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Differential Response of Myeloid-Derived Suppressor Cells to the Nonsteroidal Anti-Inflammatory Agent Indomethacin in Tumor-Associated and Tumor-Free Microenvironments

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Myeloid-derived suppressor cells (MDSCs) are key regulatory cells that control inflammation and promote tumor-immune escape. To date, no specific immunomodulatory drug has proven efficacy in targeting the expansion and/or function of these cells in different pathophysiologic settings. In this study, we identified a context-dependent effect of the nonsteroidal anti-inflammatory drug indomethacin (IND) on MDSCs, depending on whether they were derived from tumor microenvironments (TME) or from tumor-free microenvironments (TFME). Treatment of mice bearing the LP07 lung adenocarcinoma with IND inhibited the suppressive activity of splenic MDSCs, which restrained tumor growth through mechanisms involving $CD8^+$ T cells. The same effect was observed when MDSCs were treated with IND and conditioned media from LP07 tumor cells in vitro. However, in the absence of a tumor context, IND enhanced the intrinsic suppressive function of MDSCs and amplified their protumoral activity. In a model of autoimmune neuroinflammation, IND-treated MDSCs differentiated in TFME attenuated inflammation, whereas IND-treated MDSCs differentiated in TME aggravated clinical symptoms and delayed resolution of the disease. Mechanistically, IND reduced arginase activity as well as NO and reactive oxygen species production in MDSCs differentiated in TME but not in TFME. Moreover, expression of the C/EBP-β transcription factor isoforms correlated with the suppressive activity of IND-treated MDSCs. Our study unveils the dual and context-dependent action of IND, a drug that serves both as an anti-inflammatory and anticancer agent, which differentially affects MDSC activity whether these cells are derived from TME or TFME. These results have broad clinical implication in cancer, chronic inflammation and autoimmunity. ***The Journal of Immunology*****, 2015, 194: 000–000.**

The intimate connections between inflammation and cancer have been documented extensively (1). Chronic inflammation can promote tumor growth and metastasis through multiple mechanisms, including the induction of a particular population of regulatory cells called myeloid-derived suppressor cells (MDSCs) (2). MDSCs are composed of a heterogeneous population of immature macrophages, granulocytes, and dendritic cells (DCs) as well as other myeloid cells at early stages of differentiation (2). In mice, MDSCs are typically characterized by the expression of the myeloid differentiation Ags Gr1 and CD11b (3). In healthy individuals, immature myeloid cells rapidly differentiate into mature granulocytes, macrophages, or DCs. In pathological conditions including sepsis, trauma, and autoimmune diseases, an aberrant expansion of MDSCs may occur to promote the resolution of chronic inflammation and to avoid the deleterious effects of a persistent immune response (4, 5). However, in cancer the abnormal expansion of MDSCs may contribute to thwart antitumor responses, leading to tumor-immune escape (3, 6, 7). Moreover, these cells also contribute to tumor aggressiveness by increasing angiogenesis and tumor cell motility (4, 8). In tumor-bearing mice and cancer patients, MDSCs accumulate in lymphoid organs, infiltrate the tumor, and circulate within the peripheral blood and tissues (9–11). The release of soluble factors by the tumor microenvironment (TME) may influence the state of MDSCs activation (12), which, in turn can upregulate the expression of effector mechanisms including arginase (ARG)1 (9), reactive oxygen species (ROS) (13, 14), NO (15), and regulatory cytokines (16, 17).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used to treat inflammatory conditions primarily because of their ability to interfere with cyclooxygenase (COX)-1 and -2 activity (18, 19). However, NSAIDs also display anticancer activities through mechanisms that are independent of COX-1 or COX-2 (20–22). Interestingly, PGE_2, a product of COX-2 activity, can stimulate MDSC induction via the EP_2 receptor (21), highlighting a mechanism of COX-2 and PGE_2-induced tumorigenesis. In a metastatic mouse breast cancer model, COX-2 inhibitors have been shown to

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Abbreviations used in this article: ARG, arginase; BM, bone marrow; BM-MDSC, bone marrow myeloid–derived suppressor cell; COX, cyclooxygenase; DC, dendritic cell; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; IND, indomethacin; MDSC, myeloid-derived suppressor cell; NSAID, nonsteroid anti-inflammatory drug; TDLN, tumor-draining lymph node; TFME, tumor-free microenvironment; TME, tumor microenvironment; Treg, regulatory T cell.

reduce the accumulation of MDSCs and to delay tumor growth (21). Moreover, the NSAID indomethacin (IND) suppressed tumor progression and metastasis of the LP07 mouse lung tumor (23), which recapitulates the features of non-small cell lung carcinoma including paraneoplastic syndromes like cachexia, leukocytosis, and hypercalcemia. In this model, we found that IND treatment attenuated chronic inflammation and suppressed tumor progression and metastasis (24).

Given the central role of MDSCs in the regulation of tumor progression and the control of chronic inflammatory disorders, we studied the impact of IND in the MDSC compartment. Particularly, we investigated how the diverse microenvironments prevailing during MDSC differentiation can differentially drive MDSC responses to this agent. Our results provide a rational explanation for the dual anti-inflammatory and anticancer activities of IND in clinical settings.

Materials and Methods

Animals and cell lines

BALB/c female mice (8–12 wk old) from the animal facilities of the Institute of Oncology Ángel H. Roffo (Buenos Aires, Argentina) were used for tumor models. C57BL/6 mice (3 mo old), bred at the Institute of Biology and Experimental Medicine (Buenos Aires, Argentina), were used for induction of experimental autoimmune encephalomyelitis (EAE). All animals studies were conducted in accordance with the highest standards of animal care as outlined in the National Institutes of Health guide for the *Care and Use of Laboratory Animals*. The BALB/c LP07 adenocarcinoma cell line was grown as described previously (23). The B16 mouse melanoma cell line was grown in DMEM supplemented with 10% FBS and antibiotics/antimycotics. The 4T1 mouse mammary carcinoma cell line was grown in RPMI 1640 medium supplemented with 10% FBS and antibiotics/antimycotics. GM-CSF was obtained from supernatants of J588L cells (GM-CSF–transfected myeloma cells) grown in RPMI 1640 medium supplemented with 10% FBS and antibiotics/antimycotics. The LP07, B16, and J588L cells were grown without FBS overnight, and supernatants were centrifuged 10 min at 300 $\times$ *g* to obtain conditioned media. All culture media, FBS, and antibiotics/antimycotics were from Life Technologies. The 4T1 and B16 cell lines were acquired from the American Type Culture Collection.

In vivo tumor model

BALB/c mice were inoculated s.c. in the left flank with LP07 cells (3×10^5 cells) in serum-free RPMI 1640 medium. IND (stock solution: 0.05 g/10 ml ethanol) (Sigma-Aldrich) was given orally through drinking water (1 ml stock solution in 500 ml water) from the day of tumor inoculation up to the day of sacrifice. The final concentration reached in plasma was 10 μM as described previously (22). Tumor volume was calculated as $X^2 \times L/2$, where X = width and L = length. Mice were sacrificed on days 10, 20, or 33 (when tumors reached a volume of 1000 mm^3).

Analysis and sorting of MDSCs

Tumor, spleen, and tumor-draining lymph nodes (TDLN) were collected from LP07 tumor-bearing mice and from spleens and lymph nodes from mice with EAE. Cell suspensions from the abovementioned tissues were prepared mechanically and RBCs were lysed with red blood lysis buffer (Sigma-Aldrich). Cells were stained for 30 min at 4°C with allophycocyanin-conjugated anti-CD11b, PE-conjugated anti-Gr1 and FITC-conjugated anti-CD11c or PerCP-Cy5.5–conjugated anti-F4/80 mAb or with appropriate isotype control Ab (all from eBioscience) and analyzed on a FACSAria (BD Biosciences) using a FlowJo software (Tree Star). For sorting, cell suspensions from spleens of tumor-bearing mice were stained for CD11b and Gr1, yielding a purity higher than 95%.

Analysis of the regulatory T cell and CD8 T cell compartments

Tumor and TDLN were collected from tumor-bearing mice, IND-treated tumor-bearing mice and adoptively transferred tumor-bearing mice. Regulatory T cells (Tregs; $CD4^+CD25^+Foxp3^+$) and effector $CD8^+$ T cells were analyzed by flow cytometry using PE-conjugated anti-CD4, FITC-conjugated anti-CD25, and allophycocyanin-conjugated anti-Foxp3 Ab for Tregs and PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 Ab for CD8 T cells. All Ab were from eBioscience.

ARG activity

Spleen, tumors, and lungs were minced in PBS buffer. Cell suspensions from collected tissues as well as sorted splenic MDSCs were lysed with radioimmunoprecipitation–EDTA buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA). ARG activity was determined as described previously (25).

Delayed-type hypersensitivity

Formalized 5×10^5 LP07 cells were injected s.c. into the footpad of tumor-bearing BALB/c mice 14, 22, and 35 d after tumor inoculation. Saline injected in the contralateral footpad was used as a control. Before injection and 24 h later, the footpad diameter (swelling) was measured with a pneumatic caliper

Bone marrow–derived MDSCs

Progenitor hematopoietic cells were obtained from the femur bone marrow (BM) of female BALB/c mice (6–8 wk old). Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-ME (Life Technologies), 1 mM HEPES (Life Technologies), and antibiotics/antimycotics and exposed to 1) tumor-free microenvironment (TFME): 10% GM-CSF (BM-MDSC); 2) TFME in the presence of IND: 10% GM-CSF + 10 μM IND ($BM\text{-}MDSC_{IND}$); 3) TME: 10% GM-CSF + 10% LP07 conditioned medium ($BM\text{-}MDSC_{TME}$); and 4) TME in the presence of IND: 10% GM-CSF + 10% LP07 conditioned medium + 10 μM IND ($BM\text{-}MDSC_{TME+IND}$) for 4 d. Cell purity (analyzed by flow cytometry) was >90%. To assess IND effects on terminally differentiated MDSCs, hematopoietic progenitor cells were cultured under the abovementioned conditions, except that IND was added after the differentiation period (4 d) and cultured for 4 additional days in the absence of GM-CSF or conditioned medium.

Mixed leukocyte reaction

BALB/c splenocytes (5×10^5) were cultured with 1×10^5 irradiated C57BL/6 splenocytes in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-ME, 1 mM HEPES, and antibiotics/antimycotics. Either 1×10^5 splenic MDSCs or BM-derived MDSCs were washed twice with saline and added to cell culture. After 4 d, [^{3}H]thymidine was added, and 18 h later, its incorporation was measured by a scintillation counter (Perkin-Elmer).

Adoptive transfer of MDSCs

BALB/c mice were inoculated with LP07 tumor cells and 3 d later received either 1) 5×10^5 MDSCs isolated from the spleen of 33-d tumor-bearing mice treated or not with IND or 2) with BM-derived MDSCs exposed in vitro to IND and/or LP07 conditioned medium (TME). Prior to inoculation, MDSCs were incubated with 2 μl CFDA-SE (Molecular Probes, Life Technologies) for 15 min at 37°C in PBS and resuspended for 30 min at room temperature in differentiation medium. Tumor growth was monitored as described above. To evaluate the involvement of CD8 T cells in the antitumor effect, anti-CD8 mAb (clone YTS 169.4; 0.2 mg; American Type Culture Collection) or the same concentration of normal mouse IgG was injected at days −1, 1, 8, 15, and 22 relative to tumor inoculation as described previously (26).

In vivo labeling with BrdU

Mice were injected with LP07 cells as described above. At day 25 post-inoculation, a BrdU saline solution (50 mg/kg) was injected i.p., and 30 min or 48 h later, BM, tumor, spleen, and TDLN were collected and stained with PCy7-labeled anti-Gr1, allophycocyanin-labeled anti-CD11b Ab, and FITC-conjugated anti-BrdU Ab (eBioscience) following DNase treatment (Invitrogen).

EAE induction and treatment

For EAE induction, mice were immunized s.c. in the left and right flanks with 150 μg MOG_{35-55} peptide (M-E-V-G-W-Y-R-S-P-F-S-R-V-V-H-L-Y-R-N-G-K) (Biopolymer Laboratory University of California, Los Angeles, CA) emulsified in CFA (Sigma-Aldrich) containing 200 μg *Mycobacterium tuberculosis* (Difco Laboratories). Mice received 200 ng pertussis toxin (List Biological Laboratories) in 0.2 ml PBS by i.p. injection at the time of immunization and 48 h later. Control mice were immunized with CFA, followed by pertussis toxin. Mice were scored daily as follows: 0, no disease; 1, loss of tail tone; 1.5, poor righting ability; 2, hind limb weakness; 3, hind limb paralysis; 4, quadreparesis; and 5, moribund (27). BM-MDSCs (5×10^5 cells/ 300 μl saline) were injected i.p. into mice that reached a clinical score of 1.

Immunoblot analysis

Expression of C/EBP-β isoforms (C/EBP-β, LAP, and LIP) and COX-2 was assessed in total cell extracts by immunoblotting using mouse anti–C/EBP-β

(3 isoform detection; BioLegend), rabbit anti–α-tubulin (Cell Signaling Technology), rabbit anti–COX-2 (Cayman Chemical), and mouse anti-actin (Santa Cruz Biotechnology) Abs. Briefly, total cell extracts were prepared by cell lysis in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 20 mM EDTA, and 1% Nonidet P-40) supplemented with a mixture of protease inhibitors (Sigma-Aldrich). Equal amounts of protein extracts (50 μg) were denatured in Laemmli buffer, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane (GE Healthcare Life Sciences). After saturation of nonspecific binding sites by incubating the blot for 1 h in TBS containing 20 mM Tris-HCl, 5% nonfat dry milk, and 0.1% Tween 20, membranes were probed with the primary Ab, followed by staining with the appropriated secondary HRP-conjugated Ab (Vector Labs) and processed by chemoluminiscence (Millipore).

Determination of NO and ROS

The Griess reagent was used for NO detection in culture supernatants, and dihydroethidium (Calbiochem) was used for ROS determination as described previously (28, 29).

Statistical analysis

Data were analyzed with GraphPad Prism 5.0 (GraphPad Software). Bars represent mean ± SEM. One-way ANOVA, followed by Bonferroni posttest, was conducted to assess for significant differences in MLR assay and MDSC percentages. Two-way ANOVA, followed by Bonferroni posttest, was conducted to assess for significant differences in tumor volume and EAE scoring. A p value < 0.05 was considered statistically significant.

Results

IND prevents tumor-induced MDSC accumulation

The LP07 lung adenocarcinoma secretes GM-CSF, IL-6, IL-1β, parathyroid hormone-related peptide (PTHrP), and PGE_2, which together contribute to generate a systemic inflammatory response in tumor-bearing mice (23, 30). Because these soluble factors can promote the expansion of MDSCs (4, 31) and IND treatment suppresses tumor progression (Supplemental Fig. 1), we first investigated whether growth of LP07 tumors was accompanied by an increased accumulation of MDSCs and whether administration of IND may impact the fate of this regulatory cell population. For this purpose, we excised tumors, spleens, and TDLN at early (day 10), intermediate (day 20), or late (day 33) stages of tumor growth from BALB/c mice treated or not with IND. We found a considerable increase in the percentage of MDSCs in the spleen and TDLN of tumor-bearing mice compared with normal mice at day 33 posttumor inoculation (Fig 1A, 1B). Notably, IND treatment of tumor-bearing mice prevented the accumulation of MDSCs in the spleen, TDLN (Fig. 1A, 1B), and tumor parenchyma (Fig. 1C). Moreover, IND administration not only reduced the percentage of MDSCs at late stages of tumor development but also favored a shift in the composition of this myeloid cell population in the

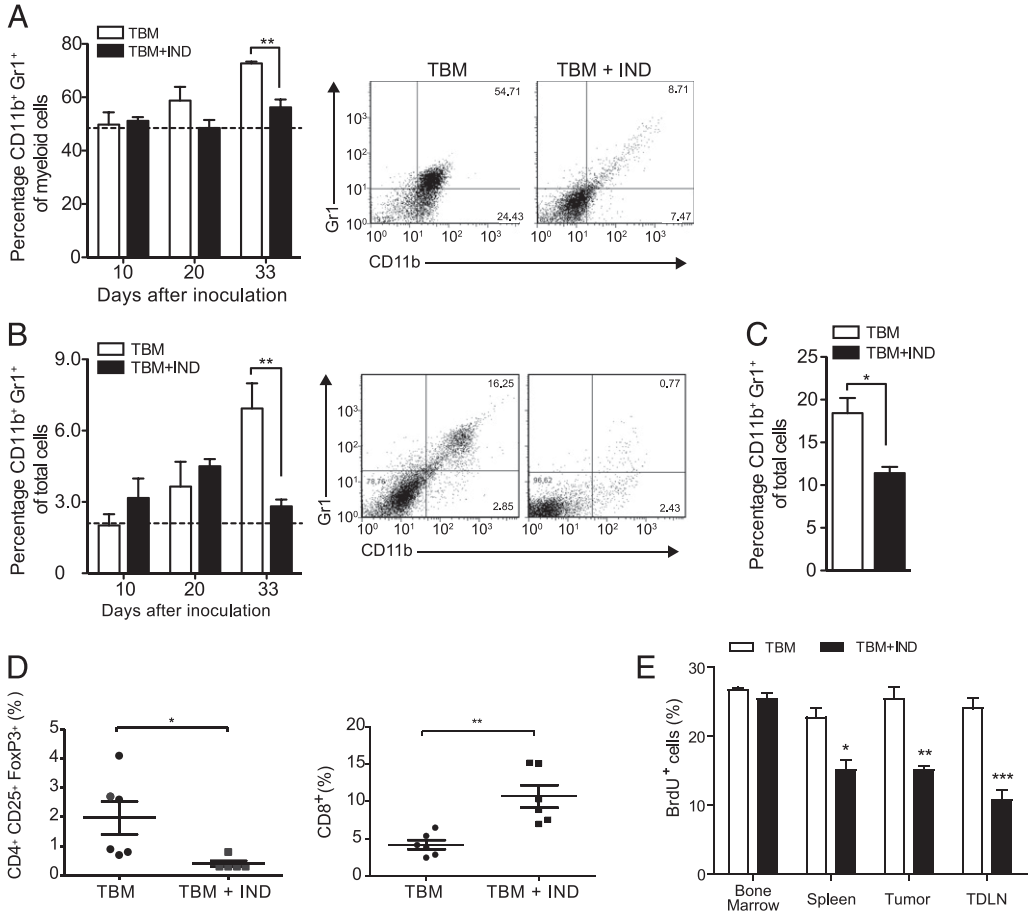

FIGURE 1. IND prevents tumor-induced MDSC accumulation. The number of MDSCs was evaluated by analyzing Gr1 and CD11b expression in splenocytes (n = 12) (**A**) and TDLN (n = 12) (**B**) purified from mice bearing LP07 tumors treated or not with IND sacrificed at days 10, 20, and 33 postinoculation (splenic MDSCs: day 33 tumor-bearing mice [TBM] versus control $^{**}p < 0.01$; TDLN MDSCs: day 33 TBM versus control $^{**}p < 0.01$). Splenocytes and TDLN from tumor-free mice were used as controls (n = 11). (**C**) Tumors from day 33 isolated from TBM or from TBM treated with IND were evaluated for MDSC content (n = 12); $^{*}p < 0.05$. Control values are represented by a horizontal dotted line. (**D**) The number of Tregs and CD8 T cells was evaluated in TDLN from tumors obtained on day 33 from TBM or IND-treated TBM (n = 6); $^{*}p < 0.05$, $^{**}p < 0.01$. (**E**) $Gr1^+CD11b^+BrdU^+$ cells were tested in the bone marrow (BM), spleen, TDLN, and tumors from TBM or from IND-treated TBM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (A) and (C) show percentage of $CD11b^+Gr1^+$ cells from the myeloid cell gate, and (B) and (D) show percentage from total TDLN cells. (E) shows $BrdU^+$ cells from $CD11b^+$ $Gr1^+$ cells. Results represent the mean ± SEM (A and B, *left panels*; C and E) or are representative (A and B, *right panels*) of four experiments.

spleen toward a $Gr1^{high}$ phenotype (Fig. 1A). In addition, IND treatment reduced the frequency of $CD4^+CD25^+Foxp3^+$ Tregs and augmented the population of $CD8^+$ T cells in TDLN (Fig. 1D). Thus, in tumor-bearing mice, IND may counteract tumor-induced immunosuppression by targeting MDSCs and shifting the Treg/T effector cell balance. To determine whether IND treatment reduced MDSC number through impaired recruitment from the BM, we injected BrdU in tumor-bearing mice and IND-treated tumor-bearing mice at day 20 posttumor inoculation. Thirty minutes or 48 h after BrdU injection, we analyzed the number of $BrdU^+$ MDSCs in the BM, spleen, and tumors of both experimental groups. We found that IND treatment prevented MDSC accumulation in the spleen, tumor, and TDLN, suggesting that this drug may act by inhibiting the recruitment of MDSCs from the BM compartment. This effect was confirmed by the lack of differences in $BrdU^+$ MDSCs in BM of IND-treated versus untreated tumor-bearing mice (Fig. 1E).

IND alleviates tumor-induced immunosuppression

MDSCs control effector T cell responses, at least in part, through induction of ARG activity (32). We determined the activity of ARG in the tumor, spleen, and lungs of tumor-bearing mice treated or not with IND. ARG activity increased in response to tumor growth in tumor tissue (Fig. 2A), spleen (Fig. 2B), and lungs (the metastasis target organ) (Fig. 2C). The rise in ARG activity was prevented by IND treatment (Fig. 2A–C). Likewise, splenic MDSCs purified from IND-treated tumor-bearing mice showed lower ARG activity, compared with MDSCs sorted from untreated tumor-bearing mice (Fig. 2D). To evaluate whether the increased MDSC number and the augmented ARG activity reflected a state of general immunosuppression (33, 34), we evaluated tumor-specific T cell responses in a delayed-type hypersensitivity (DTH) reaction. Although mice bearing advanced tumors were unable to respond to formalized LP07 cells, IND treatment allowed the development of a tumor-specific response even at late stages of tumor progression (Fig. 2E). Thus, IND treatment blunts ARG activity during tumor growth and counteracts tumor-induced systemic immunosuppression.

IND treatment mitigates the suppressive capacity of MDSCs from tumor-bearing mice

To investigate whether accumulation of MDSCs contributes to systemic immunosuppression, we examined the capacity of splenic MDSCs from tumor-bearing mice to inhibit T cell proliferation in MLR assays. MDSCs isolated from tumor-bearing mice inhibited allogeneic T cell proliferation (Fig. 3A). This suppressive activity was mitigated in MDSCs obtained from IND-treated tumor-bearing mice (Fig. 3A). To study further the effect of IND treatment on the in vivo suppressive potential of splenic MDSCs, we performed an adoptive transfer experiment using MDSCs from IND-treated or untreated tumor-bearing mice as donors and evaluated their effect on recipient mice bearing the LP07 tumor. Notably, only MDSCs isolated from IND-treated tumor-bearing mice restrained growth of LP07 tumors when adoptively transferred to tumor-bearing recipient mice (Fig. 3B). This effect was mediated by CD8 T cells as administration of an anti-CD8 mAb prevented the antitumor response induced by MDSCs from IND-treated tumor-bearing mice (Fig. 3C). Notably, this antitumor effect was also observed in the 4T1 mouse mammary carcinoma model (Supplemental Fig. 2). Moreover, exposure of splenic MDSCs purified from tumor-bearing mice to IND ex vivo counteracted their immunosuppressive capacity (Fig 3D). Thus, IND treatment attenuates the suppressive potential of MDSCs and negatively regulates their tumor-promoting activity.

Opposite effects of IND on MDSCs exposed to TME or TFME

Because MDSCs participate in a wide range of inflammatory responses to restore immune cell homeostasis, we next investigated the effect of IND on the suppressive capacity of MDSCs exposed to TFME. BM progenitors from normal mice were differentiated in vitro with GM-CSF as previously described (35) in the absence or presence of IND, and the resultant population was used for MLR and adoptive transfer experiments. Assessment of the purity of the BM-MDSC preparation revealed no CD11c expression and only low reactivity with the anti-F4/80 Ab (Supplemental Fig. 3), as previously described for the heterogeneous MDSC population (36). Surprisingly, when tumor-free BM-MDSCs were differentiated in vitro in the presence of IND, its suppressive capacity was greater compared with cells differentiated with GM-CSF alone (Fig. 4A). However, when we differentiated BM-MDSCs in the presence of conditioned medium from LP07 cells, mimicking a TME, we found a robust suppressive capacity of these cells, which was abolished when IND was incorporated to the culture medium (Fig. 4B). Interestingly, when we analyzed Gr1 expression, we found that

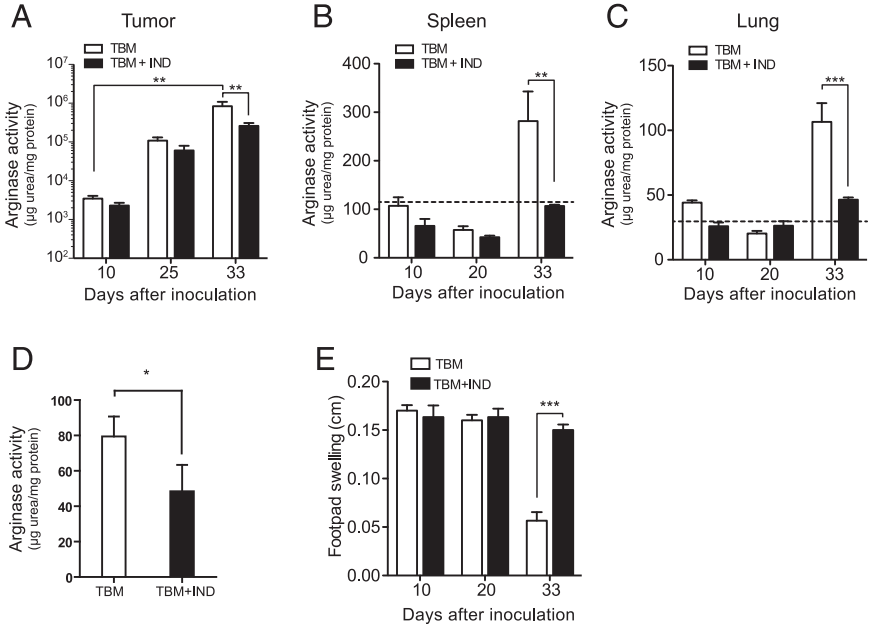

FIGURE 2. IND alleviates tumor-induced immunosuppression. ARG activity was evaluated by measuring urea production in tumors (**A**), spleen (**B**), and lungs (**C**) collected at days 10, 20, and 33 from LP07 tumor-bearing mice (TBM) or IND-treated TBM ($n = 10$). Spleen and lungs from normal mice were used as controls ($n = 8$). Control values are represented by a horizontal dotted line. (A–C) $^{**}p < 0.01$, $^{***}p < 0.001$. (**D**) ARG activity of sorted splenic MDSCs from TBM or IND-treated TBM. $^{*}p < 0.05$. (**E**) DTH in TBM or IND-treated TBM. Tumor cells (LP07; 3×10^5) were injected s.c. to syngeneic mice, and IND was given orally beginning at the day of tumor inoculation. At the indicated time points, LP07 cells or saline were injected s.c. into the footpad, and swelling was measured before inoculation and 24 h later. Data are expressed as diameter (T24h–T0). $^{***}p < 0.001$. (A–E) Results represent the mean ± SEM of three experiments.

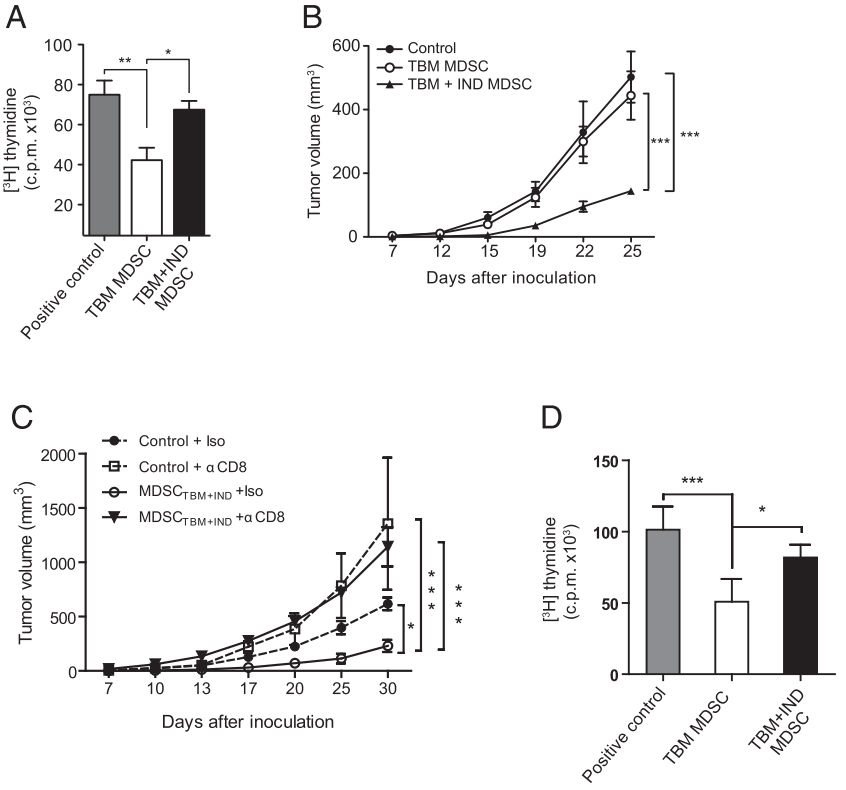

FIGURE 3. IND treatment mitigates the suppressive capacity of MDSCs from tumor-bearing mice. (**A**) MDSCs sorted at day 33 from spleen of LP07 tumor-bearing mice (TBM) or IND-treated TBM ($n = 9$) were cocultured with normal BALB/c splenocytes in the presence of irradiated C57BL/6 splenocytes as an allogeneic stimulus (ratio: 1:5:1; MDSCs: BALB/c splenocytes: C57BL/6 splenocytes). $*p < 0.05$, $**p < 0.01$. Positive control includes responder splenocytes in the presence of allogeneic splenocytes. Negative controls include responder splenocytes only, allogeneic splenocytes only, and MDSCs only, which showed no changes in proliferation (data not shown). (**B**) Tumor growth of mice injected i.p. with MDSCs isolated from TBM or IND-treated TBM ($n = 8$) ($***p < 0.001$). (**C**) Splenic MDSCs sorted from TBM or IND-treated TBM were adoptively transferred into recipient TBM receiving the anti-CD8 or control isotype Ab as indicated in *Materials and Methods*. $*p < 0.05$, $***p < 0.001$. (**D**) MLR of sorted splenic MDSCs from TBM treated or not ex vivo with IND. $*p < 0.05$, $***p < 0.001$. Data are the mean ± SEM (A and D) or are representative (B and C) of three experiments.

TME increased the $Gr1^{low}$ population, whereas the combination of TME and IND increased the $Gr1^{high}$ phenotype (Fig. 4C).

Adoptive transfer of either BM-MDSCs differentiated in TME alone or BM-MDSCs differentiated in TFME in the presence of IND had no effect on LP07 tumor growth with tumors growing progressively as the control group (LP07 alone) (Fig. 4D). However, adoptive transfer of MDSCs differentiated in TME in the presence of IND inhibited LP07 tumor growth in recipient mice (Fig. 4D). These mice showed an increased frequency of $Gr1^{high}$ MDSCs in the spleen as well as reduced number of MDSCs (Fig. 4E) and higher percentage of CD8 T cells (Fig. 4F) in TDLN. When mice were transferred with MDSCs previously exposed to a TFME, tumor growth was not statistically different compared with controls but was significantly lower compared with tumor growth observed in mice injected with MDSCs differentiated in the presence of a TFME and IND (Fig. 4D). These data further substantiate the paradoxical action of IND on MDSCs whether they were derived from tumor-associated or from tumor-free settings. Transferred MDSCs were labeled with the CFDA-SE dye, which allowed their identification in the spleen, TDLN, and tumor parenchyma. We found that MDSCs differentiated in a TME and treated with IND maintained an increased expression of Gr1 following adoptive transfer, as shown by analysis of $CFDA\text{-}SE^{+}$ cells in the spleen of recipient mice (Fig. 4G). Notably, long-term exposure of differentiated MDSCs to IND (4 d) induced, although at a lesser extent, a similar paradoxical effect depending on whether cells were exposed to a TME or a TFME (Fig. 4H). Furthermore, when we evaluated the immunoregulatory profile of $Gr1^{high}$ and $Gr1^{low}$ populations following IND treatment, we found that $Gr1^{low}$ MDSCs were more sensitive to IND treatment than $Gr1^{high}$ MDSCs when generated in a TMFE, although the opposite effect was observed when MDSCs were generated in a TME (Fig. 4I). Thus, IND differentially controls the phenotype and suppressive activity of MDSCs depending on whether they are exposed to TME or TFME.

IND-treated MDSCs derived from TME or TFME differentially influence the course of autoimmune neuroinflammation

The ability of IND to enhance the suppressive potential of MDSCs when exposed to a TFME prompted us to investigate the consequences of this effect in EAE, a mouse model of CNS inflammation that recapitulates some of the features of multiple sclerosis (27). Transfer of mice with MDSCs differentiated in a TME considerably decreased the clinical severity of the disease, whereas IND-treated MDSCs differentiated in a TME aggravated the inflammatory disease and prevented its resolution (Fig. 5A). Notably, injection of MDSCs differentiated in a TFME alone delayed the onset of EAE, but once the maximal clinical score was reached, resolution was comparable to the control group. On the contrary, transfer of IND-treated MDSCs differentiated in a TFME suppressed EAE (Fig. 5A).

Injection of IND-treated MDSCs differentiated in a TME led to a substantial decline in total MDSCs in the spleen and TDLN of EAE mice (Fig. 5B, 5C), supporting the immunosuppressive potential of these cells. Interestingly, the low proportion of splenic MDSCs occurring in mice receiving IND-treated $MDSC_{TME}$ displayed a prominent $Gr1^{high}$ phenotype (Fig. 5B). Thus, MDSCs exposed to a TFME are endowed with greater immunosuppressive potential when treated with IND, as reflected by their ability to attenuate CNS inflammation. However, MDSCs differentiated in a TME show the opposite behavior upon IND treatment, as evidenced by aggravation of EAE symptoms.

IND alters suppressive pathways in MDSCs

To further characterize the molecular events triggered by IND treatment on MDSCs from TME or TFME, we assessed the expression of C/EBP-β, a transcription factor associated with MDSC suppressive capacity (35). Because C/EBP-β occurs in three different isoforms (C/EBP-β, LAP, and LIP), we sought to examine whether IND could act by altering their expression. We found a positive correlation between C/EBP-β expression and the suppressive activity of MDSCs. In

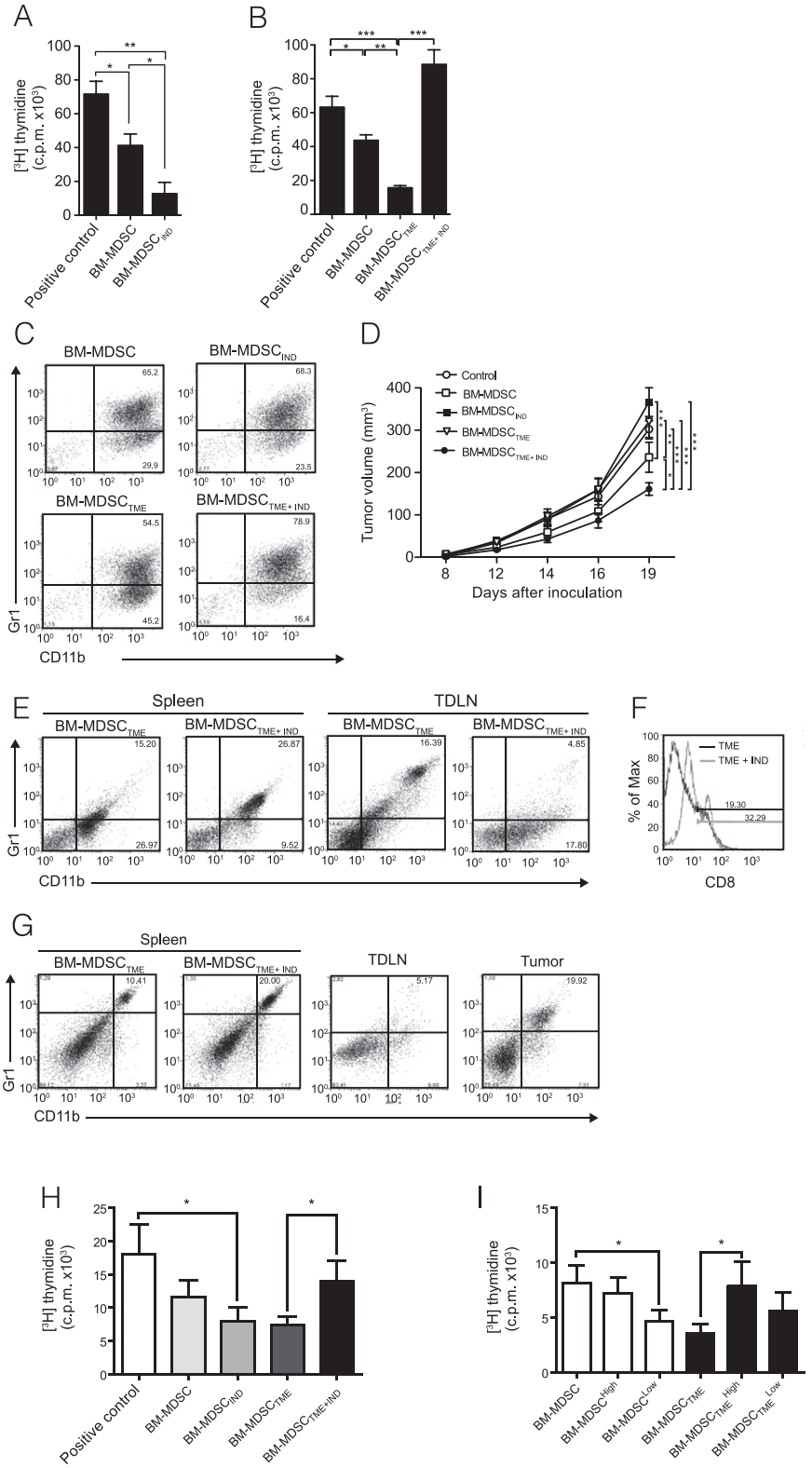

FIGURE 4. Opposite effects of IND on MDSCs exposed to TME or TFME. (**A** and **B**) Bone marrow progenitors were differentiated in vitro with GM-CSF and treated (BM-MDSC$_{IND}$) or not (BM-MDSC) with IND (A) or GM-CSF and exposed to tumor-conditioned medium in the presence (BM-MDSC$_{TME + IND}$) or absence (BM-MDSC$_{TME}$) of IND [(B) $n = 9$]. The resultant BM-MDSC populations were cocultured with normal BALB/c splenocytes in the presence of an allogeneic stimulus (ratio: 1:5:1; MDSCs: BALB/c splenocytes: C57BL/6 splenocytes). T cell proliferation was assessed by [^{3}H]thymidine incorporation. BALB/c splenocytes cultured in the presence of allogeneic stimulus were used as positive control. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (**C**) Flow cytometry of BM-MDSCs following differentiation in vitro. Gr1^{high} and Gr1^{low} quadrants are shown within the myeloid cell gate. (**D**) Tumor growth in adoptively transferred mice. Untreated BM-MDSCs cultured in a TFME (BM-MDSC), BM-MDSCs cultured in a TFME in the presence of IND (BM-MDSC$_{IND}$), untreated BM-MDSCs cultured in a TME (BM-MDSC$_{TME}$), or BM-MDSCs cultured in a TME in the presence of IND (BM-MDSC$_{TME + IND}$) were transferred on day 3 to LP07 tumor-bearing mice (TBM) ($n = 8$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Tumor-bearing mice not receiving MDSCs were used as controls ($n = 8$). (**E**) The percentage of MDSCs in the spleen and TDLN of recipient TBM. Spleen: Gr1$^+$CD11b$^+$ cells within the myeloid cell gate are shown. TDLN: Gr1$^+$ CD11b$^+$ cells from total TDLN cells are shown. (**F**) CD8 T cells in TDLN from recipient mice. CD8 T cells within the total lymphocyte gate are shown. (**G**) CD11b$^+$ Gr1$^+$ cells from the CFDA-SE$^+$ gate are shown. MDSCs were stained with CFDA-SE and transferred i.p. into mice bearing LP07 tumors (day 3). Forty-eight hours later, spleen, TDLN, and tumors were collected and examined for MDSCs by analyzing Gr1, CD11b, and CFDA-SE by flow cytometry. (**H**) Fully differentiated BM-MDSCs were treated for 4 d with IND, and MLR was performed as described above. $*p < 0.05$. (**I**) Sorted Gr1^{high} and Gr1^{low} populations from BM-MDSC and BM-MDSC$_{TME}$ were treated ex vivo with IND, and their function was analyzed in MLR assays. $*p < 0.05$. Data are the mean ± SEM (A, B, H, and I) or are representative (C, D, E, F, and G) of three experiments.

fact, MDSCs differentiated in a TFME in the presence of IND as well as MDSCs differentiated in a TME alone exhibited higher expression of C/EBP-β, LAP and LIP, compared with MDSCs differentiated in a TFME alone. Remarkably, MDSCs differentiated in a TME in the presence of IND showed a dramatic downregulation of the three C/EBP-β isoforms (Fig. 6A). Furthermore, IND treatment lowered ARG activity in MDSCs exposed to a TME but not in those differentiated in a TFME (Fig. 6B). In addition, MDSCs exposed to a TME exhibited lower NO and ROS production in response to IND, whereas in a TFME, IND increased the synthesis of these reactive molecules in MDSCs (Fig. 6C, 6D). However, we could find no differences in COX-2 expression among the different experimental groups analyzed (Fig. 6E). Thus, the differential effects of IND on MDSCs isolated from TME or TFME involve modulation of critical effector molecules responsible for the regulatory activity of these cells.

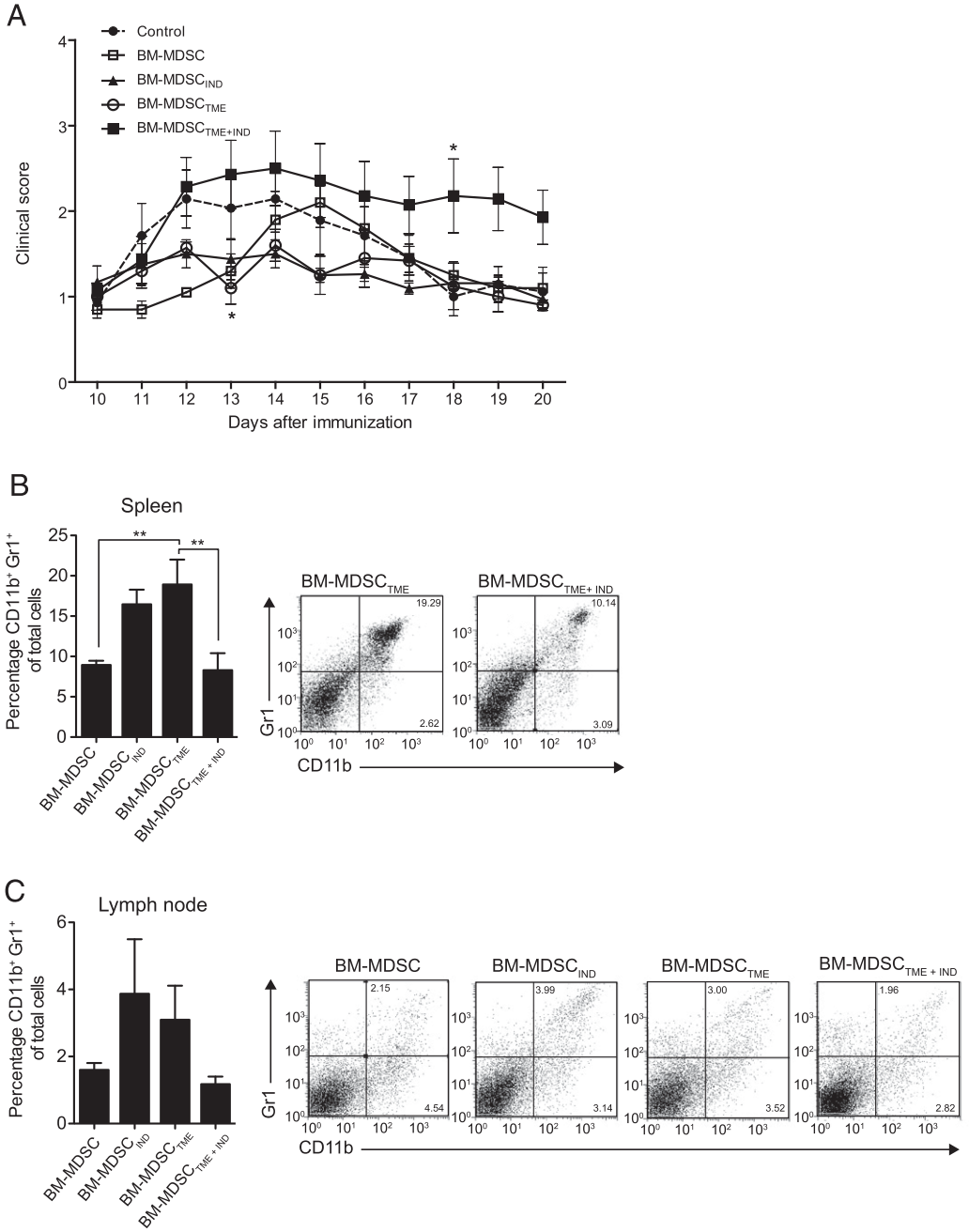

FIGURE 5. IND-treated MDSCs derived from TME or TFME differentially influence the course of autoimmune neuroinflammation. (**A**) Clinical scores of C57BL/6 mice (n = 13) injected at day 10 with BM-MDSCs cultured in a TFME in the absence (BM-MDSC) or presence (BM-MDSC$_{IND}$) of IND or injected with BM-MDSCs cultured in a TME in the absence (BM-MDSC$_{TME}$) or presence (BM-MDSC$_{TME + IND}$) of IND (*$p < 0.05$ control versus BM-MDSC$_{TME+IND}$; *$p < 0.05$ BM-MDSC versus BM-MDSC$_{TME + IND}$; *$p < 0.05$ BM-MDSC$_{IND}$ versus BM-MDSC$_{TME + IND}$; *$p < 0.05$ BM-MDSC$_{TME}$ versus BM-MDSC$_{TME + IND}$). Mice immunized with MOG$_{35–55}$ but not injected with MDSCs were used as controls (n = 11). Spleen (**B**) and inguinal LN (**C**) were collected at day 20 after MDSC injection and the percentage of MDSCs was determined by flow cytometry. Spleen: MDSCs from total myeloid cell gate are shown; TDLN: MDSCs within total TDLN cells are shown. **$p < 0.01$. Data represent the mean ± SEM (A–C, *left panels*) or are representative (B and C, *right panels*) of three experiments.

Discussion

Although the use of NSAIDs for the treatment of inflammatory diseases is widely accepted, they have recently emerged as alternative promising adjuvant therapies for neoplastic processes (20). However, the mechanisms of action of these drugs in the tumor microenvironment and their activity on regulatory immune cell populations, is largely unknown. MDSCs comprise a heterogeneous cell population which contributes to foster immunosuppressive circuits in cancer, autoimmune inflammation and infection (2). Hence, targeting MDSC function represents a major challenge for immunologists. The present study shows that IND can differentially influence the regulatory activity of MDSCs depending on whether these cells are derived from TME or TFME. Briefly, IND treatment enhanced the suppressive capacity of MDSCs when these cells were derived from a TFME; this effect is in agreement with the well-known anti-inflammatory action of this drug. Conversely, when applied to tumor-bearing hosts or added to MDSCs cultured in a TME, IND instructed MDSCs to differentiate into a cell population with reduced suppressive activity, which favored the development of a more efficient antitumor response. This

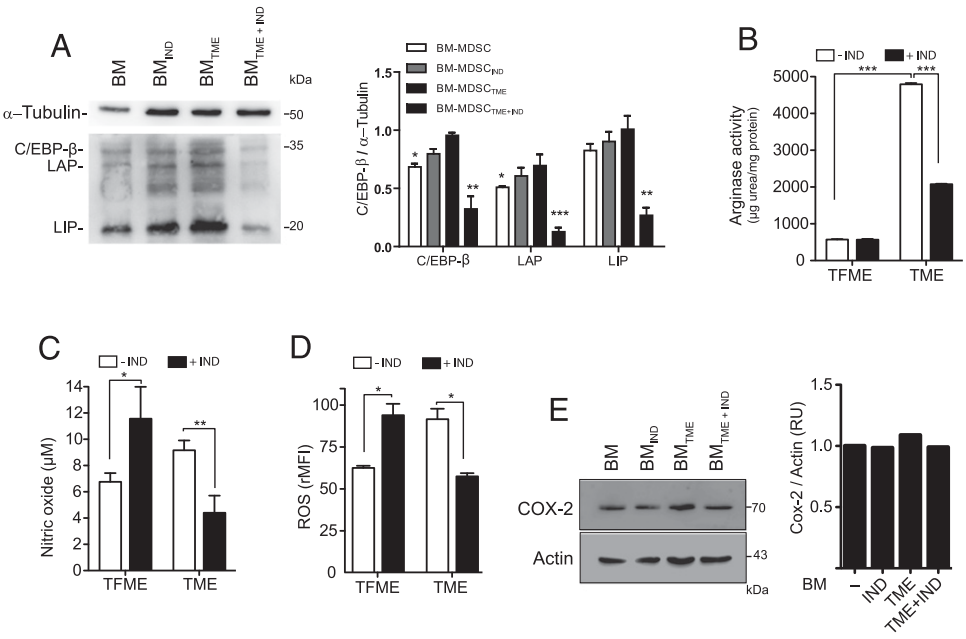

FIGURE 6. IND alters suppressive pathways in MDSCs. (**A**) Immunoblot analysis of C/EBP-β isoforms (C/EBP-β, LAP, and LIP) (n = 6) in BM-MDSCs cultured in a TFME in the absence (BM-MDSC) or presence (BM-MDSC$_{IND}$) of IND or cultured in a TME in the absence (BM-MDSC$_{TME}$) or presence (BM-MDSC$_{TME}$ + IND) of IND. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (**B**) ARG activity of BM-MDSCs exposed to TME or TFME and treated or not with IND (n = 6). ***$p < 0.001$. (**C**) NO content of BM-MDSCs exposed to TME or TFME and treated or not with IND (n = 6). *$p < 0.05$, **$p <$ 0.01. (**D**) ROS production by BM-MDSCs exposed to TME or TFME and treated or not with IND (n = 6); *$p < 0.05$. (**E**) Immunoblot of COX-2 expression of BM-MDSCs exposed to TME (BM$_{TME}$) or TFME (BM) and treated with IND (BM$_{TME + IND}$; BM$_{IND}$) (n = 6). Data represent the mean ± SEM (A and E, *right panels*; B–D) or are representative (A and E, *left panels*) of three experiments.

context-dependent function of IND may explain, at least in part, the dual anti-inflammatory and anticancer activities of some NSAIDs. The relevance of the TME in shaping the myeloid cell compartment has been widely recognized as illustrated by the ability of tumor cells to instruct the polarization of DCs toward a tolerogenic phenotype (37).

Here we identified a novel strategy of pharmacologic modulation of MDSCs, which may contribute to reverse tumor tolerance and to potentiate immunotherapeutic approaches. In this regard, conversion of MDSCs toward a proinflammatory phenotype by IND treatment may boost DC vaccination protocols and cooperate with other immunotherapeutic regimens (1). Furthermore, IND treatment of tumor-bearing hosts may limit MDSC function and stimulate tumor immunity in an Ag-independent manner which avoids studies of the patient´s HLA phenotype, a problem that is frequently faced in DC or peptide vaccination protocols (38). Interestingly, in an experimental model of mouse breast cancer metastasis, IND was able to restore the anti-metastatic effect of adoptively transferred macrophages, thus supporting the effects of this drug on other tumor-associated myeloid cell populations (39). Moreover, preclinical studies have demonstrated the beneficial effects of chemotherapeutic drugs such as gemcitabine and receptor tyrosine kinase inhibitors such as sunitinib in the regulation of the MDSC compartment (40–42). We showed here that administration of IND reversed tumor-induced systemic immunosuppression, as evidenced by an enhanced DTH response associated with a higher frequency of infiltrating CD8 T cells, decreased percentage of Tregs in TDLN and tumor parenchyma, as well as inhibition of MDSC accumulation in the spleen, tumor and TDLN. In this regard, adoptive transfer of MDSCs differentiated in a TME in the presence of IND resulted in increased CD8 T cell responses in TDLN and a considerable decline of the MDSC population. Of note, CD8 T cells were crucial for this antitumor effect, since elimination of this T cell population completely abrogated the beneficial effect of transferred MDSCs. Our results suggest the possibility of shifting the function of MDSCs toward a dominant proinflammatory profile to foster an efficient anti-tumor immune response, instead of eliminating this regulatory cell population in tumor microenvironments. In fact, IND treatment of tumor-bearing mice did not suppress BM cell proliferation, but rather decreased MDSC accumulation in secondary lymphoid organs and tumor tissues and counteracted the suppressive potential of these cells. In this sense, previous studies emphasized the importance of polarization of tumor-associated macrophages toward a proinflammatory M1-type phenotype as a potential anti-tumor strategy (43–45). We demonstrate here that the effects of IND treatment on MDSC function are durable as transfer of MDSCs differentiated in a TME in the presence of IND suppressed tumor growth and facilitated EAE progression for prolonged time periods of up to 25–30 d. Thus, modulation of MDSC phenotype toward a proinflammatory profile in cancer or an anti-inflammatory phenotype in autoimmune diseases might contribute as an adjuvant strategy for already approved clinical therapies. Moreover, as IND can shape the function of fully-differentiated BM-MDSCs and sorted splenic MDSCs, this approach could be used to manipulate MDSCs activity ex vivo.

Interestingly, we found an increased percentage of splenic MDSCs displaying a Gr1^{high} phenotype (even if the total percentage of MDSCs was eventually reduced) in: a) IND-treated tumor-bearing mice; b) tumor-bearing mice transferred with MDSCs differentiated in the presence of a TME and IND and c) EAE mice transferred with MDSCs differentiated in a TME in the presence of IND. Moreover, in vitro MDSCs differentiated in a TME and treated with IND exhibited a similar Gr1^{high} phenotype. These results are in agreement with other studies showing that, within the heterogeneous MDSC population, the Gr1^{high} subset appears to be less suppressive compared with Gr1^{low} MDSCs (12, 46, 47). In this study we found a link between the

$Gr1^{high}$ phenotype and the diminished suppressive activity of MDSCs, as evidenced by reduced tumor growth and exacerbated EAE in adoptively transferred mice.

During autoimmune diseases, MDSCs play a key role in the resolution of inflammation preventing collateral damage induced by aberrant T-cell responses (4, 12). As IND typically serves as an anti-inflammatory agent, we hypothesized that this NSAID may restrict inflammation by increasing MDSC suppressive potential. Adoptive transfer of MDSCs differentiated in the presence of a TFME and IND attenuated the severity of autoimmune disease in the EAE model, suggesting that, in nontumoral conditions IND enhances the MDSC suppressive activity. As MDSCs are precursors of macrophages, DCs and neutrophils, these cells intrinsically possess the genetic information to function as inflammatory leukocytes. However the interplay between arginine metabolism and peroxynitrites skews the balance of the MDSC population toward a regulatory phenotype (5). Interestingly, an increased suppressive activity of MDSCs differentiated in a TFME in the presence of IND correlated with elevated NO and ROS production together with increased expression of the three C/EBP-β isoforms, whereas decreased suppressive activity of IND-treated MDSCs differentiated in a TME was accompanied by downregulation of all studied suppressive pathways (Fig. 7). Surprisingly, no changes were observed in the expression of COX-2 on MDSCs among different experimental groups, suggesting that IND could modulate MDSC activity through COX-2-independent mechanisms (48). To gain a more complete understanding of the relevance of COX-2 in IND effects, future studies are warranted to examine the impact of this drug on MDSC phenotype and function in COX-2–deficient mice. In this regard, inhibition of tumor growth by IND could also involve mechanisms that are independent of tumor COX-2, as previously observed in mice injected with COX-2 knockdown LP07 cells and treated orally with IND; these mice evidenced a more pronounced inhibition of tumor growth than mice receiving COX-2 knockdown LP07 tumor cells alone (A. Blidner, S. Klein, and M. Jasnis, unpublished observations).

The question of whether tumor-derived MDSCs may differ from MDSCs originating during acute and chronic inflammatory responses is still a matter of debate (5). In our study, we provide experimental evidence that MDSCs generated in TME respond completely different to the same pharmacological agent than MDSCs differentiated in tumor-free conditions. Our data identify the plasticity of an immature cell population which can adopt immunostimulatory or inhibitory profiles in response to NSAID treatment, depending on whether they are derived from TME or TFME. These findings highlight the importance of the microenvironment in which a certain cell population resides or differentiates, as it can modify its phenotype to such an extent of dramatically altering its response and sensitivity to a pharmacological agent. Further studies are needed to elucidate the identity of the tumor-derived factors responsible of conferring such plasticity. In this regard, Marigo et al. documented an increased suppressive potential of BM- MDSCs following exposure to IL-6 and GM-CSF (35). Moreover, Ostrand-Rosenberg and colleagues reported an association between IL-1β and the tumor-promoting activity of MDSCs (49–51). Interestingly, both IL-6 and IL-1β

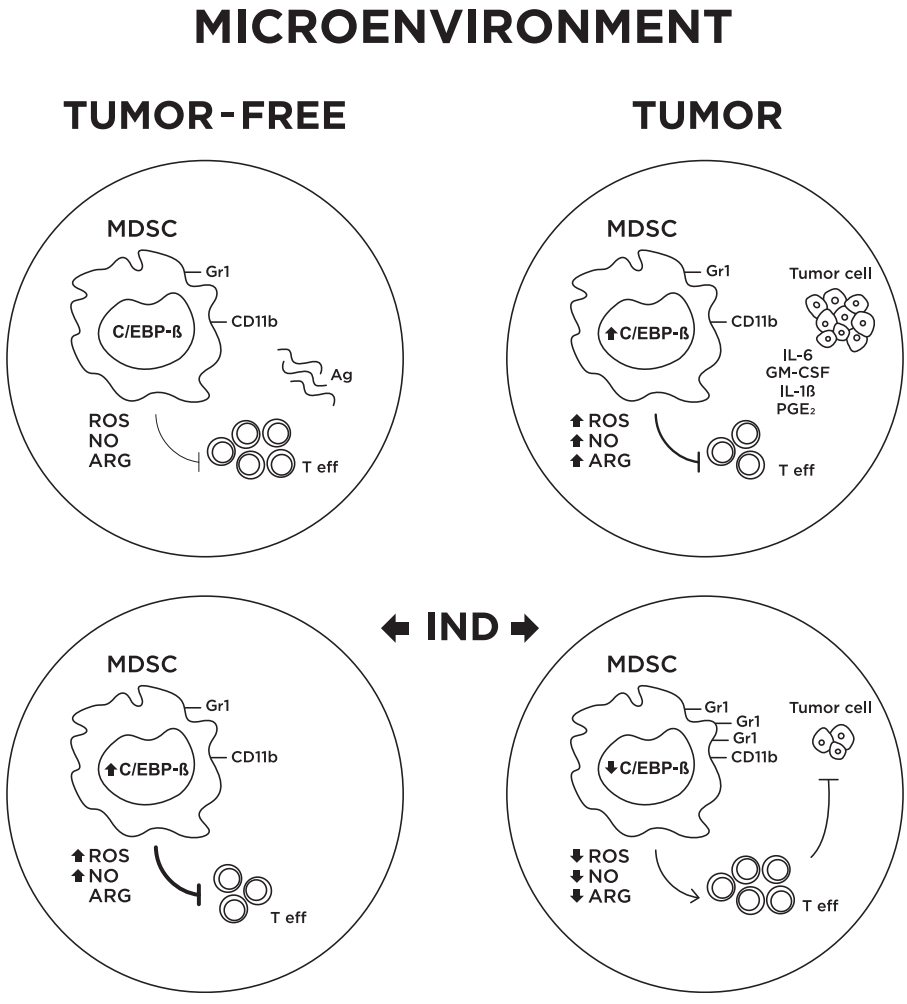

FIGURE 7. Model: differential modulation of MDSCs by IND in tumor-associated or tumor-free microenvironments. In a TFME, MDSCs control T cell responses through mechanisms involving ROS, NO, and ARG, thus facilitating the resolution of inflammation. IND treatment increases C/EBP-β expression, NO synthesis, and ROS production in MDSCs, which contributes to amplify the immunosuppressive capacity of these cells. In a TME, soluble factors produced by tumor cells promote accumulation of MDSCs with high expression of C/EBP-β isoforms, increased NO and ROS production, and higher ARG activity. This inhibitory microenvironment impairs antitumor immunity. Under these conditions, IND counteracts the suppressive pathways used by MDSCs and increases Gr1 expression. This effect contributes to unleash an otherwise repressed antitumor response.

were found to be abundant in LP07 conditioned media (23, 52) and IND treatment decreased their serum levels in tumor-bearing mice (24). Hence, the impact of IND treatment in MDSC recruitment to secondary lymphoid organs and peripheral tissues could reflect changes in cytokines and chemokines differentially produced in inflammatory and tumor microenvironments (23, 24, 52).

The possibility to modulate the function of regulatory cell populations using pharmacological agents offers novel opportunities for treating a wide variety of pathologic conditions, without the need of depleting these cells from the host. Our study identifies a dual role for the NSAID IND in facilitating the resolution of inflammation or suppressing tumor growth through differential modulation of the MDSC population. Moreover, it reveals an unexpected role of the microenvironment in shaping the phenotype of myeloid regulatory cells and imprinting selective responses to pharmacological agents.

Disclosures

The authors have no financial conflicts of interest.

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