Research Article

Cancer Immunology Research

Mast Cells Boost Myeloid-Derived Suppressor Cell Activity and Contribute to the Development of Tumor-Favoring Microenvironment

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Abstract

Inflammation plays crucial roles at different stages of tumor development and may lead to the failure of immune surveillance and immunotherapy. Myeloid-derived suppressor cells (MDSC) are one of the major components of the immune-suppressive network that favors tumor growth, and their interaction with mast cells is emerging as critical for the outcome of the tumor-associated immune response. Herein, we showed the occurrence of cell-to-cell interactions between MDSCs and mast cells in the mucosa of patients with colon carcinoma and in the colon and spleen of tumor-bearing mice. Furthermore, we demonstrated that the CT-26 colon cancer cells induced the accumulation of $CD11b^+Gr1^+$ immature MDSCs and the recruitment of protumoral mast cells at the tumor site. Using *ex vivo* analyses, we showed that mast cells have the ability to increase the suppressive properties of spleen-derived monocytic MDSCs, through a mechanism involving IFNγ and nitric oxide production. In addition, we demonstrated that the CD40:CD40L cross-talk between the two cell populations is responsible for the instauration of a proinflammatory microenvironment and for the increase in the production of mediators that can further support MDSC mobilization and tumor growth. In light of these results, interfering with the MDSC:mast cell axis could be a promising approach to abrogate MDSC-related immune suppression and to improve the antitumor immune response. *Cancer Immunol Res; 3(1); 85–95. ©2014 AACR.*

Introduction

In the tumor microenvironment (TME), cancer progression is often associated with an inflammatory response, which is mediated by numerous cytokines, chemokines, and growth factors produced by cancer and stromal cells. These factors support the tumor-induced immune suppression and constitute an impediment to immunotherapy and immunosurveillance in patients as well as in experimental animals with malignant tumors (1). Among the stromal cells infiltrating the tumor, myeloid-derived suppressor cells (MDSC) are one of the major components of the immunosuppressive network responsible for T-cell nonresponsiveness and for the generation of the immunosuppressive microenvironment that favors tumor growth. MDSCs have a very rapid turnover and accumulate in large numbers in lymphoid tissues of tumor-bearing mice as well as in mice with infectious diseases, sepsis, and trauma. These cell populations have been described in human cancer and have been found circulating in the blood of patients with cancer (reviewed in ref. 2). They are a heterogeneous population of early myeloid progenitors, immature granulocytes, macrophages, and dendritic cells at different stages of differentiation. In mice, these cells are broadly defined as $Gr1^+CD11b^+$ cells (3), and at least two major subsets, the so-called granulocytic polymorphonuclear ($CD11b^+Ly6G^+Ly6C^{low}$) and the highly immunosuppressive monocytic ($CD11b^+Ly6G^-Ly6C^{high}$) types, are described (4, 5). The main feature of these cells is their ability to suppress T-cell responses in Ag-specific or nonspecific manners depending on the status of T-cell activation (reviewed in ref. 6). However, available information is growing on the role of MDSCs in the modulation of innate immune cell activity that is present in the TME.

Despite their well-known role in allergy and anaphylaxis, mast cells are emerging as general antennae of the microenvironment regulating physiologic and pathologic immune responses (7, 8). In the context of the TME, mast cells are recognized as an early and persistent infiltrating cell type involved in angiogenesis, tissue remodeling, and immune modulation (9). Mast cells may have both antitumor and tumor-promoting roles, potentially also in the same context (10, 11).

Mast cells interact with and functionally modulate different cells in the TME; however, relatively little is known about their possible functional interaction with MDSCs. Using a transplanted hepatocarcinoma model, Huang and colleagues (12) have recently demonstrated that mast cells can accumulate in the TME and exacerbate inflammation and immunosuppression via the SCF/c-kit signaling pathway. Moreover, they discuss a close loop between mast cells,

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

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MDSCs, and regulatory T cells (Treg) in creating a potent suppressive TME. Protumor mast cells are able to mobilize the infiltration of highly suppressive MDSCs, likely by increasing CCL-2 levels within the tumor lesion, and to induce these cells to produce IL17 that indirectly attract Tregs and enhance their suppressor function (13). Other findings by Cheon and colleagues (14) suggest that in the APCΔ468 murine model of colon polyposis, mast cells could support and mobilize MDSCs via 5-lipoxygenase activity to drive immune escape with an increase in polyp development. The idea that mast cells can enhance the immunosuppressive function of MDSCs is further supported by recent *in vivo* data showing that MDSCs depend on mast cells to exert their protumor suppressive function (15, 16). Herein, we demonstrate the relevance of the interplay between mast cells and monocytic MDSCs in a colon cancer model and describe the molecular mechanisms involved.

Materials and Methods

Human tissue samples

Formalin-fixed, paraffin-embedded bioptic tissue specimens of patients with colon carcinoma were retrospectively gathered from the archives of the Department of Human Pathology of the University of Palermo, Italy, per institutionally approved protocols.

Immunohistochemistry and immunofluorescence

Double-marker immunohistochemistry was performed by two sequential rounds of single-marker staining. Tissue samples were incubated with the following primary antibodies: mouse anti-human CD33 (clone PWS44; Novocastra), mouse anti-human mast cell tryptase (clone AA1; Dako), anti-mouse IL4R (Bioss), anti-mouse CD117/c-kit (Acris antibodies), or anti–mouse-FcεRI (mouse anti-rat FcεRI clone JRK, which also recognizes mouse FcεRI, a kind gift from Dr. Juan Rivera, NIH). The staining was detected by specific HRP- and AP-conjugated secondary antibodies (Sigma). 3-amino-9-ethylcarbazole (AEC) and 5-Bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (Bcip/NBT) were used as substrate chromogens (DAKO).

Slides were analyzed under a Leica DM2000 optical microscope, and microphotographs were collected using a Leica DFC320 digital camera.

For immunofluorescence staining, primary antibody incubation was performed overnight at 4°C in the case of Gr1 (rat anti-mouse Ly6c clone RB6-8C5; Life Technologies; 1:100) and FcεRI staining, and 2 hours at 37°C for CD11b staining (rabbit polyclonal anti-mouse; Abcam; 1:100). To detect primary antibodies, A488, A555 (both from Molecular Probe, Invitrogen), and Dylight 649 (Jackson Immunoresearch) dye–labeled secondary antibodies, diluted 1:600, were employed 1 hour at 37°C. Finally, Vectashield (Vector) added with 0.1 μg/mL DAPI (Sigma) was used as mounting medium. Epifluorescence images were obtained utilizing a live cell imaging–dedicated system consisting of a Leica DMI 6000B microscope connected to a Leica DFC350FX camera (Leica Microsystems) and equipped with a 63× oil immersion objective (numerical aperture: 1.40) or a 40× oil immersion objective (numerical aperture: 1.25). Adobe Photoshop software was utilized to compose, overlay the images, and to adjust contrast (Adobe).

Mice, cell lines, and MDSC purification

BALB/c mice were purchased from Harlan Laboratories. All mice were maintained in our animal facility and used from 8 weeks of age. Gamma knock-out (*gko*), *cd40l*$^{-/-}$, and *tnf-α*$^{-/-}$ mice on Balb/c background were kindly provided by Dr. M. Colombo (Milan).

For the induction of colitis-associated carcinoma, mice (ages 10–12 weeks) were given a single intraperitoneal injection of azoxymethane (AOM; 10 mg/kg body weight diluted in saline). Seven days after, mice were exposed to drinking water containing 1.5% dextran sodium sulfate for a period of 7 days, followed by normal drinking water. Mice were observed daily for any relevant clinical signs and were sacrificed 90 days after AOM injection. Colonic carcinogen AOM was purchased from Sigma-Aldrich; DSS with a molecular weight of 40,000 to 50,000 was from Affymetrix and was dissolved in water to a concentration of 1.5% (w/v).

CT-26 tumor cells were cultured in DMEM (Euroclone) supplemented with 10% FCS (Sigma-Aldrich). CT-26 cells (2×10^5) were injected s.c. in the mouse flank. Within 3 weeks of inoculation, CT-26 tumor–bearing mice were sacrificed, and MDSCs were purified from spleens with mouse Myeloid-Derived Suppressor Cell Isolation Kit (Miltenyi Biotec) developed for the isolation of Gr1^{high}Ly-6G^{+} (indicated as PMN-MDSC) and/or Gr1^{dim}Ly-6G^{-} (indicated as M-MDSC) CD11b^{+} myeloid cells, according to the manufacturer's instructions. Where specified, tumor growth was monitored three times per week and recorded as [longest diameter × (shortest diameter)^2/2] (mm^3). Where indicated, tumor-bearing mice were treated with cromolyn (Sigma) by daily i.p. injection of 10 mg/kg (in 1× PBS) per mouse from day +5 to day +15 after cancer cell injection.

BMMC differentiation and IgE-dependent activation

Bone marrow–derived mast cells (BMMC) were obtained by *in vitro* differentiation from *wt*, *gko*, *cd40l*$^{-/-}$, and *tnf-α*$^{-/-}$, Balb/c mice as described (17); their maturation was monitored for c-kit and FcεRI expression by flow cytometry after 4 to 5 weeks with purity usually more than 95%.

For IgE-dependent activation, BMMCs were sensitized in medium without IL3 for 3 hours with 1 μg/mL of dinitrophenol (DNP)-specific IgE and then eventually treated with 100 ng/mL of DNP (Sigma-Aldrich).

Migration assay

Chemotaxis was performed in a 96-transwell insert (Corning) with 5-μm pore-size filters. Cells (7.5×10^4) were plated in the upper chamber and different stimuli were added in the lower chamber. After 4 hours, cells that migrated into the lower chamber were counted. Results were expressed as fold induction compared with number of cells migrated in response to nonconditioned medium. Assays were done in duplicate in four separate experiments. Where indicated, the leukotriene inhibitor Montelukast (Merck) was added to the culture medium.

Flow cytometry

PE-Cy7–conjugated anti-CD4 (L3T4 clone), PE-Cy7–conjugated anti-CD8 (53–6.7 clone), FITC-conjugated anti-CD11b (M1/70 clone), PE-conjugated anti-Gr1 (RB6-8C5 clone), PerCp-Cy5.5–conjugated anti-Ly6C (HK1.4 clone), FITC-conjugated anti-CD117 (c-kit, 2B8 clone), and PE-conjugated anti-FcεRIα (MAR-1 clone) were from eBioscience, and BD Horizon V450–conjugated anti-CD11b (M1/70 clone) and PE-Cy7–conjugated anti-Ly6G (1A8 clone) were from BD Biosciences.

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PE-conjugated anti-CD40L (clone MR-1 clone) was by Biolegend. Surface staining reactions were performed in PBS supplemented with 0.05% BSA on ice for 30 minutes. Flow cytometry data were acquired on a FACSCalibur (BD Biosciences) or on a LSRFortessa cell analyzer (BD Biosciences), and analyzed with FlowJo software (TreeStar).

Proliferation assay

Responder cells (1.5×10^5) consisting of whole splenocytes from untreated *wt* or *gko* Balb/c mice, labeled by incubation with 5 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) for 15 minutes at 37°C, were stimulated with 5 μg/mL of anti-CD3 (145-2C11 clone; eBioscience). Responder cells were cultured with 1×10^5 purified splenic MDSCs derived from CT-26 tumor–bearing mice with or without 1×10^5 BMMCs in RPMI 1640 medium (Sigma-Aldrich) containing 10% FCS, 2 mmol/L L-glutamine, 200 U penicillin, and 200 mg/mL streptomycin (Sigma-Aldrich). Where indicated, IgE-sensitized BMMCs were treated with 100 ng/mL DNP (100 ng/mL; Sigma-Aldrich; IgE/Ag). In some experiments, the effects of L-NG-monomethyl-arginine (L-NMMA; 0.5 mmol/L; Sigma-Aldrich), neutralizing antibody anti-IFNγ (10 μg/mL; XMG.1 clone; Harlan), anti-TNFα (Miltenyi Biotech), anti-IL6R [15A7 clone, a gift from Dr. M. Colombo (Milan)], phorbol 12 myristate 13 acetate (PMA; 100 ng/mL; Sigma-Aldrich), and lipopolysaccharide (LPS; 5 μg/mL; Sigma-Aldrich) were tested on coculture system. In some coculture experiments, BMMCs and/or MDSCs were separated from responder cells by a transwell polyester membrane using 96-well plates (Costar; Euroclone) with 0.4-μm pore size, following the manufacturer's recommendations. For transwell experiments, the number of origin cells used has been doubled. After 72 hours, CFSE dilution on gated $CD4^+$ and/or $CD8^+$ responder T cells was evaluated by flow cytometry as a function of proliferation, and results were expressed as percentage of divided cells.

Nitric oxide detection

Equal volumes of culture supernatants were collected after 72 hours and mixed with Griess reagent (Sigma-Aldrich). After incubation at room temperature for 15 minutes, the absorbance at 560 nm was measured using a microplate plate reader (Bio-Rad Laboratories). Nitrite concentrations were determined by comparing the absorbance values for the test samples to a standard curve generated by serial dilution of 100 μmol/L sodium nitrite.

Statistical analysis

Results are expressed as mean plus SD (or SEM where specifically indicated). Data were analyzed using the two-tailed Student *t* test. $P < 0.05$ was considered statistically significant.

Results

Mast cells potentiate monocytic MDSC–suppressive properties in the CT-26 mouse model of colon carcinoma

A preliminary investigation of a cohort of patients with colon carcinoma revealed a spatial interaction between $tryptase^+$ mast cells and $CD33^+$ myeloid cells (18), suggesting a potential mast cell–dependent role on colon cancer–derived MDSCs (Fig. 1A). Similarly, immunohistochemical analysis of AOM/DSS-induced colitis-associated carcinoma in BALB/c mice indicated that mast cells were in close proximity with MDSCs (Fig. 1C and D), suggesting the possibility of a cross-talk between these two cell types in the development of an immunosuppressive microenvironment. Indeed, when compared with normal epithelium, dysplastic glands showed a more intense inflammatory infiltrate characterized by a higher number of c-kit–expressing mast cells interacting with IL4R-expressing myeloid cells, namely MDCSs (5), in the lamina propria (black arrows). Of note, respectively, $44.1\% \pm 7.0\%$ and $42.9\% \pm 7.0\%$ of total mast cells were found in contact with MDSCs in both human and mouse samples (Fig. 1B and E).

Because the key role of the spleen for tumor-induced tolerance mediated by MDSCs has been recently demonstrated in a thymoma and mammary mouse model (19), we focused our attention on the splenic microenvironment of AOM/DSS-treated tumor-bearing mice, in which the two cell populations also were found in close proximity (Fig. 1F).

To elucidate and analyze the possible functional relevance of the MDSC–mast cell interaction, the cross-talk between these two immune cell populations was further examined in tumor-bearing mice. Animals were injected s.c. with 2×10^5 CT-26 colon cancer cells, and all the mice used were sacrificed at development of similar-sized tumors (i.e., 1.5 cm in diameter) within 3 weeks of inoculation. Mast cells were recruited to the CT-26 growing tumor (Fig. 2A) and exerted a protumor role because mice treated with cromolyn, a specific mast cell inhibitor (20), showed a significant tumor growth reduction compared with that in untreated mice (Fig. 2B).

Considering the rate of variability in MDSC frequency between different tumor models (5), we took advantage of the CT-26 colon carcinoma model because it showed a relatively strong accumulation of MDSCs in the bone marrow, blood (Supplementary Fig. S1A), and spleen (Fig. 2C). As in the spleen of AOM/DSS-induced colon carcinoma–bearing mice (Fig. 1F), $FcεRI^+$ mast cells were found to localize in the proximity of accumulating $IL4R^+$ MDSCs (Fig. 2D) Consistently, immunohistochemical analysis of spleens of CT-26 tumor–bearing mice revealed $FcεRI^+$ cells in close proximity of $Gr1^+$ or $CD11b^+$ cells (Supplementary Fig. S1B).

MDSCs consist of two major subsets with distinct phenotype, morphology, and preferential suppression mechanisms: the polymorphonuclear $CD11b^+Gr1^{high}Ly6G^+Ly6C^{low}$ (PMN-MDSC) and the monocytic $CD11b^+Gr1^{dim}Ly6G^-Ly6C^{high}$ (M-MDSC) cells (4). Both subsets were present in the spleens of CT-26 tumor–bearing mice 3 weeks after tumor inoculation (percentage of $CD11b^+Gr1^+$ MDSCs: $13.5\% \pm 1.0\%$ in CT-26 tumor–bearing mice vs. $3.8\% \pm 0.8\%$ in control mice; PMN- and M-MDSCs represented $76.9\% \pm 0.7\%$ and $23.0\% \pm 0.8\%$ of total MDSCs, respectively) and were separately purified (Supplementary Fig. S1C).

Because mast cells have been described for their ability to mobilize MDSCs at the TME (13, 14), we decided first to verify this mast cell capability in our colon cancer model. We performed in *vitro* migration assays and confirmed that supernatants from IgE/Ag-activated BMMCs induced the migration of both purified PMN- and M-MDSCs, and these had respectively an additive or synergistic effect with CT-26–conditioned culture media–induced migration. We further validated that the effect was mediated partly by mast cell–derived leukotrienes by using the specific inhibitor Montelukast (Supplementary Fig. S1D).

To assess whether the role of mast cells in the TME is limited to the recruitment of MDSCs or if it can impact their immunosuppressive activity, we analyzed the interplay between these two cell populations on T-cell proliferation *in vitro*. CFSE-

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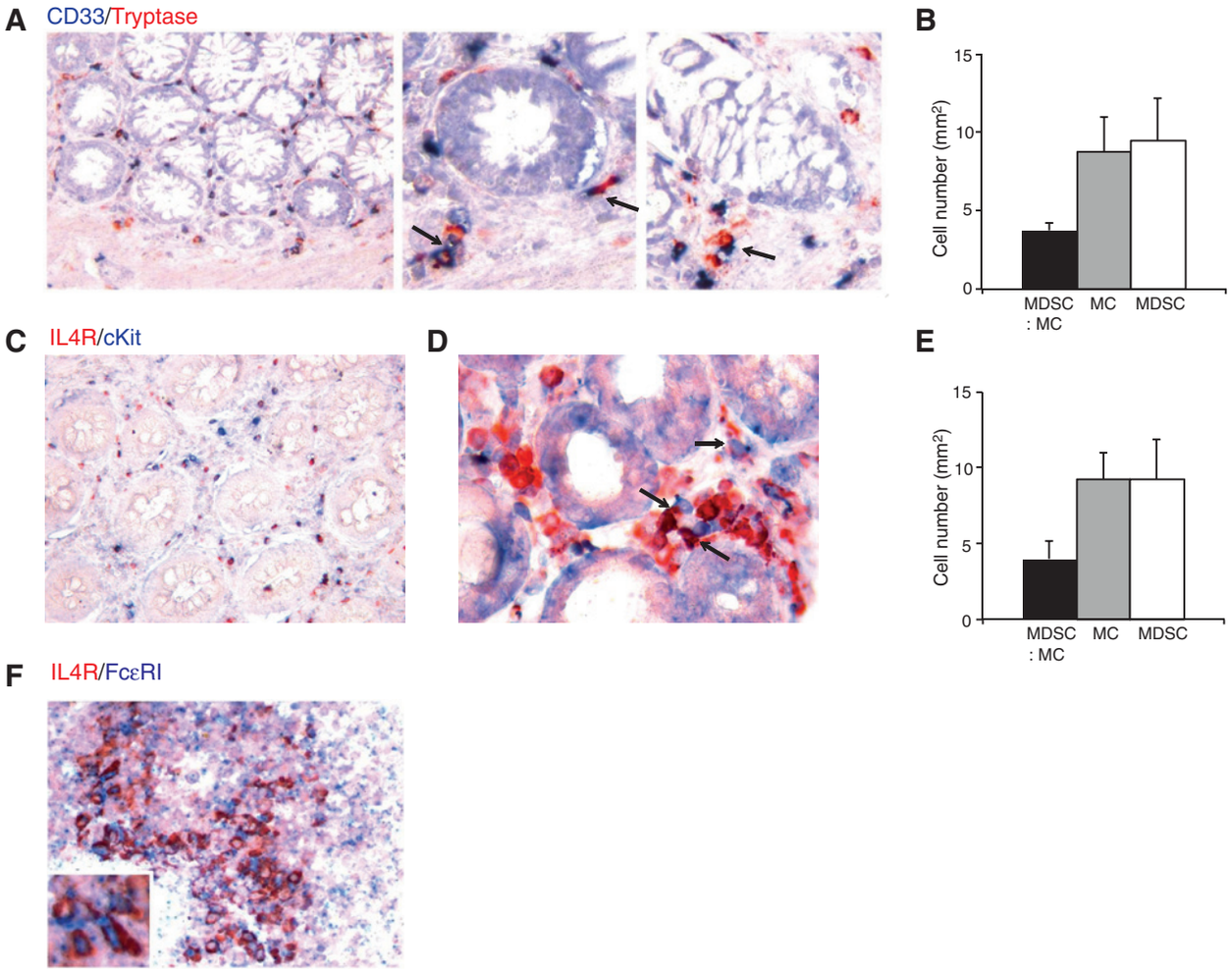

Figure 1.
Interacting MDSCs and mast cells (MC) in human colon carcinoma and in the colon of AOM/DSS-induced tumor-bearing mice. Double immunohistochemistry for CD33 (blue) and tryptase (red) in the human mucosa of a patient with colon carcinoma (A); for IL4R (red) and c-kit (blue) in normal (C) and in dyplastic (D) colonic mucosa of AOM/DSS-induced colon carcinoma–bearing mice; for IL4R (red) and FcεRI (blue) in the spleen of AOM/DSS-treated mouse. Arrows indicate tryptase-positive mast cell interacting with CD33 myeloid cells (in A) and c-kit–positive mast cells interacting with IL4R-expressing lymphocyte (in D), respectively. Original magnification, ×200 (A, left, and C) and ×400 (A, intermediate and right plots, and D). Histograms represent the mean + SD count (in ×400 fields) of interacting MDSCs and mast cells compared with total MCs and MDSCs in at least 4 different samples from patients with human colon carcinoma (B) and AOM/DSS-treated mice (E).

labeled total splenocytes from naïve Balb/c mice were polyclonally activated with an anti-CD3 mAb and were used as responder cells in a proliferation assay in the presence of M-MDSCs, PMN-MDSCs, or mast cells alone or in coculture. In our experimental conditions, only M-MDSCs revealed relatively weak suppression ability, whereas mast cells alone did not perturb non–antigen-specific $CD4^+$ T-cell proliferation (Fig. 3A and data not shown). As shown in Fig. 3A and B, resting mast cells significantly enhanced the ability of M-MDSCs to suppress the proliferation of $CD4^+$ T cells but had a slight and not statistically significant effect on $CD8^+$ T-cell proliferation. Moreover, when we used IgE/Ag-activated mast cells, the M-MDSC–inhibitory potential was further amplified for $CD4^+$ T cells and not so much for the $CD8^+$ cell compartment (Fig. 3B). By contrast, in these experimental conditions, PMN-MDSCs were not suppressive and their inhibitory potential was not affected by mast cells (Fig. 3A and B).

M-MDSC:mast cell–suppressive axis is nitric oxide mediated and IFNγ dependent

Different mechanisms of MDSC-mediated suppression of T-cell function have been described mostly based on the release of soluble mediators such TGFβ, arginase 1, and reactive oxygen species. Among the suppressive molecules, nitric oxide (NO) acts as a key factor (21). Thus, as the activity of M-MDSCs is typically NO dependent (2), culture media from proliferation assays were tested for nitrites production, using a method based on the Griess reaction. Both resting and activated mast cells were able to increase basal NO release in the coculture (Fig. 4A), proportionally to the inhibition of T-cell proliferation (Fig. 3). Considering that in M-MDSCs NO production is dependent on the inducible nitric oxide synthase (iNOS), we assessed the specificity of NOS signaling on M-MDSC activity by using NMMA, a nitric oxide synthase inhibitor. As shown in Fig. 4B, NMMA completely abolished the production of nitrites and reestablished T-cell

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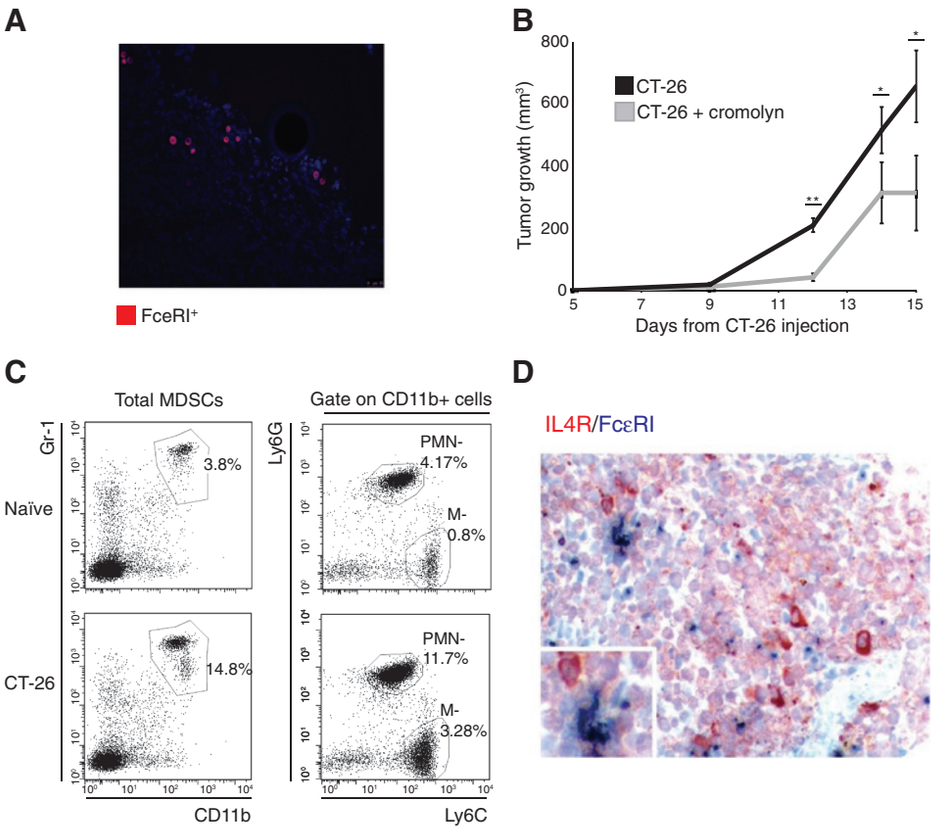

Figure 2.
CT-26 colon cancer induces the recruitment of protumor mast cells and the accumulation of MDSCs. CT-26 colon carcinoma cells (2×10^5) were injected s.c. into Balb/c mice. After 3 weeks, spleens and/or tumors were collected. A, FcεRI-positive mast cells (in red) infiltrate CT-26 tumors. B, tumor size (mean + SEM) in Balb/c mice injected s.c. with 2×10^5 CT-26 cells and 1×10^5 BMMCs, eventually treated with cromolyn ($n = 5$) from day 5 to day 15 after cell injection. *, $P < 0.05$ and **, $P < 0.01$. C, $Gr1^+$ $CD11b^+$ MDSCs were evaluated as percentage of total splenocytes (left). Alternatively, splenocytes from naive and CT-26 tumor–bearing mice were stained with CD11b, Ly6G, and Ly6C Abs. A representative flow cytometry analysis of PMN-MDSC and M-MDSC subset is shown (right). Spleens of untreated mice were used as controls for the definition of the MDSC population. Numbers represent the percentage of gated cells compared with the total live population of splenocytes. D, IL4R-expressing MDSCs (red) in close proximity with FcεRI (blue) in the spleen of CT-26 tumor–bearing mice.

proliferation. We also tested arginase activity, and reactive oxygen species and TGFβ production, but did not record any statistically significant regulation in the production of these mediators modulated by MDSC:mast cell interaction (data not shown).

Because freshly isolated MDSCs from tumor-bearing mice required IFNγ to become fully suppressive and to activate the iNOS machinery (22, 23), we investigated the possible contribution of this cytokine in the MDSC:mast cell axis. As expected, anti–CD3-polyclonally activated splenocytes produced IFNγ but, interestingly, a significant increase in IFNγ levels was found when the responder population was cocultured with resting or IgE/Ag-activated mast cells either alone (as already described in ref. 24) or together with M-MDSCs (Fig. 4C). To understand the importance of IFNγ in the coculture system, a neutralizing anti-IFNγ antibody was used. This addition significantly inhibited the acquisition of mast cell–dependent M-MDSC–suppression properties (Fig. 4B).

To discriminate the contribution of IFNγ production from the different cell populations, we isolated responder T-effector cells, BMMCs, and MDSCs from *gko* mice. In the presence of M-MDSCs derived from *gko* tumor-bearing mice, NO production was not affected, and consequently they still suppressed proliferation of $CD4^+$ T cells (Supplementary Fig. S2A). In addition, *gko*-derived mast cells were still able to potentiate M-MDSC nitrites production similarly as that of the *wt* counterpart (Supplementary Fig. S2B). Instead, IFNγ deficiency in responder cells reduced almost completely the mast cell–dependent M-MDSC activation, emphasizing the important role of IFNγ in NOS machinery activation (Fig. 4D). To confirm the importance of T cells as a source of IFNγ, $CD4^+CD25^-$ T cells were purified from spleens of untreated *wt* mice, CFSE-labeled, and used as responder population in the presence of *wt* mytomicin–treated APCs. In these conditions, $CD4^+$ T-cell proliferation was inhibited at high levels by M-MDSCs alone, independent from mast cell presence (Fig. 4E, left). Despite the fact that mast cells did not play a crucial role in T-cell suppression, resting or IgE-activated mast cells continued to increase NO levels when cocultured with M-MDSCs (Fig. 4E, right). To exclude IFNγ production by other non–T cells, proliferation of *wt* $CD4^+$ T cells was performed in the presence of *gko*-derived APCs. Figure 4E shows that M-MDSCs, either alone or together with mast cells, were still competent in the suppression of $CD4^+$ T-cell proliferation, indirectly confirming that anti–CD3-activated $CD4^+$ T cells represent the crucial initial source of IFNγ required to fully activate mast cell–MDSC-suppressive axis.

Moreover, if mast cells and M-MDSCs were cultured either alone or together for 72 hours in the absence of anti–CD3-activated populations, resting or IgE/Ag-activated mast cells alone were not sufficient to switch on M-MDSC activation, but activated mast cells contributed to the generation of NO-competent M-MDSCs only in the presence of soluble IFNγ (Fig. 4F).

To investigate whether the M-MDSC-suppressive efficacy was affected by the activation status of the distinct cellular components representative of the TME, different stimulatory pathways were investigated. Proliferation assays were performed in the presence of LPS and PMA, respectively a known activator of both mast cells and MDSCs (25–29), and mitogen for T-cell proliferation (30). Both LPS and PMA stimulation raised the basal level of M-MDSC suppression in terms of both T-cell proliferation inhibition and NO secretion, phenomena that were completely (for PMA) or partially (for LPS) reverted when using the anti-IFNγ

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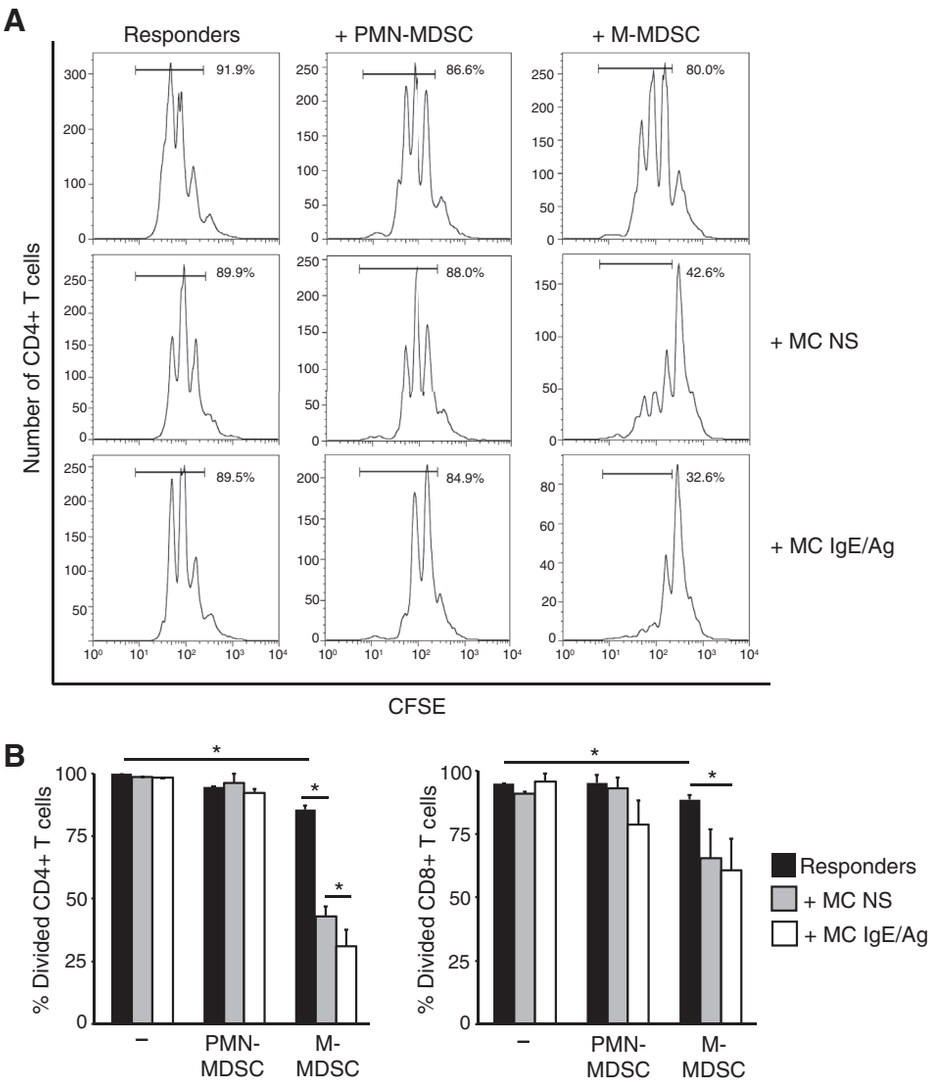

Figure 3.
Mast cells potentiate monocytic MDSC–suppressive functions. A and B, 1.5×10^5 total splenocytes (responders) from untreated mice were CFSE-labeled and anti-CD3 activated in the presence of 1×10^5 purified PMN-MDSCs or M-MDSCs isolated from CT-26 tumor–bearing mice, and/or of the same number of resting (+ mast cell NS) or IgE/Ag-stimulated BMMCs (+ mast cell Ag). After 72 hours, CFSE dilution in gated $CD4^+$ and $CD8^+$ T cells was evaluated by flow cytometry as a function of proliferation. Results are expressed as percentage of divided T cells. A, representative histogram plots show CFSE dilution in $CD4^+$ T cells in the different conditions. Percentages indicate the fraction of divided $CD4^+$ T cells. B, histograms represent the percentage of divided $CD4^+$ T (left) or $CD8^+$ T cells (right). Data (mean + SEM) are from at least three independent experiments. *, $P < 0.05$. MC, mast cell.

antibody (Supplementary Fig. S2C). For IgE-dependent activation, LPS- or PMA-activated mast cells boosted M-MDSC production of NO and suppression of T-cell proliferation. Addition of neutralizing anti-IFNγ Ab demonstrated that the mechanism of mast cell–dependent M-MDSC suppression was mediated by IFNγ for both IgE-dependent and IgE-independent mast cell activation (Supplementary Fig. S2C).

Reciprocal influence of M-MDSC:mast cell via CD40:CD40L axis on cytokine release

So far we have shown that mast cells are able to recruit MDSCs and that they are able to influence their suppressive activity in an experimental setting that involves T cells. To investigate the cross-talk between these two immune cell populations, equal numbers of mast cells and M-MDSCs were cultured either alone or together in the absence of anti–CD3-activated populations, and mast cell degranulation and cytokine production were assessed.

Although mast cell degranulation, measured both as release of β-hexasominidase and histamine production, was not affected by coculture with M-MDSC (data not shown), when IgE/Ag-activated mast cells were cocultured with M-MDSCs, we observed an increase in TNFα production (Fig. 5A). This cytokine has a fundamental role in promoting MDSC activity in an inflammatory setting (31) and is required for MDSC survival and accumulation (32). M-MDSCs may be an important source of TNFα. Indeed, even though their basal secretion levels were almost undetectable, these cells released a large amount of TNFα upon LPS activation (data not shown). To identify the source of TNFα and to elucidate the mechanism of the synergism in TNFα production, the coculture experiment was performed with BMMCs derived from TNFα-deficient (*tnf-α*$^{-/-}$) mice. In M-MDSC and IgE/Ag-activated *tnf-α*$^{-/-}$ mast cell coculture, TNFα was undetectable, suggesting that mast cell–derived TNFα is crucially involved in the upregulation of this cytokine (Fig. 5A).

Because it has been suggested that TNFα promotes the suppressive activities of MDSCs via TNFR2 (33), a neutralizing anti-TNFα antibody was used in culture to study the role of this cytokine in supporting the suppressive activity of the M-MDSC on anti–CD3-activated $CD4^+$ T cells. A decreased response of mast cell–dependent M-MDSC–suppressive activity was observed following TNFα

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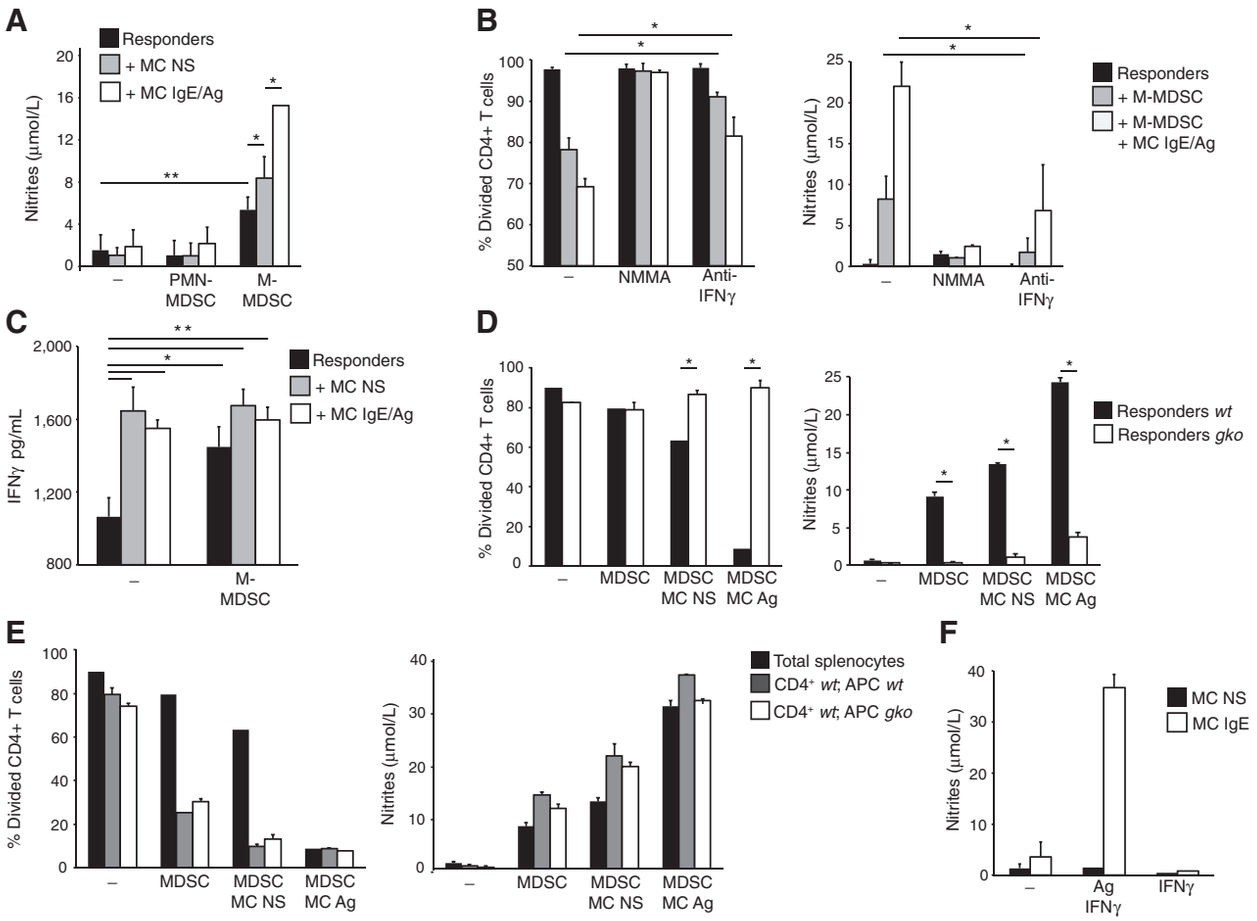

Figure 4.

Role of NO and IFNγ in mast cell–dependent MDSC-suppressive activities. A, supernatants from coculture experiments of proliferation assay (as in Fig. 3) were collected and tested for nitrites levels with Griess reagent. *, $P < 0.05$ and **, $P < 0.01$; $n = 3$ experiments. B, total splenocytes (responders) were anti-CD3 stimulated in the presence of M-MDSCs with (+ M-MDSC + mast cell Ag) or without (+ M-MDSC) IgE/Ag-stimulated BMMCs. Where indicated, an iNOS inhibitor (0.5 mmol/L NMMA) or a neutralizing anti-IFNγ antibody (10 μg/mL) was added. Results from one representative of three independent experiments are expressed as percentage of divided $CD4^+$ T cells (left) and nitrites production (right). C, supernatants, as in A, were analyzed for the production of IFNγ by ELISA. *, $P < 0.05$ and **, $P < 0.01$ versus anti-CD3-stimulated responder cells alone. Mean + SEM (*, $P < 0.05$) of three independent experiments are shown. D, proliferation assay was performed using total splenocytes from naive *gko* Balb/c mice (responders *gko*), whereas the other cellular components were from *wt* Balb/c mice. Results (mean + SEM) expressed as percentage of divided $CD4^+$ cells and/or nitrites production of three independent experiments are shown and compared with the levels of proliferation of the *wt* system (responders *wt*). *, $P < 0.05$. E, 1.5×10^5 $CD4^+$ $CD25^-$ T cells, purified from *wt* Balb/c mice, were anti-CD3 stimulated in the presence of 7.5×10^4 mytomicin-treated APCs derived from *wt* (gray bars) or *gko* (white bars) mice, with or without M-MDSCs and resting or IgE/Ag-stimulated mast cells. Data from one representative of two experiments were shown and compared with proliferative response of total splenocytes from *wt* mice (black bars). F, 2×10^5 M-MDSCs were cultured with an equal number of resting (mast cell NS, black histograms) or IgE-sensitized (mast cell IgE, white histograms) BMMCs without additional stimuli (−) or in the presence of 20 ng/mL IFNγ (IFNγ), and eventually 100 ng/mL IgE-specific Ag DNP (Ag IFNγ). After 72 hours, nitrites levels in supernatants were measured with Griess method. MC, mast cell.

blocking (Fig. 5B). Notwithstanding, *tnf-α*$^{-/-}$ mast cells showed no difference in their ability to amplify M-MDSC–inhibitory activity compared with *wt* mast cells, and the addition of the neutralizing anti-TNFα antibody inhibited M-MDSC activity (corresponding NO levels are shown in Fig. 5C), suggesting that in the proliferation assay, the source of TNFα responsible for MDSC activity could not be directly represented by mast cells.

The finding of a synergism between mast cells and MDSCs in the context of TNFα production led us to investigate whether a generalized increase in cytokine production is the outcome of this cellular interaction. Similar to results shown by Saleem and colleagues (15) and Morales and colleagues (16), whereas resting mast cells, either alone or together with M-MDSCs, released low or undetectable levels of the investigated mediators, coculture of activated mast cells and M-MDSCs resulted in a significant increase of IL6 and CCL-2 levels (Fig. 6A), similar to that observed for TNFα production. Neither resting or IgE/Ag-activated mast cells, or M-MDSC alone, produced detectable levels of CCL-3, IL2, IL4, IL10, or IL17, and a synergistic effect in the production of these cytokines was not observed in the M-MDSC:mast cell coculture (data not shown).

Considering the role of CD40 expression and ligation in the activation and in the immunosuppressive function of MDSCs (34) and knowing the constitutive expression of its ligand on mast cells (ref. 35; Supplementary Fig. S3A), the requirement of CD40:CD40L axis in the activation of M-MDSC in this setting was

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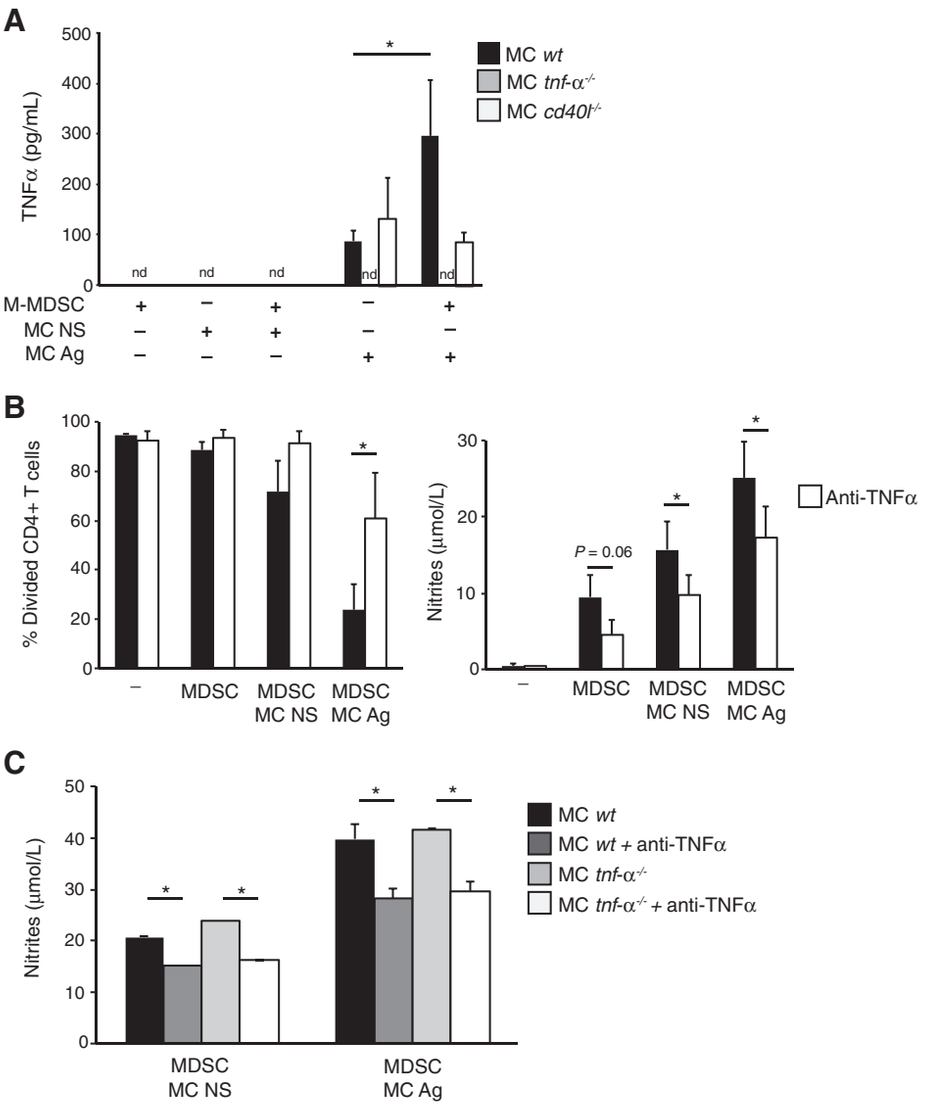

Figure 5.
M-MDSC:mast cell cross-talk in the regulation of TNFα production. A, 2×10^5 M-MDSCs were cultured with an equal number of resting (mast cell NS) or IgE/Ag-activated (mast cell Ag) BMMCs derived from *wt* (black bars), *tnf-α*$^{-/-}$ (gray bars), or *cd40l*$^{-/-}$ (white bars) mice. TNFα levels in the supernatants collected after 24 hours of coculture were measured by ELISA. Data are the mean + SEM of three independent experiments. nd, not detectable. B, anti–CD3-stimulated responder splenocytes were cocultured with M-MDSCs and/or resting or IgE/Ag-activated BMMCs. The proliferation assay was performed in the presence of 10 μg/mL of neutralizing anti-TNFα antibody. Mean + SEM of three independent experiments are shown as percentage of divided $CD4^+$ T cells (left) and nitrites production (right). *, $P < 0.05$. C, proliferation assay (as in B) was performed in the presence of *tnf-α*$^{-/-}$ BMMCs, and results were compared with the ones obtained with *wt* BMMCs. One representative of three independent experiments is shown in terms of nitrites levels. *, $P < 0.05$. MC, mast cell.

evaluated. When we used *cd40l*$^{-/-}$ mast cells, the increased release of TNFα, IL6, and CCL-2 seen in the M-MDSC:*wt* mast cell coculture was completely lost (Figs. 5A and 6A). Therefore, these results suggest that the M-MDSC:mast cell axis, through the CD40:CD40L interaction, induces a selective and preferential production of proinflammatory cytokines and chemokines, such as TNFα, IL6, and CCL-2, that could sustain an immune response supporting tumor promotion.

The importance of the CD40:CD40L axis in affecting T-cell proliferation was further evaluated taking advantage of *cd40l*$^{-/-}$ mast cells. Of note, no reduction was measured in terms of M-MDSC NO induction by *cd40l*$^{-/-}$ mast cells compared with *wt* cells in the presence of IFNγ (Supplementary Fig. S3B). As shown in Fig. 6B, no significant differences in terms of MDSC-dependent T-cell suppression or NO production was observed compared with *wt* mast cells. Similarly, no differences in T-cell proliferation and NO levels were found when the proliferation assay was performed in the presence or absence of a transwell that physically separates mast cells from the other cell populations (Supplementary Fig. S3C). It is also plausible that the importance of CD40, and similarly, of other possible membrane-bound molecules involved, could be lost in the proliferation assay because of the presence, in the splenocytes preparation, of other $CD40L^+$ cells besides mast cells, such as T cells and macrophages with a potential redundant role in CD40 activation of MDSCs.

Discussion

In tumor-bearing hosts, cancer growth results in the accumulation of MDSCs in peripheral lymphoid organs and at the tumor site. This tumor-induced cell population acts to suppress on-going antitumor immune response, either directly or by interacting with other immune cells present in the tumor-associated microenvironment (6). In terms of immunomodulation, the existence of a closed loop between mast cells and MDSCs provides a new insight into the relationship between inflammation and immunosuppression in the tumor

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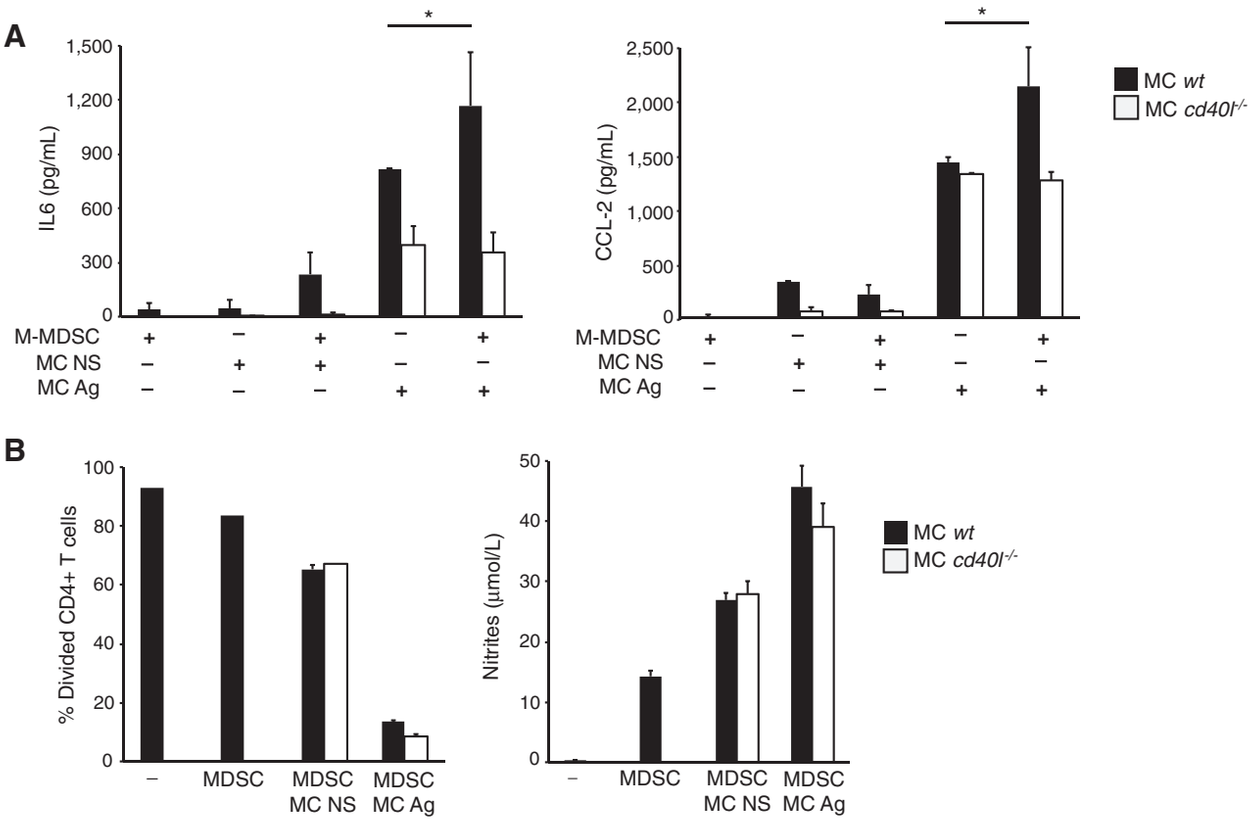

Figure 6.
CD40:CD40L–interacting MDSCs and mast cells acquire an enhanced activation state. A, 2×10^5 M-MDSCs were cultured with an equal number of resting (MC NS) or IgE/Ag-activated (mast cell Ag) BMMCs derived from *wt* (black bars) or *cd40l*$^{-/-}$ (white bars) mice, and supernatants were collected 24 hours after coculture. IL6 and CCL-2 levels were measured by ELISA. Data are mean + SD of at least two independent experiments. B, proliferation assay was performed comparing the effect of *wt* (black bars) and *cd40l*$^{-/-}$ (white bars) BMMCs on MDSC-dependent T-cell suppression. One representative of three experiments is shown, and results are (mean + SD) expressed as percentage of divided $CD4^+$ T cells (left) and levels of nitrites (right). MC, mast cell.

microenvironment (36). Saleem and colleagues (15) demonstrated that the suppression of antitumor immune response mediated by monocytic MDSCs is mast cell dependent and that the M-MDSC:mast cell interaction determines a synergistic Th2-skewed immune response, deleterious in the context of cancer immune response. Furthermore, mast cell–derived histamine has been recently indicated as the potential mediator involved in the immunoregulation of histamine receptor-1– and -2–expressing MDSCs (37).

Our findings herein demonstrate that mast cells not only had the potential to induce MDSC recruitment, but they could contribute to the acquisition of suppressive functions by MDSCs. In fact, by using BMMCs and MDSCs derived from the spleens of CT-26 tumor–bearing mice, we showed that the suppressive function of monocytic $Ly6G^-Gr1^{dim}CD11b^+$ cells was significantly increased when this population was cocultured with resting or stimulated mast cells. The described mechanisms of mast cell–dependent modulation of MDSCs seemed to be relevant only for the monocytic subset of the immunosuppressive populations, because PMN-MDSCs responded to mast cell–derived migratory signals but their activity was not affected. M-MDSCs are known to be a highly immunosuppressive population compared with PMN-MDSCs that inversely are traditionally represented as the predominant subset that accumulates during cancer progression (38).

The immune system is considered a double-edge sword for cancer development, and it is known that immune cells, such as $CD8^+$ and $CD4^+$ T-helper cells or natural killer cells, along with their characteristic cytokine IFNγ, function as the major antitumor effector cells (discussed in ref. 39). Despite the traditional view of IFNγ as a tumor-suppressor cytokine, results from several studies suggest that IFNγ could be involved in the recruitment and activation of MDSCs, and that IFNγ-induced signaling is crucial to activate NO production in M-MDSCs (22). Results from a recent article suggest that in a mouse model of melanoma, the efficacy of transgenic cytotoxic T lymphocyte (CTL) transfer was limited due to the consequent massive accumulation of M-MDSCs in the tumor. Their accumulation and suppression activity were caused by IFNγ produced by the effector CTLs (40). Similarly, a recent report showed that the low efficacy of vaccination in melanoma-bearing mice was associated with IFNγ production by early attacking and invading $CD8^+$ T cells, resulting in the upregulation of immunosuppressive processes that suppressed local T-cell activation (41). In our *ex vivo* system, we showed that IFNγ production by activated $CD4^+$ T cells was required to sustain the initial activation of M-MDSCs and render functional the

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M-MDSC:mast cell–suppressive axis. IFNγ may become a detrimental response in tumor-bearing hosts because the cytokine cascade could contribute to MDSC functional activation, further sustained by signals derived from infiltrating mast cells.

Classically, the bone marrow represents the primary site of myelopoiesis, but suppressive cells accumulate in the spleens of tumor-bearing mice. In fact, the spleen has been identified as a key organ in the regulation of the frequency of MDSCs and inflammatory monocytes with a T-cell tolerating potential that is described in the splenic marginal zone during cancer development (19). Despite draining lymph nodes are "historically" considered the primary site for tumor-antigen presentation and tolerance induction (42), recent data underlined that MDSC-dependent immune tolerance to tumor antigens occurs efficiently in specialized sites of the spleen (19). In light of this consideration, the evidence that in the spleen mat cells can interact with and further amplify the potential of M-MDSCs to switch off the antitumor response of $CD4^+$ T cells, it could be seen as an effective novel mechanism to amplify tumor-induced tolerance. This possibility does not exclude that, once activated, M-MDSCs, driven by mast cells, could migrate toward lymph nodes and the tumor mass and exert their suppressive functions directly in these sites. Our findings are further supported by the possibility of a previously not investigated interaction between mast cells and MDSCs in the spleen of CT-26 tumor-bearing mice, as well as in the spleens and colons of AOM/DSS-induced colitis-associated carcinoma tumor–bearing mice. These findings are supported by a similar physical colocalization between $tryptase^+$ mast cells and $CD33^+$ myeloid cells in a cohort of patients with colon carcinoma under preliminary investigation, suggesting a specific mast cell–dependent influence on colon cancer MDSC biology.

Mast cells express costimulatory molecules for innate and adaptive immune cells, and are therefore able to replace some T-cell functions. This prompts us to investigate the CD40:CD40L cross-talk between M-MDSCs and mast cells. This axis was shown to be responsible for shaping a proinflammatory microenvironment, with an exacerbation in the production of mediators (TNFα, IL6, CCL-2) that could further support MDSC activation and tumor growth. In particular, the production of CCL-2 has been linked with MDSC migration and activation in the cancer microenvironment (43). Recently, it has been suggested that endocannabinoid-activated mast cells produce CCL-2, resulting in the massive recruitment of M-MDSCs (44). mast cells have the ability to induce the trafficking of MDSCs, which cause immunosuppression that may lead to immune evasion by cancer cells, or downregulation of inflammation in the context of autoimmunity or other inflammatory diseases.

In light of these results, interfering with the MDSC:mast cell axis could be a promising approach to abrogate MDSC-related immune suppression and to improve antitumor immune response.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L. Danelli, G. Gri, C. Tripodo, M.P. Colombo, C.E. Pucillo

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Danelli, G. Gri, F. Mion, C. Tripodo, L. Mariuzzi, C.E. Pucillo

Writing, review, and/or revision of the manuscript: L. Danelli, B. Frossi, G. Gri, C. Tripodo, M.P. Colombo, C.E. Pucillo

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Guarnotta, L. Bongiovanni, L. Mariuzzi, S. Marzinotto, C.E. Pucillo

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Cancer Immunology Research

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