Tumor-Produced Interleukin-8 Attracts Human Myeloid-Derived Suppressor Cells and Elicits Extrusion of Neutrophil Extracellular Traps (NETs)

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Abstract

**Purpose:** Myeloid-derived suppressor cells (MDSC) are considered an important T-cell immunosuppressive component in cancer-bearing hosts. The factors that attract these cells to the tumor microenvironment are poorly understood. IL8 (CXCL8) is a potent chemotactic factor for neutrophils and monocytes.

**Experimental Design:** MDSC were characterized and sorted by multicolor flow cytometry on ficoll-gradient isolated blood leukocytes from healthy volunteers (n = 10) and advanced cancer patients (n = 28). In chemotaxis assays, sorted granulocytic and monocytic MDSC were tested in response to recombinant IL8. IL8 derived from cancer cell lines, and patient sera. Neutrophil extracellular traps (NETs) formation was assessed by confocal microscopy, fluorimetry, and time-lapse fluorescence confocal microscopy on short-term MDSC cultures.

**Results:** IL8 chemoattracts both granulocytic (GrMDSC) and monocytic (MoMDSC) human MDSC. Monocytic but not granulocytic MDSC exerted a suppressor activity on the proliferation of autologous T cells isolated from the circulation of cancer patients. IL8 did not modify the T-cell suppressor activity of human MDSC. However, IL8 induced the formation of NETs in the GrMDSC subset.

**Conclusion:** IL8 derived from tumors contributes to the chemotactic recruitment of MDSC and to their functional control.

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Introduction

IL8 (CXCL8) is a chemokine originally discovered for its ability to attract neutrophils to inflammatory foci (1). In cancer its role in promoting angiogenesis is well characterized. IL8 does not only attract neutrophils but also monocytes to inflamed tissues (2). Tumor cells constitutively produce IL8 (2–9) and hence plasma concentrations of IL8 reflect tumor burden (2) and determine prognosis (2–9). IL8 is functional on CXCR1 and CXCR2 where it induces chemotactic responses. There is no IL8 analogue in the mouse genome (10, 11), but IL8 derived from cancer cell lines, and patient sera. Neutrophil extracellular traps (NETs) formation was assessed by confocal microscopy, fluorimetry, and time-lapse fluorescence confocal microscopy on short-term MDSC cultures.

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Translational Relevance

IL8 is a chemokine produced by cancer cells and whose serum concentration correlates with poor prognosis. In this study, we demonstrate that IL8 chemotactically attracts myeloid-derived suppressor cells (MDSC) sorted from the peripheral blood of advanced cancer patients and that this chemotactic activity can be pharmacologically disrupted in vivo. Surprisingly, we also found that IL8 activates granulocytic MDSC to extrude DNA-forming neutrophil extracellular traps (NET). These IL8-mediated mechanisms are likely to be relevant in shaping a protumoral leukocyte microenvironment in cancer.

between higher levels and worse prognosis (21, 26–28). We have previously reported that IL8 attracts dendritic cells and discovered that tumors producing IL8 were chemotactically retaining dendritic cells injected intratumorally, thereby avoiding their outmigration to draining lymph nodes (10, 29). In this study, we explored if IL8 could attract and functionally modulate MDSC. To this end, human MoMDSC and GrMDSC were identified and FACS-sorted from the peripheral blood of a series of advanced cancer patients. It was found that IL8 readily attracts all these cell subsets, although it does not regulate their T-cell suppressive activity. However, IL8 induces the formation of neutrophil extracellular traps (NET) with projected DNA as T-cell suppressive activity. However, IL8 induces the formation of neutrophil extracellular traps (NET) with projected DNA as T-cell suppressive activity. Therefore, the role of NETs in shaping the immune microenvironment remains to be determined.

Materials and Methods

Cell lines and tumor grafting

HT29 was obtained from ATCC in 2011 and retrieved from the verified master cell bank and CT26GM-CSF transfectants were a kind gift of Mario P. Colombo (Milano; ref.40). For tumor engraftment, 5 × 10^5 tumor cells were injected onto the flank of BALB/c mice (Harlan) or Rag-1-/- IL2R-/- mice bred in our pathogen-free animal facility. Animal experiments were conducted in accordance to Spanish laws and under approval by the Ethics Committee of animal experimentation at the University of Navarra (study number 070-15).

FACS analyses and sorting of MDSC

Twenty milliliters of peripheral blood from a sequential series of advanced prostate cancer and NSCLC patients or from healthy volunteers were collected in 10 mL heparin-containing tubes and were mixed with 10 mL of ice-cold PBS. Blood samples from sequential patients were collected upon visit to our outpatient clinics. Patients provided informed consent under a protocol approved by the institutional ethics committee. The cell suspension was laid over 3 mL of Ficoll-Hypaque (GE Healthcare Europe GmbH) gradients in a 15 mL tube and spun 2,200 rpm for 20 minutes at 4°C. The leukocyte layer was recovered into a 50 mL tube and spun at 1,200 rpm for 12 minutes at 4°C. The leukocyte layer was recovered into a 50 mL tube and spun at 1,200 rpm for 12 minutes at 4°C. The leukocyte layer was recovered into a 50 mL tube and spun at 1,200 rpm for 12 minutes at 4°C. The leukocyte layer was recovered into a 50 mL tube and spun at 1,200 rpm for 12 minutes at 4°C. The leukocyte layer was recovered into a 50 mL tube and spun at 1,200 rpm for 12 minutes at 4°C. The leukocyte layer was recovered into a 50 mL tube and spun at 1,200 rpm for 12 minutes at 4°C.

Transwell migration assays

In vitro MDSC and neutrophil migration was measured in Transwell chambers (5 μm; Corning Costar). Human and mouse MDSC (10^5) or human neutrophils were seeded onto the top chamber and migration stimuli were placed in the bottom chamber. In this experiment, rhIL-8 (R&D Systems) was used at different concentrations. Duplicate experiments were performed with or without IL8-neutralizing mAb or IgG as control (BD Pharmingen) at 20 μg/mL that were added to the bottom chamber. Transmigrated cells recovered from the bottom chamber were manually quantified using a Neubauer chamber. The chemotactic index was calculated as the number of migrated cells in each
IL8 ELISA

IL8 concentrations in HT29 carcinoma culture supernatants (10⁶ cells/mL) and serum of patients were tested using a commercially purchased ELISA Kit used according to the manufacturer's instructions (BD Biosciences).

Hydrodynamic IL8 gene transfer

The human IL8 expression plasmid (pUC19/human IL8) was kindly given to us by Joost Oppenheim (Addgene). On day 14 following subcutaneous tumor cell inoculation (2 × 10⁶ HT29 cells), Rag2⁻/⁻/IL2Rγ⁻/⁻ mice received an intravenous injection (tail vein) of 10 μg of plasmid in a volume of 100 mL/kg using a 27-G needle at a rate of 0.4 mL/seconds (41). Reparixin (20 μg) was administered subcutaneously daily between days 12 and 18. At day 18, mice were sacrificed to obtain liver and spleen cell suspensions. Organs were disaggregated to assess absolute numbers of MoMDSC (CD11b⁻ Ly6G⁻ Ly6C⁻) and GrMDSC (CD11b⁺ Ly6G⁺ Ly6C⁺) by referring to total cells measured by manual counting on a Neubauer chamber.

Proliferation assay

Sorted peripheral blood GrMDSC or MoMDSC (10⁵) were cocultured at different MDSC to T-cell ratios with autologous T cells (10⁵) derived from the same subject. T cells were stained with carboxyfluorescein succinimidyl ester (CFSE). Cells were plated on flat-bottomed wells precoated overnight with anti-CD3 (2.5 μg/mL) and anti-CD28 (1.2 μg/mL) mAb in PBS. In some conditions, IL8 (1,000 pg/mL) was added to the culture.

After 5 days, lymphocytes were harvested and analyzed by flow cytometry using a FACSCalibur Flow Cytometer (Becton Dickinson). Lymphocyte division was measured by CFSE dilution (10).

NET formation

To quantify NETs, 5 × 10⁴ neutrophils, GrMDSC, or MoMDSC in 100 μL of HBSS (Invitrogen) without CaCl₂/MgCl₂ were seeded in a black 96-well plate (Cayman Chemical). The cells were treated with 1 μg/mL PMA (Sigma-Aldrich), 100 ng/mL LPS (Sigma-Aldrich), IL8 (R&D Systems) at different concentrations or left untreated. An equivalent cell sample was also lysed with 0.1% Triton X-100 (Union Carbide Chemicals) or the sample was frozen as a control for the total DNA in each well. Cells were incubated at 37°C for 2 to 4 hours. The membrane-impermeable DNA-binding dye, Sytox Green (Invitrogen) was added (0.1 μmol/L) at each experimental time-point to bind extracellular DNA, and captured fluorescence was quantified using a fluorescence spectrophotometer (Fisher Scientific). In some experiments, HT29 cells labeled with PKH26 were added together with neutrophils or MDSC. To digest and destroy extracellular NET-DNA in samples, cells were incubated with 200 μg/mL of RNase-free DNase I (Roche Life Sciences) for 2 hours at 37°C. Photographs of representative fields were then taken under confocal microscopy.

LPS detection test

LPS concentrations in IL8 batches purchased from R&D Systems were below the detection limits of a sensitive commercial kit that detects up to 0.15 EU/mL (Cambrex limulus amebocyte lysate QCL-1000).

Time lapse microscopy of MDSCs NET formation

GrMDSCs were FACs sorted and stained for 15 minutes in RPMI media at 37°C with DRAQ5 (ThermoFisher) at a final concentration of 5 μmol/L. Cells were washed twice with HBSS and a total of 5 × 10⁴ cells were plated in eight-well imaging chambers (IBIDI) in a volume of 100 μL. HT29 cells were stained with PKH26 (Sigma) according to manufacturer's instructions, and added to cultures in additional 100 μL. Immediately prior to imaging Sytox Green (Invitrogen) was added in the media at a final concentration of 0.1 μmol/L and 10 ng/mL of IL8 (R&D) were added. Imaging was performed with a Zeiss Axiovert 200 microscope (Zeiss) equipped with a Perkin Elmer Spining disk unit, a Hammamatsu C9100-50 CCD camera, and a 40 × Plan Apochromat Objective (N.A. 0.95), in an incubation chamber at 37°C and 5% CO2. Twenty to twenty-four micro-meters of confocal stacks were acquired with a Z slice spacing of 2 μm. Stacks were taken every 60 seconds for a total of 5 hours. Image acquisition, Z maximum projections, video generations, and gamma adjustments were generated with UltraVIEW ERS software (Perkin Elmer). Videos were further edited and compressed using FinalCutPro software and are shown at a speed of 10 fps.

Statistical analysis

Results were presented as mean ± SD or mean ± SEM and experimental groups were compared by Mann–Whitney U test and Student t tests as indicated in figure legends.

Results

Mouse MDSC are responsive to human IL8

Mice engrafted with CT26 tumors expressing GM-CSF are known to accumulate high numbers of MDSC (40). IL8 is known to cross-react to the CXCR1 mouse receptor (10, 11). In this regard, the spleens of mice subcutaneously engrafted with CT-26 transfected to express GM-CSF (CT26-GM) contained abundant MDSC. This effect actually leads to evident splenomegaly as shown in Supplementary Fig. S1. Such splenic MDSC were gated and FACs sorted as indicated in Fig. 1A. Immunostaining with specific mAb shows that both GrMDSC and MoMDSC express CXCR1 and CXCR2 on their plasma membrane up to weak but detectable levels (Fig. 1B). In line with this finding, both the MoMDSC and the GrMDSC from the spleen of such mice migrated in response to human recombinant IL8 in a dose-dependent fashion, even though IL8 does not exist in the mouse genome. Such murine MDSC also chemotactically responded to mouse Gro-α (CXCL1), tested as a natural isospecies agonist for CXCR1 and CXCR2 (Fig. 1C).

Reparixin can block the chemoattraction of IL8 on mouse MDSC in vivo

As the IL8-mediated chemo-attraction could be advantageous to tumors, we explored the possibility of therapeutic inhibition of such a function. To do so, in immunodeficient Rag2⁻/⁻/IL2Rγ⁻/⁻ mice-bearing HT29 tumor xenografts to raise their MDSC numbers, we performed hydrodynamic gene experiments under different conditions divided by number of migrated cells in the negative control, which was serum-free RPMI medium.
transfer of IL8 to the liver. This procedure achieves transient
expression of IL8 in the liver of mice without detectable
expression in other organs (42). Indeed, hydrodynamic gene
transfer of IL8 augmented the absolute number of MDSC in
the liver of mice in the course of experiments described in
Fig. 2A. As can be seen in Fig. 2B and C, the numbers of

Figure 1.
Mouse MDSC express CXCR1 and CXCR2 and chemotactically respond to IL8. A, MDSC gating strategy following immunoﬂuorescence and FACS
analysis. B, surface immunostaining for CXCR1 and CXCR2 on MoMDSC and GrMDSC derived from the spleen of mice bearing subcutaneous
CT26-GMCSF-derived tumors for 7 days (n = 10), indicating the percentage of positive cells. The mean ﬂuorescence intensity ± SEM is also given for
each group. C, dose–response chemotaxis to IL8 and mouse Gro-α of sorted GrMDSC and MoMDSC. Results are shown as the chemotactic index.
Statistical comparisons were performed by Mann–Whitney U test.
Mo-MDSC and Gr-MDSC were clearly increased in the IL8-gene-transferred livers but not in the spleens, indicating chemotraction to the IL8-producing organ, as a result of hydrodynamic gene transfer.

Reparixin is a described pharmacological inhibitor of CXCR1 and CXCR2 (39, 43). Injection of this compound into mice around the time of IL8 gene transfer almost completely abrogated the attraction of MDSC to the liver (Fig. 2B and C). We

Figure 2. Reparixin blocks chemotaxis of MDSC to IL8. A, description of the in vivo experiment involving HT29 tumor-bearing Rag2-/- IL2Rγ-/- mice which were treated with reparixin from day 12 to 18. On day +14, mice received a hydrodynamic gene transfer procedure with an IL8-encoding expression cassette (HD IL8) or control empty plasmid. B, C, show the liver and spleen absolute numbers of MoMDSC or GrMDSC in the indicated conditions determined by flow cytometry referred to total cell counts (Neubauer chamber). Student t tests were used for statistical comparisons. D, experiment performed in BALB/c mice bearing CT26-GMCSF–derived tumors for 10 days and which were treated with control vehicle or reparixin (20 μg per dose) during the last 4 days prior to sacrifice. Total numbers of GrMDSC and MoMDSC retrieved from cell suspensions from eight tumors per group. Two repetitions of the experiments were performed and Mann-Whitney U tests were used for statistical comparisons.
performed similar experiments in tumor-free mice, which showed increases in Ly6G$^+$ CD11b$^+$ cell in the liver following IL8 gene transfer (Supplementary Fig. S2), albeit in this case such cells are likely normal granulocytes because they do not express CD124 (IL4R$\alpha$), which is considered a feature of Gr-MDSC (44).

In immunocompetent mice-bearing CT26-GM-derived subcutaneous tumors, we tested the effect of four daily repeated doses of reparixin on the intratumoral content of MDSC as assessed by FACS on tumor-derived cell suspensions. As can be seen in Fig. 2D, reparixin treatment decreases the numbers of Gr-MDSC, whereas it did not affect the numbers of Mo-MDSC in this setting.

Our results confirm that IL8 chemoattraction of MDSC can take place in in vivo settings of tumor-bearing mice and show that pharmacological inhibition of CXCR1 and CXCR2 is capable of neutralizing such a function.

**Human MDSC are attracted by IL8**

Peripheral blood from advanced cancer patients is frequently characterized by the relative abundance of myeloid populations defined as lymphoid lineage negative, CD11b$^+$, HLA-DR$^-$low, and CD33$^+$ (25). These cells are considered equivalent to mouse MDSCs (16). Although increases in number among PBMCs in cancer patients have been extensively reported, the functional effects of the human MDSC counterparts on T lymphocytes are not well explored.

In a series of metastatic stage cancer patients suffering from non–small cell lung cancer ($n = 11$) and carcinoma of the prostate ($n = 7$), the relative number of these MDSC was studied among peripheral blood leukocytes collected from centrifuged ficoll gradients. Multiple color flow cytometry permitted analyses on gated HLA-DR$^-$low, lineage negative cells, that were characterized by CD33 and CD11b-positive staining and which we considered MDSC. Among these cells, subsets were characterized by mutually exclusive coexpression of CD14bright or CD15. These cells correspond to the so-called monocytic (Mo-MDSC) and granulocytic (Gr-MDSC) MDSC subtypes. Figure 3A shows the strategy of FACS staining for sorting and Fig. 3B the relative numbers of these cells in the peripheral blood of cancer patients ($n = 18$) and healthy volunteers ($n = 10$). Relative numbers of total MDSC were much higher in this sequential series of heavy burden cancer patients ($P < 0.001$). Similar high numbers of circulating MDSC were assessed by analyzing blood leukocytes following osmotic lysis of erythrocytes without ficoll separation (Supplementary Fig. S3).

We next sought to determine if the known receptors for IL8, CXCR1 and CXCR2 were expressed on human MDSC. As can be...
Effects of IL8 on MDSC

T-cell suppressor activity of peripheral blood human MDSC is not modified by IL8

Reportedly, MDSC in mice have the ability to downregulate T-cell activation and proliferation through a number of molecular mechanisms that are exerted by cell-to-cell contact (16). Human MDSC have not been consistently tested for such an immunosuppressive activity on T-cell cultures (25). Hence, we set up co-cultures of sorted peripheral blood MoMDSC and GrMDSC from four advanced cancer patients assessing the response to their own serum or to serum from a healthy donor. Supplementary Figure S4 shows that although both subsets of MDSC migrated toward the serum of the cancer patient, this was not the case with serum of the healthy volunteers.

IL8 induces formation of NETs in human MDSC

In the recent years it has been discovered that a key mechanism in the defense against bacteria and fungi is the ability of PMN granulocytes to extrude their DNA that is decorated with antimicrobial polypeptides to entrap and kill microorganisms (30). These structures are termed NETs (31). We studied if IL8 can modify these functions.

To our surprise, freshly isolated PMN from healthy volunteers extruded NETs upon exposure to endotoxin-free recombinant IL8. In fact, the degree of NET formation was comparable to that elicited by incubation in the presence of LPS (Fig. 6A). We carefully excluded contamination of endotoxin in the rhIL8 commercial batches (see Materials and Methods).

Next, we investigated if NETs could be induced among human FACS-sorted MDSC from cancer patients. As can be seen in Fig. 6B and C, GrMDSC formed NETs in short-term cultures in the presence of endotoxin-free IL8, whereas MoMDSC failed to do so.

Figure 6D shows representative images of NETs formation as induced by the autologous serum of a cancer patient diluted 1:5. As can be seen in the figure, the extent of NETs formation induced by serum was similar to that achieved by LPS. However, in the case of patient sera, the IL8 neutralizing antibody failed to inhibit NETs formation indicating that the patients sera contains additional factors involved in NETs induction, which are absent in sera from healthy volunteers (Fig. 6D). In this setting, a series of experiments indicated that IL8 induced GrMDSC netosis in a dose-dependent manner (Supplementary Fig. S6A) and was abrogated by an anti-IL8 mAb that could not inhibit netosis induced by LPS (Supplementary Fig. S6B).

Formation of NETs as induced by IL8 in human GrMDSC was also documented by time-lapse fluorescence confocal microscopy that observed GrMDSC whose DNA had been prelabeled with DRAQ5 in medium that contained Sytox-green (a non–cell-penetrant DNA probe) to stain extracellular DNA. As can be seen in Supplementary Video S1, exposure to IL8 (10 ng/mL) results in extrusion of DNA that formed double-stained NETs in a 2- to 3-hour time frame. Such an effect was not seen if IL8 were not added to the cultures (data not shown).

In cocultures of stimulated Gr-MDSC and red-labeled HT29 cancer cells, we observed that elongated NETs seemed to entrap tumor cells when that GrMDSC extruded NETs in response to IL8 or LPS (Supplementary Fig. S6C). Supplementary video 2 (right) shows similar cocultures with time-lapse confocal microscopy clearly indicating that the formed NETs upon response to IL8 were able to entrap tumoral HT29 cells (prelabeled in red). This phenomenon of NET formation did not take place if IL8 was not added to the cultures (Supplementary Video S2, left).

According to these results, it is conceivable that both GrMDSC and neutrophils of cancer patients under the pathophysiological conditions of exposure to IL8 may be induced to extrude TRAPs with potential involvement in disease-relevant mechanisms.

Discussion

Decades ago it was discovered that the myeloid compartment of tumor-bearing mice contained subsets of cells capable of suppressing T-cell proliferation and activation (45), determining a worse course of the transplanted malignancies (21). The field of study of myeloid cells in experimental cancer has extensively shown that both tumor-associated
MDSC express CXCR1 and CXCR2 and chemotactically respond to IL8. A, surface immunostaining for CXCR1 and CXCR2 on human MDSC derived respectively from peripheral blood of advanced cancer patients (n = 6). Representative FACS histograms are included as insets. Background immunostaining was controlled by fluorescence minus 1. B, chemotactic response of GrMDSC and MoMDSC from patients or dextran-gradient purified neutrophils (PMN) from healthy donors in response to the indicated stimuli including tissue culture supernatant of colon cancer HT29 cells, IL8-containing serum from cancer patients, and recombinant IL8 used at different concentrations. When indicated, an IL8 neutralizing mAb was added to the lower transmigration chamber. C, the IL8 concentration of HT29 supernatants and patient sera used in the experiments. Results are representative of four independent repetitions. Statistical comparisons were performed by Mann–Whitney U test.
macrophages and MDSC exert protumor activities that include depressing the cellular antitumor immune response using a variety of mechanisms (16). Indeed, therapies that selectively damage such myeloid cell subsets exert therapeutic effects (18, 46) and are actively pursued in the clinical setting (47).

In humans, abundant data indicate that the accumulation of MDSC (21) and M2-polarized macrophages (15) correlates with a worse clinical outcome of the disease in a variety of cancer types (25). MDSC have also been proposed to worsen the efficacy of ipilimumab immunotherapy in melanoma, but it is unclear whether this is predictive or prognostic (21, 25).

Figure 5.
Human MoMDSC suppress T-cell proliferation in culture whereas GrMDSC do not. A, coculture experiments of CFSE-labeled PBLs and MoMDSC or GrMDSC from the same cancer patient and assessment of the level of T-cell proliferation in response to anti-CD3 and anti-CD28 mAb by CFSE dilution. Experiments represent three independent cases. B, representative histograms of a case of MoMDSC T-cell coculture in which rhIL8 failed to modify the level of suppression. This experiment was repeated three times with similar results. Mann–Whitney U tests were performed for statistical comparisons.
IL8 induces NETs formation in human neutrophils and GrMDSC. A, PMN from healthy subjects or (B) GrMDSC and (C) MoMDSC from cancer patients were exposed in culture to LPS or rhIL8. Results show representative confocal microscopy photographs of NETs and fluorimetric assessment of extracellular DNA fluorescence staining per well. Statistical comparisons were performed with Mann-Whitney U test. D, representative NET formation in GrMDSC and lack of formation in Mo-MDSC following addition of LPS, IL8-containing patient serum or serum from a healthy volunteer. Of note anti-IL8 mAb failed to completely inhibit the patient-serum induction of NETs, suggesting the presence of other factors absent in healthy serum.
Recent deconvoluted transcriptomic evidence also indicates that the presence of neutrophils (indistinguishable from GrMDSC in this large series of data) in the tumor microenvironment is a major factor correlating with worse overall survival prognosis in human cancer (48).

In this study we sought to investigate if IL8, as abundantly produced by human cancer cells across malignant diseases (1), could be a chemotactic factor attracting MDSC. In humans suffering from advanced metastatic cancer it is well known that distinct subsets of myeloid cells accumulate in peripheral blood (23). This is reminiscent of the mouse MDSC counterparts and these cells can also be classified as granulocytic and monocytic based on morphology and surface markers (25). Our data conclusively demonstrate that IL8 can attract these circulating cells expressing CXCR1 and CXCR2 on their surface, and it is tempting to speculate that the chemotactic gradient of IL8 can guide such MDSC into the tumor microenvironment. In our hands, neutrophils from healthy volunteers transmigrate in response to IL8 better than MDSC. This could be due to differences in the levels of receptor expression but also to a more fit cell motility machinery. It is also clear that other chemotactic factors could be present in the conditioned culture media and the serum of the patients resulting in incomplete inhibitions by the IL8 neutralizing antibody.

We were able to model that scenario by means of genetically enforcing the production of IL8 in mouse liver and attracting mouse MDSC to this organ. Importantly, this activity can be therapeutically interfered with using the CXCR1/2 antagonist reparixin (39, 43), thus hinting at a potential cancer immunotherapy approach. This was also seen in experiments showing the effect of reparixin at reducing the numbers of GrMDSC in C1226GM tumors implanted in immunocompetent mice. In this case, other CXCR1/2 agonist chemokines ought to be involved because mouse tumors cannot produce IL8, which is absent from the murine genome.

Apart from its effects on MDSC, IL8 has been found to attract and retain dendritic cells in the tumor (29) in a disorienting manner that eventually decreases antitumor immunity (10). Therefore, IL8 could be a multifaceted inflammatory mediator used by the tumor to simultaneously promote angiogenesis (1, 3–9), co-opt immunosuppressive populations (1, 3–9), and disrupt the routes of migration of professional antigen-presenting dendritic cells (29).

In our series of cancer patients, only MoMDSC from peripheral blood showed a T-cell suppressive activity that was not modulated by IL8. In our hands, peripheral blood GrMDSC did not exert any suppressive effect on T lymphocytes, at least in cocultures. We do not have them a clear mechanistic explanation to this is in spite of the fact that both subsets express similar levels of arginase and reactive oxygen species (data not shown). Other mechanisms remain to be explored to understand suppression by human primary MDSC and differences between subsets. These may encompass mechanisms acting both in cell-to-cell contacts and at longer distance. It is important to highlight that IL8 and the other chemokines acting via CXCR1/2 are unlikely to be the only chemotactic factors used by tumors to attract and retain MDSC.

We were able to observe T-cell suppression in culture as mediated by MoMDSC. However, on per-cell basis IL8 did not modify suppression albeit by simply attracting higher MDSC numbers, IL8 should result in stronger local T-cell suppression. In experiments performed in vitro, GrMDSC failed to suppress T-cell cultures but this does not preclude that such an activity could be exerted in vivo once conditioned by the tumor microenvironment.

In a search of activities modulated by IL8 in MDSC, we discovered that IL8 can induce the formation of NETs by neutrophils. Moreover, similar experiments performed on sorted GrMDSC from cancer patients indicated that this cell subset also protruded its DNA in a comparable fashion. We ruled out artifacts potentially related to microbial contaminants and, importantly, observed the same effect with IL8-containing serum samples from cancer patients. Of note, healthy donor serum did not induce NETs whereas cancer patient serum did. Interestingly, the IL8 neutralizing antibody did not completely block this action; thus, suggesting the presence of other NET-inducing factors in the serum of the patients.

IL8-induced formation of NETs might have profound implications for microbial defense (31). In the context of our studies, NETs may be involved in the propensity of cancer patients to develop thromboses (49) and in favoring the adhesive mechanisms of metastasis (16, 37). Indeed, it has recently been described that neutrophil-extruded NETs favor the nesting of circulating tumor cells in the liver sinusoids of mice (37, 50). In our ex vivo experiments, we observed that MDSC extruded NETs in response to IL8 and thereby could entrap cancer cells ex vivo by adhesive mechanisms in coculture (Supplementary Fig. S6C). Supplementary Video S2 shows similar entrapping events in cocultures with time-lapse confocal microscopy. It is early to speculate about the functional and pathological in vivo consequences of these findings. Indeed, nothing is known in cancer patients of what the consequences of MDSC attraction and activation to form NETs could be. However, our results further argue in favor of the convenience of pharmacologically inhibiting IL8 as previously carried out in xenografted mouse models and currently in clinical trials with neutralizing antibodies (51) and small drug inhibitors (52).

As a whole, our study unambiguously shows that IL8 is a powerful chemotactic stimulus for peripheral patient-derived MDSC and that, to our surprise, IL8 directly induces the formation of NETs out of GrMDSC.

Disclosure of Potential Conflicts of Interest
I. Melero is a consultant/advisory board member for Boehringer Ingelheim, Bristol-Myers Squibb, Incyte, Novartis, and Roche-Genentech. No potential conflicts of interest were disclosed by the other authors.

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