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Inflammatory Blood Monocytes Contribute to Tumor Development and Represent a Privileged Target To Improve Host Immunosurveillance

Séverine Augier,†,*‡ Thomas Ciucci,†,*‡ Carmelo Luci,†,*‡ Georges F. Carle,†,*‡ Claudine Blin-Wakkach,†,*‡ and Abdelilah Wakkach*†

Progressing tumors in humans and mice are frequently infiltrated by a highly heterogeneous population of inflammatory myeloid cells that contribute to tumor growth. Among these cells, inflammatory Gr-1⁺ monocytes display a high developmental plasticity in response to specific microenvironmental signals, leading to diverse immune functions. These observations raise the question of the immune mechanisms by which inflammatory monocytes may contribute to tumor development. In this study, we found that adoptive transfer of normal inflammatory Gr-1⁺ monocytes in tumor-bearing mice promotes tumor growth. In this tumoral environment, these monocytes can differentiate into tolerogenic dendritic cells (DCs) that produce IL-10 and potently induce regulatory T cell responses in vivo. Moreover, diverting the differentiation of Gr-1⁺ monocytes into tolerogenic DCs by forced expression of IL-10 soluble receptor and IL-3 in tumor cells improves host immunosurveillance by reducing the regulatory T cell frequency and by inducing immunogenic DCs in the tumor. As a consequence, tumor growth is strongly reduced. Our findings indicate that Gr-1⁺ monocytes represent a valuable target for innovative immunotherapeutic strategies against cancer. The Journal of Immunology, 2010, 185: 7165–7173.

The link between inflammation and promotion of cancer was first observed in the 1800s, and recent epidemiological studies strongly support this concept (1, 2). However, although it is generally accepted that inflammation enhances tumor progression, the cellular mechanisms involved in the link between inflammation and cancer are not well understood.

Monocytes are critical for both innate and adaptive immunity, and are a population of mononuclear leukocytes that develop in the bone marrow from dividing monoblasts, and are released in the bloodstream as nondividing cells. The recruitment of monocytes into tissues is a cardinal feature of inflammation (3). Two populations of monocytes have been described in the mouse. The major population is positive for the myeloid markers CD11b and Gr-1, and is considered an inflammatory monocyte population, whereas a minor population is Gr-1 negative and characterized by a CX3CR1-dependent recruitment into noninflamed tissues (4–6). Interestingly, the Gr-1⁺ monocytes are actively recruited to inflammatory locations and have in vivo the potential to differentiate into dendritic cells (DCs) (6–8). On Leishmania major infection, these monocyte-derived DCs (mo-DCs) have the ability to induce T cell immunity against pathogens (9). A more recent study by Gunn’s group reported that after viral infection or immunization, Gr-1⁺ monocytes became inflammatory DCs that produced abundant IL-12p70 and potently stimulated Th1 responses (10). Otherwise, the injection of aluminum hydroxide (alum adjuvant) promotes Ag uptake by recruited Gr-1⁺ monocytes that become inflammatory DCs inducing persistent Th2 responses (11). These observations reveal the high plasticity of Gr-1⁺ monocytes depending on different contexts. Nevertheless, the fate of Gr-1⁺ monocytes in other immunological contexts, such as cancer, remains unknown. Furthermore, the contribution of Gr-1⁺ monocytes to the induction of T cell tolerance is still a matter of debate (12).

The induction of tolerance is critical for the maintenance of immune homeostasis. Accumulating evidence suggests that CD4⁺ regulatory T cells (Tregs) are key mediators of peripheral tolerance. Two major Treg populations have been described so far, and are designated as naturally occurring CD4⁺CD25⁺ Tregs (13, 14) and inducible Tregs or IL-10–secreting T regulatory type 1 (Tr1) cells (15–17). As such, Tregs may also block antitumor immune responses. Particularly in the context of cancer, Treg frequency and function are important because they might favor tumor development (18). Furthermore, Tregs are thought to dampen T cell immunity and to be the main obstacle tempering successful immunotherapy and active vaccination (19).

Since the 1980s, accumulating evidence demonstrates that a population of myeloid cells, recently termed myeloid-derived suppressor cells (MDSCs), is expanded in spleens and bone marrows of animals bearing tumors. Moreover, several studies suggest that these MDSCs have the ability to suppress T cell responses causing immune suppression in animal models of cancer (20). MDSCs are a heterogeneous population of cells of myeloid origin that comprise myeloid progenitors and immature myeloid cells, immature granulocytes, and immature DCs (20). Nevertheless, the exact nature

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Abbreviations used in this paper: BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; MDSC, myeloid-derived suppressor cell; MHC-I, MHC class I; mo-DC, monocyte-derived dendritic cell; TBM, tumor-bearing mouse; TFM, tumor-free mouse; Tr1, T regulatory type 1; Treg, regulatory T cell.

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and origin of the cell population responsible for suppression within the MDSs remain elusive.

DCs play a pivotal role in the initiation, programming, and regulation of tumor-specific immune responses (21, 22). Nevertheless, the role of the different DC subsets in cancer initiation and progression, as well as the role of tolerogenic DCs in cancer-induced immunosuppression, have to be more deeply understood. Rather, most of the efforts have been focused on increasing the immunostimulatory power of fully activated DCs for immunotherapy of cancer (23–25). Despite this effort, a full understanding of the complex relations between tumors and their hosts, including DC-mediated regulation of host leukocyte responses, is likely to improve the precision and effectiveness of cancer immunotherapy.

DC phenotypes in cancer tissue and cancer-draining lymph nodes often correspond to resting or immature DCs, both in tumor-bearing animals and in patients with cancer. The observation that tumor-infiltrating DCs were immature may be linked to the production of IL-10, TGF-β, and PGE₂ by tumor cells that impair DC function (26). Thus, lack of activation of DCs within tumors could explain why the immune response against the tumor is switched off.

To better understand the relations between inflammation and cancer, we have explored the involvement of inflammatory Gr-1⁺ monocytes in tumor progression and T cell tolerance in a mouse model of cancer. In this paper, we report that adoptive transfer of normal inflammatory Gr-1⁺ monocytes in tumor-bearing mice (TBMs) promotes tumor growth. In this tumor context, inflammatory Gr-1⁺ monocytes were capable of differentiating into tolerogenic DCs that produced IL-10 and potently induced Treg responses and expansion. To confirm our finding, we diverted Gr-1⁺ monocytes from tolerogenic toward immunogenic DCs using a plasmid vector encoding IL-10 soluble receptor and IL-3. Interestingly, this procedure improves host immunosurveillance by reducing the Treg frequency and by inducing immunogenic DCs in the tumor. As a consequence, tumor growth is strongly reduced. Our findings indicate that Gr-1⁺ monocytes could serve as valuable targets for innovative immunotherapeutic strategies against cancer.

Materials and Methods

Mice

Six- to 8-week-old BALB/cJ were purchased from Janvier laboratory (Le Genest Saint Isle, France). DO-11-10 mice were bred and maintained in the specific pathogen-free animal facility of the Institut Fédératif de Recherche 50 (Nice, France) in accordance with institutional guidelines and under veterinary supervision.

Tumorigenicity assay in syngeneic immunocompetent mice

Syngeneic C26 colon adenocarcinoma cells were kindly provided by Dr. Mario Colombo (Milan, Italy) and maintained as previously described (27). Tumorigenic activity of C26 cells was assayed in mice injected s.c. in the left flank of BALB/c with 5 × 10⁵ cells in 0.1 ml. Tumor growth and size were recorded at 2- to 3-d intervals using calipers.

Abs, cell purification, and flow cytometric analysis

All Abs (with the exception of anti-TIRC7 and CX3CR1) were purchased from BD Biosciences (Le Pont De Claix, France): anti-CD3 (17A2), anti-CD4 (H129.19), anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-CD19 (1D3), anti-CD25 (7D4), anti-CD11c (HL3), anti-CD31 (MEC13.3), anti-CD49b (DX5), anti-CD62L (Mel-14), anti-CD80 (16-10A1), anti-CD11b (AFS98), anti-Ly6C (AL-21), B220, anti-Gr1 (RB6-8C5), anti-IAA (2G9), and anti-CD210 (1B1.3a). Polyclonal anti-TIRC7 Ab was prepared in rabbit against (Mab Trap kit; Amersham, Orsay, France). The polyclonal anti-CX3CR1 (Eurogentec, Angers, France). The rabbit antisera were purified by affinity chromatography.

Vector construction and stable transfection of C26 cells

cDNA for extracytoplasmic region of IL-10R and full cDNA for IL-3 were amplified by RT-PCR from activated splenic T cells. cDNA for extracytoplasmic region of IL-10R was ligated into AvrII and XbaI in the first mult cloning site of pVIVO2, a multigenic cloning plasmid for strong expression in vivo (InvivoGen, San Diego, CA). cDNA for IL-3 was cloned into BamHI and NcoI in the second mult cloning site of pVIVO2 containing the extracytoplasmic region of IL-10R. The pVIVO2 plasmid containing both inserts so called p310R was sequenced and then transfected stably into C26 using hygromycin B in selective medium. Single resistant colonies were isolated, expanded, and screened by ELISAs for coated magnetic beads (Invitrogen, Cergy Pontoise, France), after incubation with mAb mixture containing anti-CD1, anti-CD2, and anti-IAA. After immunomagnetic bead depletion, monocytes were sorted using FACSaria (BD Biosciences) based on their low expression of CD11c, with a purity >98%.

The sorted monocytes were labeled with red-orange calcein tracer (Molecular Probes, Cergy Pontoise, France) and 10⁶ cells were then adoptively transferred i.v. into normal and TBMs at days 3 and 7 after tumor injection, as indicated in Fig. 2A.

Purification of red-orange calcein-positive cells

Cells from spleens of BALB/cJ and TBMs were depleted by treatment for 30 min at 4°C with a mAb mixture containing anti-CD11b, anti-CD49b, and anti-CD3 and separation with anti-rat Ig-coated magnetic beads (Dynal).

The tumor tissues were collected, washed in PBS, cut into pieces, and resuspended in DMEM supplemented with 1% FCS and 1 mg/ml collagenase D for 20 min at 37°C, to isolate cells from the tumor tissue. The cell suspension was collected after 20 min, and the single-cell suspension was stained with appropriate Abs. Anti-CD11b and red-orange calcein tracer are used to analyze transferred monocytes. The combination of anti-CD3, anti-CD4, and anti-CD8 is used to sort CD8⁺ T lymphocytes from the tumor for their antitumor capacity.

FACS analysis was performed using a FACSscan (BD Biosciences) with CellQuest software (Le Pont De Claix, France). Orange calcein cells from spleens and tumor tissues were sorted on a FACS Aria (BD Biosciences). All populations were >98% pure on reanalysis.

Isolation and proliferation of Tregs

Sorted CD25⁺CD4⁺ T cells (2.5 × 10⁶) from spleen of DO-11-10 mice were labeled with CFSE (Molecular Probes) by incubating in PBS 1× (Cambrex, East Rutherford, NJ) with 10 μg/ml CFSE for 10 min at 37°C, and then transferred intravenously into BALB/c mice. Sorted orange calcein⁺ cells (5 × 10⁵) were incubated with OVA peptide at 4°C for 2 h, washed, and injected intravenously into the T cell-transferred mice. Five days later, CFSE dilution of OVA-specific T cells was analyzed by flow cytometry.

In vitro Treg functional assay

The suppressive assay was performed as previously described (28).

DCs derived from bone marrow

Bone marrow-derived DCs (BMDcs) were generated from bone marrow cells, as described previously (29). BMDcs were harvested on day 6 and were purified using CD11c microbeads (Miltenyi Biotec, Paris, France).

Cytokine assays

Sandwich ELISAs were used to measure IL-10 as described previously (28) and IL-12p70 was measured using IL-12p70 ELISA set (from BD Biosciences) following the manufacturer’s instructions.

For intracellular cytokine assays, two rounds of stimulation of 2 × 10⁵ naive OVA-specific T cells from DO11-10 mice were performed with 5 × 10⁶ DCs sorted from the spleen of tumor-free mice (TFMs) and 5 × 10⁵ total DCs or mo-DCs sorted from spleen or tumor tissue of TBMs, in the presence of OVA peptide. Fourteen days later, cells were stimulated for 4 h by PMA/ionomycin in the presence of stop Golgi (BD Bioscience) according to the manufacturer’s instructions. Cells were then labeled with an anti-CD4 Ab, fixed, permeabilized, and stained for intracellular cytokines IL-10 and IFN-γ. The results were analyzed by flow cytometry and were gated on CD4⁺ T cells.

Histological examination

After sacrifice, tumors were removed from mice and fixed in nitrogen, sectioned at 5 μm, and stained with anti-CD11c.

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IL-3 production and for IL-10 inhibition by sandwich ELISAs; sandwich ELISAs were used to measure IL-3 concentration in supernatant of different clones of C26 transfected stably with p310R using IL-3 ELISAs set according to the manufacturer’s protocol (BD Biosciences). The expression of soluble IL-10R was evaluated by the competition between the supernatant of the clones (containing soluble IL-10R) and a biotinylated anti-IL-10 Ab in sandwich ELISAs.

In vitro cytotoxic assay

Enriched tumor-infiltrating DCs (10^5 cells) were isolated from C26-p310R mice with anti-CD11c magnetic beads (Miltenyi Biotech) and injected into the footpad of BALB/c mice. Five days later, lymphocytes from popliteal lymph nodes were isolated and used as responders.

For CTL activity, irradiated C26 cells (2000 rad) were divided into two populations and labeled with CFSE (Molecular Probes) at concentrations of 2 μM (CFSE<sub>low</sub>) or 0.2 μM (CFSE<sub>high</sub>), CFSE<sub>high</sub> cells were pulsed with 1 μg/ml of the AH-1 specific peptide (SPSYVYHQF) corresponding to the immunodominant MHC class I (MHC-I) restricted Ag of C26 colon tumor (30) for 1 h at 37˚C in the dark, whereas CFSE<sub>low</sub> cells remained unpulsed. The two C26 populations were then washed, mixed at equal numbers (10^5 cells), and cultured for 3 d with T lymphocytes from popliteal lymph nodes at different ratios, as indicated, or with tumor-infiltrating CD8<sup>+</sup> T cells sorted by FACSAria as described earlier. Percentage of Ag-specific lysis was calculated with the following formula as previously described (31): % of Ag-specific lysis = (1 − %CFSE<sub>low</sub>/ %CFSE<sub>high</sub>)(%CFSE<sub>high</sub> CTL × %CFSE<sub>low</sub> CTL) × 100, where CTL is the control (corresponding to C26 populations cultured in absence of T cells).

Results

Characterization of blood Gr-1<sup>+</sup> monocytes

To characterize the phenotype of inflammatory monocytes in normal mouse peripheral blood, we depleted blood cells of MHC-II<sup>+</sup>, CD11c<sup>+</sup>, B220<sup>+</sup>, and CD3<sup>+</sup> cells, after red cell lysis. Flow cytometric analysis of monocytes on the basis of their CD11b versus CD115 expression showed that the Gr-1<sup>+</sup> monocytes were capable to reach the tumor site where they predominately differentiated into CD11c<sup>+</sup> cells (Fig. 2B, 2C). In contrast, in the same conditions, the CD11b<sup>+</sup>Gr1<sup>high</sup> and the CD11b<sup>+</sup>Gr1<sup>low</sup> cells did not differentiate into CD11c<sup>+</sup> cells (Supplemental Fig. 1).

Gr-1<sup>+</sup> mo-DCs present in the spleen and in the tumor were characterized by a low expression of MHC-II and high expression of CD80 (Fig. 2D). Furthermore, they were positive for the endothelial marker CD31 (Fig. 2D). Such an endothelial-like phenotype of tumor-infiltrating DCs has already been reported in TBMs (34). This result suggests an endothelial-like specialization of part of the mo-DCs and their potential participation in tumor angiogenesis.

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We next analyzed the influence of mo-DCs on the priming and differentiation of naive CD4\(^+\) T cells. Two rounds of stimulation of naive OVA-specific T cells were performed with DCs sorted from the spleen of normal BALB/c (TFMs) and mo-DCs sorted from spleen and tumor tissue of TBMs, in the presence of OVA peptide. Our results showed that mo-DCs from both tumor tissue and spleen of TBMs induced the differentiation of naive T cells into Tr1 cells, which secreted IL-10 and IFN-\(\gamma\) (Fig. 3B). In contrast with mo-DCs from TBMs, splenic DCs from TFMs primed Th1 cells that secreted only IFN-\(\gamma\) (Fig. 3B). These results show that, under tumor conditions, blood Gr-1\(^+\) monocytes have the potential to differentiate into IL-10–producing DCs that express low levels of MHC-II and to induce Tr1 cells. These properties are hallmark of tolerogenic DCs and suggest that the IL-10–producing mo-DCs facilitate tumor growth by providing immune suppression.

**Gr-1\(^+\) mo-DCs induced CD4\(^+\)CD25\(^+\) Treg proliferation in vivo**

The results from our study on Tr1 cell priming by mo-DCs prompted us to analyze their influence on natural Tregs (CD4\(^+\)CD25\(^+\) Treg) in vivo. FACS analysis showed an increase in the number of CD4\(^+\)CD25\(^+\) Tregs in spleen from TBMs as compared with control spleen from TFMs (Fig. 3C). As expected, the lymphocytes that accumulated in the tumor mainly consisted in CD4\(^+\)CD25\(^+\)Foxp3\(^+\) Tregs (∼70%; Fig. 3C). Analysis of their suppressive activity showed that CD4\(^+\)CD25\(^+\) Tregs from tumor tissue, as well as CD4\(^+\)CD25\(^+\) Tregs from spleen of TBMs and TFMs, were capable of suppressing the APC- and anti–CD3-driven proliferation of CD25\(^+\)CD4\(^+\) T cells in vitro (Fig. 3D), confirming their Treg function. These observations suggested that an increased Treg number also contributes to the suppression of the immune response at the tumor site.

We next addressed the relation between the mo-DCs and the accumulation of Tregs at the tumor site. To analyze the proliferation of CD4\(^+\)CD25\(^+\) Tregs, we transferred CFSE-labeled splenic OVA-specific CD4\(^+\)CD25\(^+\) Tregs from D011-10 mice into normal BALB/c (TFMs). These transferred mice were then injected with purified splenic CD11c\(^+\) orange calcine-labeled cells from TBMs, pulsed in vitro with OVA peptide, as indicated in Fig. 4A. Five days later, we analyzed the proliferation of Tregs in the spleen of transferred mice. FACS analysis showed that CD11c\(^+\) orange calcine-labeled cells from the spleen of TBMs stimulated the proliferation of CFSE-labeled OVA-specific CD4\(^+\)CD25\(^+\) Tregs after adoptive transfer in BALB/c mice (TFMs; Fig. 4B). Collectively, these data indicate that Tregs proliferate on stimulation by the mo-DCs from TBMs.

We next investigated the contribution of IL-10 on the in vitro proliferation of CD4\(^+\)CD25\(^+\) Tregs elicited by mo-DCs from TBMs. CFSE-labeled OVA-specific CD4\(^+\)CD25\(^+\) Tregs cocultured with CD11c\(^+\) orange calcine-labeled cells purified from the spleen of TBMs were capable of proliferating in the presence of OVA peptide (Fig. 4C). The addition of blocking mouse anti–IL-10R Ab decreased significantly the CD4\(^+\)CD25\(^+\) Treg proliferation. In contrast, the addition of neutralizing mouse anti–TGF-\(\beta\) Ab had no effect on CD4\(^+\)CD25\(^+\) Treg proliferation (Fig. 4C). Thus, IL-10 appears to be determinant in the costimulation of CD4\(^+\)CD25\(^+\) Treg proliferation in our model. Furthermore, transfer of MDSCs from IL-10\(^{-/-}\) mice into TBMs led to an increased survival of these mice compared with TBMs transferred with MDSCs from wild-type control mice underlying the essential role of IL-10 (Supplemental Fig. 2). Taken together, these data indicate that normal Gr-1\(^+\) monocytes, in a tumor context, were able to differentiate into IL-10–producing DCs that induce Treg proliferation and facilitate tumor growth.

**Characterization of Gr-1\(^+\) mo-DCs**

The specific function of each DC subset is dictated by distinct cytokine profiles and resulting T cell polarization. In a first set of experiments, we analyzed by ELISA the secretion of IL-10 and TNF-\(\alpha\) from spleen and tumor of TBMs. As reported in Fig. 3C, CD11c\(^+\) monocytes from TBMs secreted high levels of IL-10 and TNF-\(\alpha\) (Fig. 3C). Analysis of their suppressive activity showed that CD4\(^+\)CD25\(^+\) Tregs from tumor tissue, as well as CD4\(^+\)CD25\(^+\) Tregs from spleen of TBMs and TFMs, were capable of suppressing the APC- and anti–CD3-driven proliferation of CD25\(^+\)CD4\(^+\) T cells in vitro (Fig. 3D), confirming their Treg function. These observations suggested that an increased Treg number also contributes to the suppression of the immune response at the tumor site.

Lastly, transfer of Gr-1\(^+\) monocytes into TBMs increased the volume of the tumor (Fig. 2E). These data support the hypothesis that normal Gr-1\(^+\) monocytes from blood circulation promote tumor growth.

**Fig. 2.** Differentiation potential of blood monocytes in TBMs. A, C26 colon carcinoma cells (5 × 10⁵) were injected s.c. in the left flank of BALB/c mice. Tumor growth and size were recorded at 2- to 3-d intervals using calipers. Sorted blood monocytes were labeled with orange calcine tracer, and 10⁷ cells were then adoptively transferred i.v. into TBMs as indicated. B, Orange calcine-positive cells from spleen and tumor of TBMs were analyzed for the expression of CD11c at day 14 after C26 cell infection by cytometric analysis. C, Immunohistology analysis of CD11c expression in tumor tissue. D, Flow cytometric analysis of IA\(^\beta\), CD80, and CD31 gated on orange calcein CD11c\(^+\) cells. E, Tumor growth in TBMs injected or not with blood Gr-1\(^+\) monocytes. Results were presented as the mean of five mice per group. All experiments were repeated three times with five mice per group and with similar results.

We next analyzed the influence of mo-DCs on the priming and differentiation of naive CD4\(^+\) T cells. Two rounds of stimulation of naive OVA-specific T cells were performed with DCs sorted from the spleen of normal BALB/c (TFMs) and mo-DCs sorted from spleen and tumor tissue of TBMs, in the presence of OVA peptide. Our results showed that mo-DCs from both tumor tissue and spleen of TBMs induced the differentiation of naive T cells into Tr1 cells, which secreted IL-10 and IFN-\(\gamma\) (Fig. 3B). In contrast with mo-DCs from TBMs, splenic DCs from TFMs primed Th1 cells that secreted only IFN-\(\gamma\) (Fig. 3B). These results show that, under tumor conditions, blood Gr-1\(^+\) monocytes have the potential to differentiate into IL-10–producing DCs that express low levels of MHC-II and to induce Tr1 cells. These properties are hallmark of tolerogenic DCs and suggest that the IL-10–producing mo-DCs facilitate tumor growth by providing immune suppression.

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Diverting the differentiation of Gr-1+ monocytes into tolerogenic DCs by expression of IL-10 soluble receptor and IL-3 in tumor cells improves host immunosurveillance and reduces tumor growth

Based on these results, we have constructed a plasmid vector containing the cDNAs for IL-10 soluble receptor, to neutralize proximal IL-10 secretion, and IL-3, to promote myeloid cell development, referred to as p310R. C26 tumor cells were transfected with the p310R plasmid and selected in the presence of hygromycin. This approach was based on the observation that culture of normal splenocytes in the presence of IL-3 leads to an enrichment of CD11b+Gr1+ cells able to differentiate into DCs in the presence of OVA peptide. Seven days later, cells were stimulated, stained with CD4 Ab, fixed, permeabilized, and stained for intracellular cytokine assay using FITC- or PE-conjugated specific mAbs as indicated. One representative experiment out of three is shown. C, CD4+ T cells from spleens and tumors were enriched by depletion with a mixture of Abs specific for CD8, CD19, CD11b, and Dx5. Enriched CD4+ T cells were analyzed by flow cytometry for CD4, CD25, and Foxp3 expression. D, In vitro suppressive activity of sorted CD4+CD25+ T cells. CFSE-labeled CD25+ CD4+ responder T cells were stimulated for 4 d with anti-CD3 mAb and irradiated APCs either alone (CD25−) or in the presence of an equal number of sorted CD4+CD25+ T cells from spleen of TFM, spleen of TBMs, and tumor tissue. The results of three independent experiments are shown.

Clones obtained from antibiotic-resistant cultures were screened for IL-3 and IL-10 soluble receptor production by ELISA. We chose a stable clone with the greatest amount of IL-3 (10 ng/ml from 0.5 × 10^6 cells) and greatest percentage of IL-10 inhibition (80%). We also verified that both clones, transfected with p310R (C26-p310R) or with plasmid alone (C26-pvivo2), have the same rate of proliferation in vitro (not shown).

To assess the effect of p310R on tumor growth, we injected normal BALB/c mice s.c. with C26-p310R or C26-pvivo2 cell clones. The resultant tumor growth curves indicated that the expression of p310R had a significant effect on tumor growth in vivo, leading to a tumor inhibition in 70% of the treated mice (Fig. 5A, 5B). Equivalent results were obtained using another clone of C26-p310R (data not shown). Survival was also improved considerably with survival rate of 100% on day 60 for mice injected with C26-p310R cells, whereas 100% of C26-pvivo2–treated mice succumbed before day 28 (Fig. 5C).

Furthermore, flow cytometric analysis of immune cells from spleens of C26-p310R and C26-pvivo2 mice, showed that the percentage of Gr1+CD11b+ myeloid cells corresponding to MDSC and reflecting the immunosuppressive status (20) is dramatically reduced in the spleens of C26-p310R mice (Fig. 5D).

Next, we analyzed the myeloid cells present in the C26-p310R tumor. Interestingly, transfection of C26 cells with the p310R vector also decreased MDSC accumulation in the tumor (Fig. 6A). Thus, we wondered whether expression of this vector affected the phenotype of the Gr1+CD11b+ cells. FACs analysis of the tumor-infiltrating Gr1+CD11b+ cells from C26-p310R mice showed that the majority of cells were CD11b+high and MHC-II+high compared with tumor-infiltrating Gr1+CD11b+ cells from C26-pvivo mice (Fig. 6A). This upregulation of CD11c and MHC-II in C26-p310R mice suggested that myeloid cells had acquired an immunogenic
phenotype. Moreover, the induction by the p310R vector of a block in tolerogenic DC differentiation cannot be excluded. Recently, we have shown that the majority of tumor-infiltrating Tregs consist of effector cells characterized by the expression of TIRC7, a new surface marker, and the secretion of high amounts of IL-10 (28). As expected, analysis of tumor-infiltrating T cells revealed a high percentage of TIRC7+ Tregs in C26-pvivo control mice, whereas this population was dramatically decreased in C26-p310R mice (Fig. 6B). This result suggests a break of immunosuppression by the expression of p310R. Furthermore, TIRC7low CD25low CD4+ is accumulated in C26-p310R mice compared with the C26-pvivo mice (Fig. 6B), suggesting that these cells may participate in the antitumoral response as antitumor CD4+ Th cells.

To assess the ability of tumor-infiltrating DCs from C26 mice to prime specific CTL activity response in vivo, we injected CD11c+ cells enriched from p310R tumors in the footpad of naive BALB/C mice. Five days later, T lymphocytes were purified from the popliteal lymph nodes. These lymphocytes were cultured at a different ratio with irradiated CFSEhigh C26 cells loaded with the AH1 immunodominant peptide of C26 cells (target cells) as described previously (31). Unloaded CFSElow C26 cells served as internal control. Our results showed that these lymphocytes elicited specific cytolytic effect revealed by the diminished proportion of the CFSehigh labeling (Fig. 6C, 6D). We have also tested directly the tumor-killing properties of CD8+ T cells isolated from C26-p310R and C26-pvivo2 mice. Fig. 6E shows, indeed, that sorted CD8+ T cells from C26-p310R tumors have a lytic function compared with those from C26-pvivo2 tumors. However, we have not detected any cytolytic function of accumulated TIRC7low CD25low CD4+ T cells in C26-p310R tumors (data not shown).

Altogether, our results show: 1) blood inflammatory monocytes can promote tumor growth via differentiation into IL-10–producing mo-DCs that, in turn, induce Treg accumulation; 2) they can be diverted from this differentiation toward immunogenic myeloid cells by the expression of IL-10 soluble receptor and IL-3; and 3) this diversion is associated with improved host immunosurveillance and reduced tumor growth.

Discussion

Emerging evidence suggests that blood monocytes possess the potential to differentiate into DCs with different functions in vivo during infection and inflammatory reactions. During Listeria monocytogenes infection, blood monocytes egress massively from bone marrow to the bloodstream and differentiate via a MyD88-dependent mechanism into DCs producing TNF-α and NO, termed Tip-DCs (6, 35). The main function of these inflammatory mo-DCs is to kill bacteria rather than to regulate T cell functions. However, during Leishmania infection, monocytes were recruited to the dermis and differentiated into dermal mo-DCs that produced IL-12 and induced Th1 responses (9). And recent study by Gunn’s group indicates that inflammatory monocytes, after viral infection or immunization, became inflammatory DCs that produced abundant IL-12p70 and potently stimulated Th1 responses (10). Interestingly, the injection of aluminum hydroxide (alum adjuvant) promotes Ag uptake by recruited monocytes that became inflammatory DCs inducing a persistent Th2 responses (11). Altogether, these observations give evidence for the plasticity of inflammatory monocytes.
inflammatory monocytes with their environment during the infectious process and in inflammatory reactions.

In this study, we have shown an additional aspect of the plasticity of blood inflammatory monocytes. Our results revealed that these monocytes can reach the spleen and tumor site of TBMs, thus becoming IL-10–producing mo-DCs that potently induce Treg responses. These observations mean that conversion of monocytes into mo-DCs is not always advantageous for the host as described earlier. We noted that adoptively transferred monocytes fail to reach the spleen of TFM. A recent study showed that grafted inflammatory blood monocytes shuttle back to the bone marrow in the absence of inflammation (33). However, the mechanism whereby monocytes accumulate or expand in the spleen of TBMs remains to be determined.

Our findings suggest that the IL-10–producing mo-DCs acquire an endothelial-like specialization because they express CD31 molecules and probably participate in tumor growth and tumor angiogenesis. Consistent with this hypothesis, the transfer of blood inflammatory monocytes into TBMs directly promotes tumor growth (Fig. 2E). Our data are supported by the recent work from Lin’s group that showed the importance of Gr-1+CD11b+ immature myeloid cells or MDSCs, from spleens of TBMs, in tumor growth (36). There are two mechanisms by which MDSCs may promote tumor growth and enhance tumor angiogenesis. First, MDSCs produce high levels of matrix metalloproteinase-9, an important mediator in angiogenesis (37, 38). Second, MDSCs can directly incorporate into tumor endothelium in vivo (36).

During tumor progression, Tregs accumulate in tumors and secondary lymphoid organs (18, 28). It has been suggested that the CCL22 produced by tumor cells or associated macrophages recruits Tregs into the tumor bed (39). Treg accumulation could also result from the priming and differentiation of naive CD4+CD25 T lymphocytes into Tregs, a phenomenon well established in vitro (40) and on homeostatic proliferation in vivo (41). In this study, we demonstrate that the Treg accumulation in tumor and spleen largely resulted from their local proliferation by IL-10–producing monocytes.
mo-DCs in vitro and in vivo. These results are in line with the studies from Ghiringhelli in other tumor-bearing models (42). Contrary to this work, we found that the proliferation of Tregs is IL-10 dependent rather than TGF-β dependent in our tumor model.

Collectively, these findings strongly suggest that IL-10 was one of the factors responsible for the induction of a refractory state that favors tumor growth in vivo. Indeed, it has become increasingly clear that successful cancer immunotherapy will be possible only with a strategy that involves the elimination of suppressive factors. As Gr-1+ monocytes can differentiate into IL-10-producing mo-DCs in a tumor context, these monocytes represent a valuable target for innovative immunotherapeutic strategies against cancer. Therefore, diverting the differentiation of Gr-1+ monocytes from tolerogenic DCs toward immunogenic DCs should not only improve host immunosurveillance but reduce tumor growth.

Our findings show that expression of the p310R plasmid, containing the cDNAs for IL-10 soluble receptor and IL-3, in tumor cells had a significant antitumor effect in vivo and improved host immunosurveillance. Indeed, the expression of p310R induced a dramatic decrease of MDSC accumulation in the tumor and the spleen that caused potent suppression of tumor immunity. Interestingly, we have found that the majority of tumor-infiltrating Gr-1+ CD11b+ cells are CD11c+ and MHC-II+ from C26-p310R mice. This phenotype is characteristic of conventional and immunogenic DCs. Moreover, these CD11c+ cells are able to prime cytotoxic T lymphocytes responses (Fig. 6C, 6D). Supporting these observations, recent studies have demonstrated that CpG plus anti–IL-10R Ab treatment induced robust antitumor therapeutic activity (15, 43). Indeed, this treatment reverted ineffective tumor-infiltrating DCs into DCs expressing high levels of MHC-II and triggered de novo IL-12 production (34, 43).

Selective accumulation of Tregs in the tumor environment can be one important local suppressive factor of immune responses against a strong tumor Ag, leading to progressive growth of cancer in the immune-competent hosts (44). Recently, we have shown that the majority of tumor-infiltrating Tregs consist of effector cells characterized by the expression of TIRCD7, a new surface marker, and the secretion of high amounts of IL-10 (28). Remarkably, these tumor-infiltrating Tregs were dramatically decreased in C26-p310R mice showing a reversing immunosuppression, without having directly targeted Tregs. Indeed, targeting Tregs with anti-CD25 mAb in tumor models resulted in tumor regression but significantly increased susceptibility to autoimmune complications (19, 45). Our findings show for the first time, to our knowledge, that targeting suppressive molecules such as IL-10 modulate Tregs and may point the way to successful application for tumor immunotherapy.

In conclusion, this work has uncovered what we believe is a role for blood Gr-1+ inflammatory monocytes in tumor development through their differentiation into IL-10–producing DCs. Interestingly, diverting this differentiation by expression of IL-10 soluble receptor and IL-3 in tumor cells improves host immunosurveillance and reduces tumor growth. Our findings indicate that Gr-1+ monocytes could be used as a valuable target for innovative immunotherapeutic strategies against cancer.

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Disclosures

The authors have no financial interests of conflict.

References


Figure S1: Fate of $\text{Gr}^{1\text{high}}\text{CD}11^{b+}$ and $\text{Gr}^{1+}\text{CD}11^{b-}$ cells.

A: After labelling with calcein orange, $\text{Gr}^{1\text{high}}\text{CD}11^{b+}$ neutrophils or $\text{Gr}^{1+}\text{CD}11^{b-}$ cells were adoptively transferred into TBM. No calcein labelling was detected in endogenous dendritic cells 1 week after the second transfer (as shown in figure 2a of the manuscript), indicating that none of the transferred population differentiated into dendritic cells. B: $\text{Gr}^{1+}\text{CD}11^{b-}$ cells were labelled with CD19 and CD3 antibodies and were analyzed by flow cytometry.
Figure S2: Effect of IL-10 on the survival of mice injected with MDSC.
Mice were injected SC with $5 \times 10^5$ C26 cells. At day 3 and 7 after C26 cells injection, they received an IV injection of $10^6$ MDSCs isolated from +/- or IL-10/-/- mice tumor bearing mice. Their survival was analyzed up to 35 days. At this time, all mice from the MDSC +/- group were died, whereas no mortality was observed in the MDSC IL-10/-/- group.