that the levels of COX-2 as well as of PLC-γ2 and T-cell factor 4 (TCF4) in the β-catenin/TCF4 pathway were decreased in TW03 and CNE1 cells treated with siCOX-2 and only slightly increased after co-culture with T-MDSCs for 48 h compared with TW03 and CNE1 cells treated with scrambled siRNA.
These data indicate that changes in COX-2 expression have an effect on the upregulation of related genes, including PLC-γ2 and TCF4, in the β-catenin/TCF4 pathway during EMT progression in NPC cells. Moreover, alterations of EMT-like cellular markers, including vimentin, fibronectin, snail and E-cadherin, in siCOX-2-treated or scrambled siRNA-treated TW03 and CNE1 cells with or without T-MDSC co-culture were consistent with the above results for the EMT phenotype, as shown in Fig. 5A. Taken together, these results suggest that T-MDSC-induced EMT occurs through the upregulation of COX-2 and activation of the β-catenin/TCF4 pathways in NPC cells.

**NO and TGFβ are involved in T-MDSC-induced EMT**

To further confirm that TGFβ and other molecular pathways are involved in T-MDSC-induced EMT in NPC cells, we detected high expression levels of immune mediator molecules, including TGFβ, IL-10, iNOS, ARG-1, and ROS (NOX2), in T-MDSCs using a semi-quantitative RT-PCR array (Fig. 7A). In the co-culture system, we inhibited several highly expressed immune mediator molecules, including TGFβ, iNOS (NO), ARG-1, and NOX2 (ROS), using neutralizing antibodies or small-molecule drugs, including a TGFβ-neutralizing antibody, L-NG-monomethylarginine (LNMMA), N-hydroxy-nor-L-arginine (NOHA), and N-acetylcysteine (NAC), which are inhibitors of TGFβ, iNOS, ARG-1 and NOX2, respectively. The administration of LNMMA and the TGFβ-neutralizing antibody, but not NOHA and NAC, significantly blocked T-MDSC-mediated TW03 and CNE2 cell invasion (P < 0.05), as shown in Fig. 7B. Notably, the upregulation of COX-2 and mesenchymal markers (vimentin, fibronectin and snail) and the downregulation of the epithelial marker E-cadherin in TW03 and CNE2 cells were also reversed to varying degrees after administration of LNMMA, the TGFβ-neutralizing antibody,
NOHA and NAC, as shown in Fig. S5B. To further confirm the regulation of COX-2 and related genes in the β-catenin/TCF4 pathway in NPC cells by TGFβ and NO, we subsequently measured changes in COX-2, PLC-γ2, TCF4 and β-catenin expression in TW03 and CNE1 cells after the cells were treated with the cytokine TGFβ (40 ng/mL) and an L-arginine supplement (2 mM) for 48 h. As shown in Fig. 7C, the expression of COX-2, PLC-γ2, and TCF4 was dramatically increased in TGFβ- or L-arginine supplement-treated TW03 and CNE1 cells. Moreover, we determined that the expressions of PLC-γ2, TCF4 and β-catenin were dramatically increased in TGFβ-treated siCOX-2-TW03 and siCOX-2-CNE1 cells as well as the expression of COX-2; however, the presence of COX-2 inhibitor reversed the effect of TGFβ on the expression of PLC-γ2, TCF4 and β-catenin in siCOX-2-TW03 and siCOX-2-CNE1 cells (Fig. 7D). Taken together, our data indicate that TGFβ promotes the MET of NPC cells through the COX-2 signaling pathway.

**Discussion**

Cancer is a systemic disease, and primary tumors secrete factors that influence metastatic outcomes at distant sites. Many studies have reported that COX-2 expression is usually upregulated in human cancers, including NPC.\(^{34,44,45}\) COX-2 activity is detected throughout the progression of a premalignant lesion to a metastatic phenotype. Increased expression of COX-2 is associated with angiogenesis, decreased host immunity, enhanced invasion and metastasis in cancers.\(^{46,47}\) In the current study, we identified COX-2 as a driving factor in a molecular loop between the NPC cells and T-MDSCs that support the expansion of T-MDSCs in NPC patients and the migration and invasion of tumor cells through EMT (Fig. 8).

Although the detailed molecular mechanisms for the generation of MDSCs in a tumor-bearing host remain to be elucidated, a number of inflammatory modulators, including GM-CSF, IL-6 and TNF-α, have been suggested to contribute to the induction and expansion of MDSCs.\(^{34,45}\) Here, our data reveal for the first time that expansion of circulating and tumor-infiltrating MDSC populations in NPC patients is positively correlated with the levels of COX-2 in tumor tissues (\(P < 0.001\)). COX-2 derives bioactive PGs, particularly PGE2, which is one of the main pro-inflammatory factors.\(^{28}\) Recently, several researchers proposed that PGE2 is indispensable for the induction and maintenance of MDSCs in cancer patients.\(^{21,34,48}\) In the current study, we first revealed that the induction of T-MDSCs is hampered when COX-2 is suppressed in NPC cells in vitro. Furthermore, our data demonstrate that serum IL-6 levels are correlated with the proportion of circulating MDSCs in NPC patients, and blockade of COX-2 expression inhibits the induction of NPC-mediated T-MDSCs by decreasing the secretion of IL-6 from NPC cells in vitro.

Evidence suggests that interactions between tumor cells and stroma mediate the development of cancer and promote tumor metastasis.\(^{49}\) The tumor stroma consists of the extracellular matrix (ECM) and cellular components. In the tumor microenvironment, tumor-infiltrating immunosuppressive cells, including Treg cells, MDSCs, alternatively activated macrophages (M2), and immature/tolerogenic DC, inhibit anticancer immunity and...
play a role in tumor angiogenesis and cancer cell survival, proliferation, and metastatic potential. In the present study, we delineated a role for NPC T-MDSCs in directly promoting NPC cell migration, invasion and metastasis. First, we found that T-MDSCs increase cancer migration and invasion via the induction of EMT in NPC cells in vitro. Moreover, we showed that T-MDSCs enhance the lung metastasis of cancer cells in nude mice. In recent years, it has been shown that human tumor-associated microphages influence cancer progression and metastasis in breast cancer and human MDSCs enhance tumor cell stemness in ovarian cancer by inducing microRNA101 expression in cancer cells.37 Another report agrees with our results that tumor-infiltrating MDSCs promote cancer cell metastasis via EMT in a tumor-bearing mouse model.36 Taken together, our findings reveal for the first time that human tumor-activated MDSCs promote tumor cell migration, invasion, and metastasis via EMT in NPCs.

Multiple cancer immunoevasive and immunoinhibitory pathways have been identified. MDSCs inhibit tumor-specific T-cell responses via IFNγ-dependent NO production or the Th2-mediated IL-4/IL-13-dependent Arg1 pathway. In addition, a mechanism of ROS-mediated cell death has been proposed.52 Cell-to-cell contact appears to be essential for these mechanisms.53 However, the mechanisms by which MDSCs promote cancer cell EMT, proliferation, invasion and metastasis are unclear. Here, we found that the EMT of NPC cells induced by T-MDSCs depended on the existence of cell-to-cell contact between NPC cells and T-MDSCs, which upregulated the expression of COX-2 in tumor cells. Knocking down COX-2 expression in NPC cells abolished the T-MDSC-induced migration and invasion of the cancer cells. These results suggest that COX-2 plays a vital role in the MDSC-induced EMT in NPC cells. The molecular mechanisms underlying the distant metastasis of cancers have been intensively studied. Increasing the expression of COX-2 and its product PGE2 activates the β-catenin/TCF4 pathway and has been implicated in cancer tumorigenesis and EMT.54,55 Our findings confirmed that the expression of PLC-γ2 and TCF4 was increased after NPC cells were co-cultured with T-MDSCs but decreased in siCOX-2-treated cancer cells. Therefore, T-MDSCs induce EMT via the

Figure 7. T-MDSC-induced EMT; COX-2 expression in cancer cells induced by NO and TGFβ. (A) The expressions of immune mediator molecules, including TGFβ, IL-10, ARG-1, iNOS, and NOX, in T-MDSCs, were measured using real-time quantitative PCR. PBMCs and CD33+ cells were included as controls, and GAPDH was used as a loading control. (B) Statistical analysis of the Boyden chamber assay for TW03 and CNE2 cells co-cultured with T-MDSCs for 48 h in the presence or absence of an inhibitor of iNOS, ROS, ARG-1 or TGFβ, including LNMMA, NAC, arginine or anti-TGFβ. TW03 and CNE2 cells were incubated in medium alone as a control. * P < 0.05. (C) Immunoblotting analyses of COX-2, PLC-γ2, TCF4 and β-catenin expression in TW03 and CNE2 cells in the presence of the cytokine TGFβ, an L-arginine supplement or in medium alone for 48 h. GAPDH was used as a loading control. (D) Immunoblotting analyses of COX-2, PLC-γ2, TCF4 and β-catenin expression in siCOX-2-TW03 and siCOX-2-CNE2 cells in the presence of the cytokine TGFβ or TGFβ with the COX-2 inhibitor celecoxib.
upregulation of COX-2 and the activation of the β-catenin/TCF4 signaling pathway in NPC cells. In addition, our results confirmed the effect of NO and TGFβ on the expression of COX-2 and related genes in the β-catenin/TCF4 signaling pathway in NPC cells. Our findings are consistent with previous results showing that tumor-activated MDSCs induce EMT via multiple pathways, including TGFβ, HGF, and TGH, in melanoma.36 Importantly, we have also revealed the prognostic roles of COX-2 overexpression in the primary tumor. The COX-2 inhibitor celecoxib has been administered to cancer patients in combination with routine chemotherapy in clinical trials.56,57 Therefore, our results provide a novel mechanistic rationale for the inhibition of COX-2 expression for the treatment of patients with NPC and provide new insights into immunotherapy by inhibiting MDSCs.

In summary, the findings in the present study provide novel insights into the interactions between tumor-activated MDSCs and tumor cells and the related molecular loop between tumor-activated MDSC expansion and MDSC-induced EMT, which eventually promotes the metastasis of NPC. COX-2 plays a critical role in MDSC expansion in vivo and in vitro by promoting IL-6 secretion and driving T-MDSC-induced tumor cell EMT and metastasis through the activation of the β-catenin/TCF4 pathway, which is dependent on cell-to-cell contact to induce the production of TGFβ and NO. Finally, this network is clinically relevant because the increased T-MDSC density and tumor COX-2 levels predict a poor prognosis for patients with NPC.

Materials and Methods

Samples and cell lines

We collected 112 fresh tumor specimens, including 26 paraneoplastic tissues, 97 serum samples and 49 PBMC samples from 116 patients with NPC at the first diagnosis at the Sun Yat-Sen University Cancer Center, Guangzhou, China between March 2011 and July 2012. Thirty-two age-matched healthy donors were included as controls. The clinical details of the patients are provided in Tables S1 and S2. All patients and healthy donors provided written informed consent before being subjected to blood sampling and/or tumor biopsy. This study was conducted in accordance with the Helsinki Declaration and approved by the Research Ethics Committee of the Sun Yat-Sen University Cancer Center (GZR2013-040).

PBMCs were isolated via Ficoll-Hypaque gradient centrifugation to measure the proportion and phenotype of MDSCs. Fresh tumor and paraneoplastic tissues were separated into two pieces: one piece was fixed in formalin and embedded in paraffin for histological analysis, and the other piece was used to analyze the frequency of tumor-infiltrating MDSCs or generate TILs as previously described.58 Serum was used to measure the levels of the cytokine IL-6.

The human TW03, CNE1 and CNE2 NPC cell lines were maintained in our laboratory and were cultured in RPMI 1640 supplemented with 10% FBS and gentamicin sulfate (Guangdong Succhi Shiqi Pharmaceutical); the normal NP69 cell line was maintained in keratinocyte-SFM medium (Invitrogen).

Antibodies and fluorescence-activated cell sorting (FACS) analysis

Human monoclonal antibodies against HLA-DR, CD4, CD33, CD11b, CD8, CD14, CD15, CD39, CD73, CD117, CD66b, CXCR4, iNOS, ARG-1 and CD34 conjugated to various fluorescent dyes were purchased from BD PharMingen, Biologend or eBioscience. The oxidation-sensitive dye CM-H2DCFDA was purchased from Invitrogen (Grand Island, NY).
The expression of markers in MDSCs was investigated using FACS analysis after surface staining or intracellular staining with human-specific antibodies conjugated to various fluorochrome dyes. MDSC phenotypic analysis involved gating of the HLA-DR− cell population that expressed both the CD33 and CD11b antigens. The cells displaying positive staining were detected using a Cytomics FC 500 MPL flow cytometry system (Beckman Coulter) and were analyzed using CXP software (Beckman Coulter).

**Immunochemistry**

Paraffin-embedded tissues were sectioned continuously at a thickness of 4 μm. CD33 and COX-2 immunohistochemical staining (IHC) was performed using a primary monoclonal mouse anti-human CD33 (Abcam) or anti-human COX-2 antibody (Abcam) according to the manufacturer’s instructions. The slides were scored independently by two pathologists. The data were obtained by counting the positively stained lymphocytes in five separate 400 times high-power microscopic fields (HPFs) and calculating the mean number of positively stained cells per HPF. Mouse IgG1 (DAKO) and normal goat IgG (Santa Cruz Biotechnology) negative control stains were generated and evaluated.

**Enzyme-linked immunosorbent assay**

Enzyme-linked immunosorbent assays (ELISA) were performed using Human IL-6 (R&D Systems), human TGFβ1 SensyELISA kit (Multi Science) or GM-CSF Instant ELISA kit (eBioscience). Briefly, a 96-well plate was coated with 100 μL of coating diluent at 4°C overnight. Then, 100 μL of a standard or sample was added to the appropriate wells and incubated for 2 h at room temperature (RT). The wells were washed five times with wash buffer, and an anti-IL-6, anti-TGFβ1 or anti-GM-CSF conjugate was added to all wells followed by further incubation at RT. The plate was again washed five to seven times, and 100 μL of Avidin-HRP was added. The plate was then incubated for 30 min at RT, and 100 μL of a substrate solution was added to each well after washing again with wash buffer. The plate was subsequently incubated for 15 min at RT in the dark after which 50 μL of stop solution was added to each well, and the absorbance at 450 nm was detected using a 96-well plate reader (Bio-Rad).

**Induction of MDSCs and functional analysis of tumor-induced MDSCs in vitro**

CD33+ cells were separated from healthy PBMCs using human CD33 MicroBeads (Miltenyi Biotech) according to the manufacturer’s instructions. Isolated CD33+ cells were co-cultured with the NPC cell line TW03 in 24-well plates using a transwell system (0.4 μm pore, Corning) at a ratio of 1:5 for 48 h. CD33+ cells cultured in medium alone were evaluated as a control. A panel of harvested cells was used to analyze MDSC marker expression via FACS, and the remaining cells were subjected to a proliferation assay. In brief, PBMCs from healthy donors were labeled with CFSE (5 μM) and added at different ratios to the induced MDSCs in OKT3-coated 96-well plates. The cells were cultured for 3 d and then analyzed using FACS.

**Semi-quantitative reverse transcription (qRT)-PCR**

Total RNA from cells or from NPC cell lines was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. RT-PCR was performed using the RevertAid First-Strand cDNA Synthesis kit (Thermo Scientific) and Premix Taq™ (TaKaRa Taq™ Version 2.0 plus dye). The primer sequences are provided in Table S3. The PCR products were examined by agarose gel electrophoresis. All experiments were repeated at least five times, and GAPDH mRNA expression was used as a control.

**Immunoblotting**

For immunoblotting, cell lysates were separated by 8% or 10% SDS-PAGE, transferred to PVDF membranes (Millipore), blocked, and incubated in different primary antibodies, including specific antibodies against COX-2 (Abcam), snail (Cell Signaling Technology), vimentin (Cell Signaling Technology), fibronectin (BD Biosciences), E-cadherin (Santa Cruz Biotechnology), β-catenin (Cell Signaling Technology), TGFβ (Cell Signaling Technology), PLCγ2 (R&D Systems), and GAPDH (Santa Cruz Biotechnology). Then, the membranes were incubated with HRP-conjugated secondary antibodies (Santa Cruz). The protein bands were visualized using an ECL detection kit (PerkinElmer Life Science).

**Silencing COX-2 expression in NPC cell lines via RNA interference**

The expression of COX-2 in the TW03 (EBV+) CNE1 and CNE2 NPC cell lines was knocked down via RNA interference (siCOX-2). The sequences of siCOX-2 are as follows: siCOX-2 001 sense strand 5’ GCUGGGGAAGCCUCUCAAA dTdT 3’, anti-sense strand 3’ dTdT CGACCCUUCGGAAGAUU 5’; siCOX-2 003 sense strand 5’ GGACUUAUGGGUAUGUUA dTdT 3’, anti-sense strand 3’ dTdT CCUGAAUACCAUAU 5’. All siRNAs were designed and synthesized by Guangzhou RIBOBIO Company. The double-stranded siRNAs were transiently transfected into NPC cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After transfection for 48 or 72 h, the extent of gene silencing was measured using semi-qRT-PCR; the fold-change in COX-2 protein expression was examined using immunoblotting.

**Migration and invasion assays**

NPC cell migration was determined in tumor cells cultured in the presence or absence of TW03-induced MDSCs for 0–48 h using a pipette tip to “scratch” a line in a confluent monolayer of cells. The widths of 16 random locations along the original scratch line were measured using Photoshop CS4. For migration assays, tumor cells with or without TW03-induced MDSCs were seeded in 24-well plates containing Matrigel-coated inserts (8-μm pore; BD Falcon). After 18–24 h, the cells remaining in the upper chamber were removed, and the cells attached to the lower surface of the insert filter were counted under a microscope (Olympus Corp.) after staining with crystal violet. These experiments included a panel of samples treated with NOHA (100 μmol/mL; Sigma), L-NMMA (100 μmol/mL; Sigma), or...
NAC (1 mmol/mL; Sigma), which induce arginine starvation and inhibition of the generation of NO and ROS, respectively, or with a human TGFβ-neutralizing antibody (10 µg/mL; R&D Systems), and a panel of TW03 and CNE1 cells treated with N\textsuperscript{-}methyl-L-arginine (2 mmol/mL; Sigma), Recombinant Human Transforming Growth Factor-β 1 (RhTGFβ1, 40 ng/mL, ProSpec-Tany TechnoGene Ltd.) or a transwell insert.

Tumor formation and lung metastasis in an animal model

Female 4–5 week old nude mice were purchased from the Shanghai Slac Laboratory Animal Co., Ltd. and maintained in microisolator cages at the Animal Experiment Center of Sun-Yat-Sen University. All procedures were approved by the Animal Care and Use Committee of Sun-Yat-Sen University and conformed to the legal mandates and national guidelines for the care and maintenance of laboratory animals. For tumor formation analysis, CNE2 cells with or without MDSCs were suspended in 200 mL of PBS and inoculated subcutaneously into the left flanks of nude mice. The mice were monitored daily for palpable tumor formation, and the tumors were measured every 3 d using a vernier caliper. The mice were sacrificed after 2 weeks, and the tumors were dissected, weighed and photographed; tumor volumes were calculated using the formula: length × width\textsuperscript{2} × 0.5. For lung metastasis analysis, 1 × 10\textsuperscript{6} CNE2 cells (low metastatic activity) in 100 µL of PBS were intravenously injected into the tail veins of nude mice with or without TW03-induced MDSCs (1 × 10\textsuperscript{6}). Then, the lungs were removed for pathological examination. Transverse sections (5 mm) of the entire lungs were prepared and stained with H&E for histological assessment. Metastatic nodules were counted by the naked eyes of two observers.

Statistical analysis

All in vitro experiments were performed in triplicate and were repeated at least three times. Representative experiments are shown in the figures. All data analysis was performed using SPSS 13.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 5. The numerical data are presented as means ± standard error of the mean (SEM). Pearson’s chi-squared test was used to analyze the correlation between immunohistochemical variants, frequency of circulating or tumor-infiltrating MDSCs and the patients’ clinicopathological parameters. The relationships among the expression of COX-2, serum IL-6 levels and the frequencies of circulating or tumor-infiltrating MDSCs were assessed using Pearson’s correlation coefficient and linear regression analyses. The frequencies of circulating or tumor-infiltrating MDSCs and expression levels of COX-2 in NPC tissues in relation to the patients’ clinical prognosis were determined using the Kaplan-Meier method and log-rank survival analysis. All cut-off values were obtained using X-tile (Version 3.6.1, Yale University, New Haven, CT). A two-tailed P-value < 0.05 was considered statistically significant in this study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by grants from the National Key Basic Research Program of China (2014CB745203), the National Natural Science Foundation (Grant Nos. 81572442 and 81172164 to Li J and 81472386 and 81272340 to Qian CN), the Key Sci-Tech Program of the Guangzhou City Science Foundation (Grant No. 20111Y10036 to Li J), and the National High Technology Research and Development Program of China (863 Program) (No. 2012AA02A501 to Qian CN).

Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.

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