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CD11b$^+$/Gr-1$^+$ Immature Myeloid Cells Mediate Suppression of T Cells in Mice Bearing Tumors of IL-1β-Secreting Cells[1]

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Tumor cells secreting IL-1β are invasive and metastatic, more than the parental line or control mock-transfected cells, and concomitantly induce in mice general immune suppression of T cell responses. Suppression strongly correlates with accumulation in the peripheral blood and spleen of CD11b$^+$/Gr-1$^+$ immature myeloid cells and hematological alterations, such as splenomegaly, leukocytosis, and anemia. Resection of large tumors of IL-1β-secreting cells restored immune reactivity and hematological alterations within 7–10 days. Treatment of tumor-bearing mice with the physiological inhibitor of IL-1, the IL-1R antagonist, reduced tumor growth and attenuated the hematological alterations. Depletion of CD11b$^+$/Gr-1$^+$ immature myeloid cells from splenocytes of tumor-bearing mice abrogated suppression. Despite tumor-mediated suppression, resection of large tumors of IL-1β-secreting cells, followed by a challenge with the wild-type parental cells, induced resistance in mice; protection was not observed in mice bearing tumors of mock-transfected fibrosarcoma cells. Altogether, we show in this study that tumor-derived IL-1β, in addition to its proinflammatory effects on tumor invasiveness, induces in the host hematological alterations and tumor-mediated suppression. Furthermore, the antitumor effectiveness of the IL-1R antagonist was also shown to encompass restoration of hematological alterations, in addition to its favorable effects on tumor invasiveness and angiogenesis that have previously been described by us. *The Journal of Immunology,* **2005, 175: 8200–8208.**

Interleukin-1 is abundant at tumor sites, being secreted by the malignant cells or microenvironmental cellular elements, in response to local inflammatory signals. IL-1 has pleiotropic effects on malignant processes, ranging from promoting invasiveness and metastasis to induction of antitumor cell immunity and inhibition of tumor growth (reviewed in Refs. 1–4). The mechanisms whereby IL-1 induces these diverse effects on malignant processes are not yet understood.

IL-1 is a pleiotropic cytokine that primarily affects inflammatory responses, immune reactivity, and hemopoiesis (1–4). The potency of IL-1 stems mainly from its ability to induce the secretion of cytokines, chemokines, and proinflammatory molecules, and the expression of adhesion molecules in diverse cells, and thereby amplify and sustain its responses. The IL-1 family consists of two major agonistic proteins, namely IL-1α and IL-1β, and one major antagonistic protein, the IL-1R antagonist (IL-1Ra),[5] which binds to IL-1Rs without transmitting an activation signal. In their recombinant form, IL-1α and IL-1β bind to the same receptors and exert similar biological activities. However, IL-1α and IL-1β differ dramatically in the subcellular compartments in which they are active (1–4); IL-1α is mainly active in its cytosolic precursor form (pre-IL-1α), as a membrane-associated form, and to a limited extent in its secreted mature form, while IL-1β is only active as a mature secreted molecule. In addition, we have demonstrated that in vivo, in steady state homeostasis, and in inflammation, IL-1α and IL-1β are differentially expressed in tissues, possibly pointing to their different physiological roles (5, 6).

By using fibrosarcoma cells overexpressing active forms of the IL-1 molecules, we have demonstrated that the specific subcellular compartmentalization of IL-1 molecules, in the producing tumor cell and its microenvironment, dictates unique in vivo biological effects on the malignant process (reviewed in Ref. 1). Thus, overexpression of the precursor of IL-1α by fibrosarcoma cells, which induces expression of the cytokine in the cytosol and on the cell membrane, results in the loss of tumorigenicity due to the development of antitumor cell immunity (7–13). In contrast, IL-1β transfectants (mature form), which secrete the cytokine, grow progressively and are more invasive than the parental wild-type cells (11, 13). The highest invasiveness patterns were observed in fibrosarcoma cell lines transfected with a hybrid cDNA encoding for the mature form of IL-1β linked to a signal sequence (ssIL-1β), which results in effective secretion of the cytokine through the endoplasmic reticulum-Golgi pathway (13). In mice bearing invasive tumors of IL-1β-secreting cells, increased invasiveness correlates with enhanced angiogenesis, but also with tumor-mediated suppression that inhibits tumor cell-specific as well as polyclonal T cell mitogenic responses (13). Thus, membrane-associated

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[5] Abbreviations used in this paper: IL-1Ra, IL-1R antagonist; BM, bone marrow; CFU-C, CFU-culture; i.f.p., intrafootpad; ssIL-1β, signal sequence IL-1β.

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IL-1α efficiently activates immune cells in a juxtacrine manner, via ligation of IL-1Rs that are abundantly expressed on immune cells, and functions as a focused adjuvant. On the contrary, secretable IL-1β, which acts in broad paracrine and endocrine manners, is strongly proinflammatory, and thus potentiates tumor angiogenesis and the production of a network of invasiveness-promoting molecules, and also activates mechanisms of tumor-mediated suppression (1–4, 11, 13, 14). In accordance, the correlation between local expression of IL-1β at tumor sites and increased invasiveness has been established in experimental tumors and in cancer patients, in which it indicates bad prognosis (reviewed in Refs. 1 and 2). However, there are no reports on IL-1β as a mediator of tumor-mediated suppression.

Suppressor and regulatory cell circuits that arise in tumor-bearing subjects include $CD4^+CD25^+$ T cells and Th3 cells (15–18), tumor-associated macrophages (19), NKT cells (20–22), Gr-1^+CD11b^+ immature myeloid precursor cells (reviewed in Refs. 23–25), and possibly also other cells. Suppressor cells affect components of the immune system either by cell-to-cell interactions or by the secretion of inhibitory cytokines (i.e., IL-10 and TGF-β), acting to specifically or generally suppress immune responses, mainly cell-mediated immunity. Understanding the mechanisms of tumor-mediated immune suppression is thus essential for establishing optimal conditions for application of novel immunotherapeutic modalities in cancer patients.

There is now ample evidence that tumor growth in cancer patients and mice is associated with hematological alterations that include the accumulation of immature myeloid precursor cells in the bone marrow and spleen (reviewed in Refs. 23–25). In mice, these cells have the phenotype of granulocyte/macrophage precursors, and they express the granulocyte Gr-1 and macrophage Mac-1 (CD11b) markers, respectively. Such immature myeloid cells are an intrinsic part of the normal process of myelopoiesis, and they are present in relatively small numbers in naive hosts. Their number significantly increases in tumor-bearing subjects, but also during bacterial and parasitic infections, immunization with potent Ags in intensive immunization protocols, superantigens, and other conditions associated with impaired immune reactivity, such as cyclophosphamide treatment and lymphoid irradiation (reviewed in Refs. 23–25). Although the involvement of Gr-1^+CD11b^+ cells in tumor-mediated suppression has been established, the signals that induce the proliferation of these immature myeloid cells and the mechanisms by which they operate are not completely understood.

In this study, we show that IL-1β of tumor cell origin stimulates in tumor-bearing mice hematological alterations, including extramedullary myelopoiesis, manifested by extensive accumulation in the spleen of Gr-1^+CD11b^+ immature myeloid cells that induce tumor-mediated suppression. Our results also point to the therapeutic potential of the IL-1Ra, a physiological inhibitor of IL-1, to intervene in tumor-mediated immune suppression, and thus enable more efficient use of immunotherapeutic treatments in tumor-debulked subjects.

Materials and Methods

Mice

NFS/N mice were obtained from I. Fossar Larson (Animal Section, The Fibiger Institute, Copenhagen, Denmark) and subsequently bred at the animal facilities of the Faculty of Health Sciences, Ben-Gurion University. NFS/N mice, which are syngeneic to NIH/3T3 fibroblasts and transformed cell lines derived from them, were used in this study (26). Either female or male mice, 6–12 wk, were used in the experiments. The studies have been reviewed and approved by the Ben Gurion University Animal Committee.

Cell lines

The generation of fibrosarcoma cell lines transfected with the cDNAs of the active forms of the IL-1 molecules, i.e., the precursor of IL-1α, the mature form of IL-1β, and the mature form of IL-1β fused to a signal peptide (ssIL-1β), was previously described by us (13).

In this study, we have mainly used ssIL-1β-transfected cell lines. ssIL-1β-transfected cell lines were obtained in several transfection experiments, and displayed the same characteristics of invasive growth and induction of tumor-mediated suppression. In most experiments, the ssIL-1β1-transfected cell line was used. In experiments presented in Fig. 6, two ssIL-1β-transfected cell lines, ssIL-1β1 and ssIL-1β2, were used. ssIL-1β-transfected cell lines secrete ~3–5 ng/ml IL-1β/10^6 cells/24 h in culture. For comparison, we have used the appropriate mock-transfected cells.

Assessment of tumor development

Malignant cells (2×10^5 cells/mouse) were injected intrafootpad (i.f.p.) in 50 μl of PBS. Local tumor growth was determined 2–3 times per week by caliper measurements of the diameter of footpads.

Proliferation of murine spleen cells

For proliferation assays, single spleen cell suspensions, in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, penicillin G (100 U/ml), streptomycin (100 μg/ml), and 2-ME (2×10^{-5} M), were plated in 96-well plates (2×10^6 cells/ml) in a volume of 0.1 ml/well. All medium ingredients were purchased from Biological Industries. This medium will be subsequently termed as complete RPMI 1640. The cells were cultured in triplicates for 48 h in the presence of Con A (2.5 μg/ml; Sigma-Aldrich) and pulsed with [^{3}H]TdR (1 μCi/well, 38.0 Ci/mmol; Amersham Pharmacia) for the final 18 h of incubation. Subsequently, the cells were harvested onto glass fiber paper and radioactivity was measured using a liquid scintillation counter (Wallac).

ELISAs for cytokine measurements

Murine IFN-γ and IL-2 levels were measured using commercial ELISA kits (BD Pharmingen), according to the manufacturer's instructions. Briefly, 96-well plates were coated overnight with the relevant Abs; thereafter, the plates were washed with PBS/Tween 20 and blocked for 2 h at 37°C with PBS plus 10% FCS. After two additional washings, samples and standards were added to plates for overnight incubation at 4°C, followed by four washings. Biotinylated Abs were added to plates for 1 h of incubation at 37°C. The plates were then washed six times, and streptavidin-HRP conjugate (Jackson ImmunoReseach Laboratories) was added for 1 h at 37°C. After eight washes, the substrate tetramethylbenzidine (BD Pharmingen) was added for color development. The color reaction was stopped by adding 1 N H_2SO_4, and absorbance was scored at a wavelength of 450 nm with correction from 650 nm.

Cytofluorimetric analyses

Cells (5×10^5/sample) were blocked with anti-FcRIII (BD Pharmingen) for 30 min, followed by staining with FITC anti-mouse CD11b (clone ICRF 44, rat IgG2b) and/or PE anti-mouse Gr-1 (clone RB6-8C5, rat IgG2b) Abs (BD Pharmingen) for 1 h. After multiple washes, cells were analyzed on the FACS (BD Biosciences). Cells were kept on ice throughout the staining and analysis procedure.

Cocultures of naive spleen cells and spleen cells from tumor-bearing mice

Normal spleen cells were mixed with splenocytes from tumor-bearing mice at the ratios indicated. The mixed spleen cells were cocultured in complete RPMI 1640 medium, and proliferation and cytokine production were assessed, as described. In addition, chambers with polyvinylpyrolidone-free polycarbonate Transwell membrane filters of 6.5 mm diameter and pore size of 0.3 μm (Corning Glass) were used in some experiments. The normal spleen cells were loaded in the lower chambers, while splenocytes from tumor-bearing mice were loaded in the upper chambers. The Transwell membrane filters allow trafficking of soluble molecules, but prevent direct contact of cells in the upper and lower chambers. After coculture, splenocytes from the lower chambers were collected and replated, and Con A-induced responses were assessed, as described above.

In vitro depletion of $CD11b^+$/Gr-1^+ immature myeloid cells

Murine spleen cells, following separation on Ficoll-Hypaque, were suspended in RPMI 1640 with 0.3% BSA (Sigma-Aldrich) and adjusted to

1×10^7 cells/ml. Then, Abs against mouse Gr-1 (50 μg/ml; BD Pharmingen) were added to the cells for 1 h at 4°C. Afterward, cells were centrifuged and resuspended to the original volume in RPMI 1640 with 0.3% BSA containing rabbit complement (1:20 v/v; Cedarlane Laboratories) for 1 h at 37°C, followed by extensive washes. Depletion efficiency was monitored by cytofluorimetric analyses, following staining with anti-Gr-1 Abs.

Formation of granulocyte/macrophage colonies by myeloid precursor cells in soft agar cultures

Colony formation by granulocyte/macrophage precursors was performed in 35-mm culture plates, as described (27). Thus, plates were poured with a base agar layer (2 ml/plate, final agar concentration 0.5% w/v, Bacto-agar; Difco Laboratories) also containing 20% (v/v) conditioned medium obtained from L929 fibroblasts as a source of CSF and 20% FCS. Plates were left at room temperature to solidify and then overlaid with an upper agar layer (0.33% w/v) containing RPMI 1640, 20% FCS, and the target cells (1×10^6/ml PBMC or spleen cells and 5×10^4/ml bone marrow cells) (1 ml of upper agar layer/plate). After solidification at room temperature, plates were incubated at 37°C in a humidified incubator in an atmosphere of 5% CO_2. Colonies were counted after 7–10 days, using an inverted microscope. Only colonies consisting of 50 or more cells were counted.

In vivo treatment of mice with the IL-1Ra

Mice were injected with ssIL-1β cells, as was indicated above. Multiple injections of the IL-1Ra (100 μg/mouse, i.p.) were applied, starting 1 day before the inoculation of the malignant cells and then on days 1, 3, 5, 7, 9, 11, and 13, as previously described by us (14). Mice were sacrificed on day 15.

Statistical analyses

Each experiment was repeated at least three to five times with a similar pattern of responses. In vivo experiments consisted of 5–10 mice in each experimental group. Shown are results from pooled or single representative experiments. Shown are mean values ± SD. Significant differences in results were determined using the two-sided Student's t test; a $p < 0.05$ was considered significant.

Results

Time-course kinetics of tumor-mediated suppression in mice bearing tumors of IL-1β-secreting cells

Previously, we have reported that fibrosarcoma cell lines, transfected with hybrid cDNA encoding for the mature form of IL-1β linked to a signal sequence (ssIL-1β), which results in effective secretion of the cytokine through the endoplasmic reticulum-Golgi pathway, display increased invasiveness and metastasis, and they also induce tumor-mediated suppression in the host (13). In this study, we have assessed some of the mechanisms of tumor-mediated suppression that is induced by IL-1β derived from malignant cells.

In a time-course kinetics experiment, it was observed that Con A-induced T cell proliferation (Fig. 1*A*), IFN-γ (Fig. 1*B*), and IL-2 (Fig. 1*C*) secretion gradually decrease as tumors consisting of cells secreting IL-1β develop. Suppression starts to appear as early as 10 days after tumor cell injection, when tumors begin to develop, and is most pronounced, in this set of experiments, on days 14 and 18 (~75–90% inhibition) in mice bearing large overt tumors. In contrast, no significant suppression of Con A-induced responses was observed in mice bearing tumors of mock-transfected cells (Fig. 1) or the parental malignant cells (results not shown).

Suppression of Con A-induced T cell responses was completely ablated after tumor resection; in this set of experiments, responses were normalized 7 days after removal of the primary tumor.

Hematological alterations in mice bearing tumors of IL-1β-secreting cells

In our experimental system, tumor-mediated suppression correlated in tumor-bearing mice with splenomegaly. As IL-1β potentiates hemopoiesis, and especially myelopoiesis, we evaluated, in spleens of mice bearing tumors of IL-1β-secreting cells, immature myeloid cells that are CD11b$^+$ (Mac-1$^+$) and Gr-1$^+$ double positive. As can be seen in Fig. 2*A*, a high proportion (>40%) of CD11b$^+$/Gr-1$^+$ cells was observed in PBMCs from mice bearing tumors of IL-1β-secreting cells, whereas remarkably fewer immature myeloid cells (<10%) are present in normal mice and mice bearing tumors of mock-transfected cells. The proportion of CD11b$^+$/Gr-1$^+$ cells was ~25% in splenocytes isolated from mice bearing tumors of IL-1β-secreting cells, 15–18 days after injection of malignant cells, while only a very low proportion (<5%) of CD11b$^+$/Gr-1$^+$ immature myeloid cells was observed in spleens from normal mice or mice bearing tumors of mock-transfected cells (Fig. 2*B*).

In a time-course kinetics experiment, to characterize hematological alterations in mice bearing tumors of IL-1β-secreting cells, it was shown that the number of CD11b$^+$/Gr-1$^+$ immature myeloid cells steadily increased in the blood (Fig. 3*A*) and spleen (Fig. 3*B*) along with tumor progression. This increase was also accompanied by leukocytosis (Fig. 3*C*), anemia (Fig. 3*D*), and splenomegaly (Fig. 3*E*).

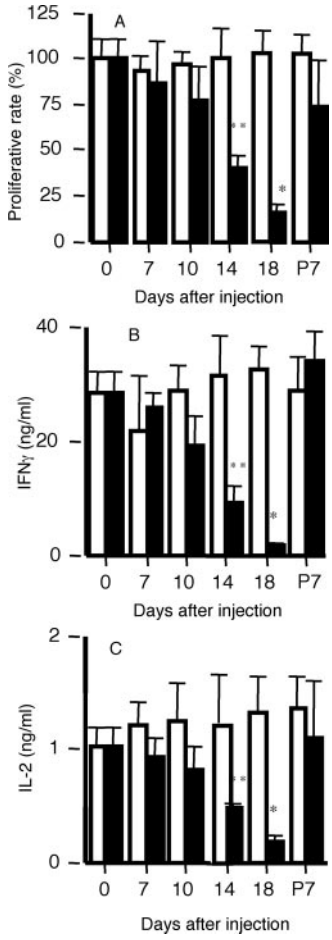

FIGURE 1. Time-course kinetics of Con A-induced responses of spleen cells from mice bearing tumors of IL-1β-secreting cells. Spleen cells (2.0×10^6/ml) from mice at different intervals after i.f.p. injection of ssIL-1β-transfected cells (■), or mock-transfected cells (□) (2×10^5/mouse), were assessed for proliferation (*A*), IFN-γ (*B*), and IL-2 (*C*) secretion upon Con A stimulation. Primary tumors were resected on day 18 after tumor cell inoculation in a group of mice, and 7 days later Con A-induced responses of spleen cells from these mice were also assessed (P7). Proliferation rates of spleen cells from tumor-bearing mice are presented as the percentage of Con A-induced proliferation of normal spleen cells (day 0). Results are presented as the mean ± SD of three experiments. *, Statistical significance: $p < 0.01$ vs the appropriate controls in each panel.

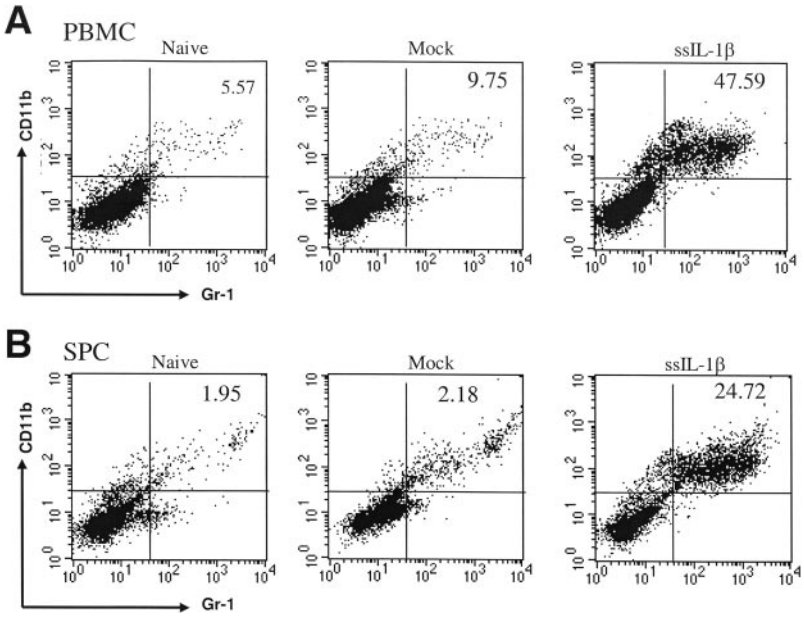

FIGURE 2. Accumulation of CD11b$^+$/Gr-1$^+$ immature myeloid cells in the blood and spleen of mice bearing tumors of IL-1β-secreting cells. PBMC (*A*) and spleen cells (SPC) (*B*) from mice 15–18 days after injection of mock- or ssIL-1β-transfected cells, as indicated in legend to Fig. 1, or naive mice were assessed by FACS analysis. The cells were stained with FITC anti-CD11b and PE anti-Gr-1 Abs. Shown are results of a single experiment of five performed.

All of these hematological alterations were not observed in untreated mice (day 0) or in mice bearing tumors of mock-transfected cells, and they reverted to control levels in mice bearing tumors of IL-1β-secreting cells after tumor resection, in this set of experiments on day 12.

Myeloid precursor cells form colonies in soft agar cultures supplemented with a source of CSF. These colonies arise from single cells, and are referred to as CFU-culture (CFU-C), which proliferate and differentiate in culture to mature granulocytes and/or macrophages. In a time-course kinetics experiment, using soft agar cultures supplemented with conditioned medium derived from L929 fibroblasts, we have assessed the levels of myelopoietic CFU-Cs in the bone marrow (BM), peripheral blood, and spleen of mice bearing tumors of IL-1β-secreting cells. As shown in Fig. 4, and similarly to other hematological alterations that are observed in mice bearing tumors of IL-1β-secreting cells, the numbers of granulocyte/macrophage CFU-Cs in the peripheral blood and spleen steadily increased along with tumor progression, whereas the number of myeloid progenitors in the BM was reduced. The latter fact indicates that concomitantly with extensive myelopoiesis in the BM, immature granulocyte/macrophage precursor cells are released at an accelerated rate into the blood and then seed in the spleen, where they possibly further expand. Granulocyte/Macrophage CFU-C levels returned to steady state levels after tumor resection. These fluctuations in the levels of granulocyte/macrophage precursor cells were not observed in control mice (day 0) or mice bearing tumors of mock-transfected cells. As already indicated, these hematological alterations were normalized ~7–12 days after resection of the primary tumor.

Secretion of IL-1β by the malignant cells, rather than an increase in tumor burden, mediates hematological alterations and immune suppression in mice bearing tumors of IL-1β-secreting cells

Secretion of IL-1β by the malignant cells increases their invasiveness and metastatic potential (13). It was thus of importance to assess whether tumor-mediated suppression is due to IL-1β secretion by the malignant cells or due to an increased tumor burden in mice bearing tumors of IL-1β-secreting cells that are more invasive than tumors of mock-transfected cells. For this purpose, we assessed, in a comparative manner, immune suppression and hematological alterations in mice with similar tumor burdens. Thus, mock-transfected tumor cells were injected into mice 10 days before injection of IL-1β-secreting fibrosarcoma cells. At intervals of 22–23 days and 13–14 days after injection of mice with mock-transfected cells and IL-1β-transfected cells, respectively, the tumor size in both groups was similar (~15 mm diameter), which indicates a similar tumor burden. In these mice, the proportion of CD11b$^+$/Gr-1$^+$ cells, hematological parameters, and colony formation in soft agar cultures by granulocyte/macrophage precursors in the BM, peripheral blood, and spleen were evaluated. The results demonstrate that in mice bearing tumors of IL-1β-secreting cells, a series of hematological alterations were observed. As already indicated, these include splenomegaly, leukocytosis, anemia, and increased numbers of CD11b$^+$/Gr-1$^+$ immature myeloid cells in the blood and spleen as well as increased levels of granulocyte/macrophage CFU-Cs in the blood and spleen, but fewer in myeloid precursors in the BM (Table I). No hematological alterations were observed in mice bearing tumors of mock-transfected cells. A marked suppression of Con A-induced proliferative responses of splenocytes from mice bearing tumors of IL-1β-secreting cells was observed, while no suppression was evident in spleen cell cultures from mice bearing tumors of mock-transfected cells or from untreated control mice (Table I).

As tumors develop locally in the footpad and hematological/immunological alterations were observed in peripheral tissues, it is reasonable that systemic secretable factors of malignant cell origin mediate tumor suppression as well as the hematological alterations. In the serum, IL-1β was detected in high levels in mice bearing tumors secreting human IL-1β, while no systemic murine IL-1β could be detected in sera of mice bearing tumors of mock-transfected cells or normal control mice (Table I). In the sera of mice, we could not detect other cytokines involved in hemopoiesis, such as GM-CSF, IL-6, murine IL-1α, and IL-1β or immune suppressive cytokines, such as IL-10 and TGF-β (results not shown).

The IL-1Ra attenuates tumor growth and hematological alterations in the spleen of tumor-bearing mice

To further substantiate the role of systemic IL-1β in tumor invasiveness and in inducing hematological alterations, mice were systematically treated with the IL-1Ra (multiple injections) during the

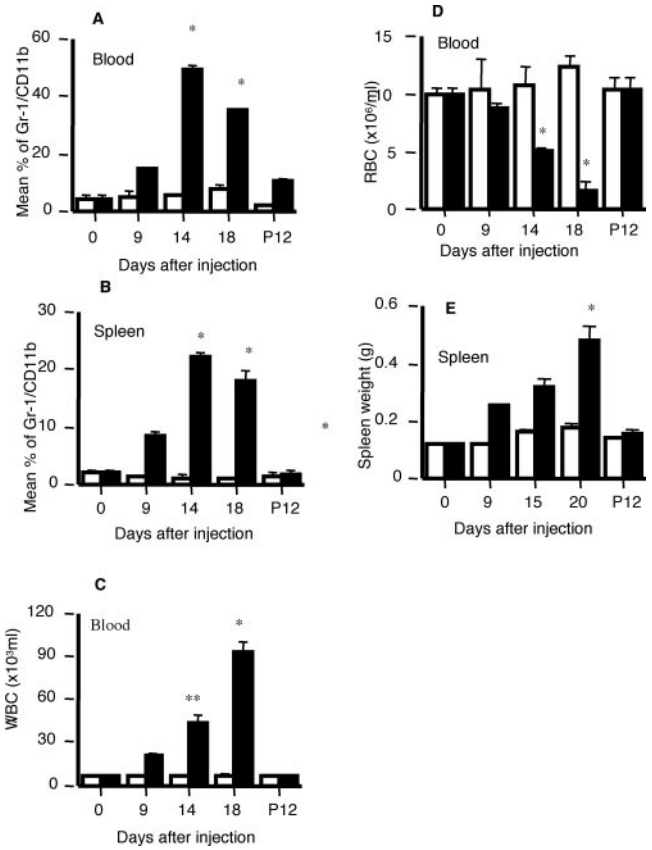

FIGURE 3. Altered hematological parameters in mice bearing tumors of IL-1β-secreting cells. Proportion of CD11b$^+$/Gr-1$^+$ myeloid cells in the peripheral blood (*A*), proportion of CD11b$^+$/Gr-1$^+$ myeloid cells in the spleen (*B*), number of white blood cells in blood (*C*), number of RBC in the peripheral blood (*D*), and weight of spleens (*E*). Hematological changes were monitored at different time intervals in mice injected with ssIL-1β-transfected cells (■) or mock-transfected cells (□), as indicated in legend to Fig. 1. Primary tumors were resected on day 18 after tumor cell inoculation in a group of mice, and 12 days later blood and spleen cells from these mice were assessed for the indicated parameters (P12). Results are presented as the mean ± SD of three experiments. Statistical significance: *, $p < 0.01$ and **, $p < 0.05$ vs the appropriate controls in each panel.

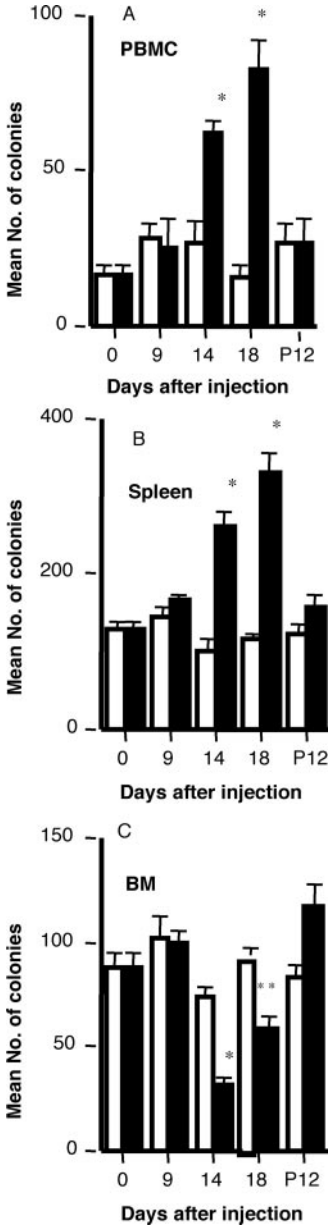

FIGURE 4. Number of granulocyte/macrophage CFU-C in the BM, spleen, and peripheral blood from mice bearing tumors of IL-1β-secreting cells. Macrophage/Granulocyte precursor cells were assessed, using the soft agar assay, in a time-course kinetics experiment in the peripheral blood (*A*), spleen (*B*), and bone marrow (*C*) from mice bearing tumors induced by ssIL-1β-transfected cells (■) or mock-transfected cells (□), under conditions described in legend to Fig. 1. Primary tumors were resected on day 18 after tumor cell inoculation in a group of mice, and 12 days later the spleen cells from these mice were also assessed for granulocyte/macrophage CFU-C content (P12). Results are presented as the mean ± SD of three experiments. Statistical significance: *, $p < 0.01$ and **, $p < 0.05$ vs the appropriate controls in each panel.

growth of tumors of IL-1β-secreting cells, according to the protocol indicated in *Materials and Methods*. This resulted in reduction in the size of the tumor, the weight of the spleen, and the levels of CD11b$^+$/Gr-1$^+$ immature myeloid cells in the spleen (Table II). The IL-1Ra did not alter the growth of mock-transfected cells (results not shown). Taking into account the very high systemic levels of IL-1β in the sera of tumor-bearing mice (~2 ng/ml) and the short $t_{1/2}$ of the IL-1Ra in the serum, we concluded that the dose of IL-1Ra used for treatment was too low. However, despite this, the IL-1Ra caused significant effects on the reduction of the invasiveness of the malignant cells and on the normalization of the hematological alterations in tumor-bearing mice.

Identification of CD11b$^+$/Gr-1$^+$ immature myeloid cells from mice bearing tumors of IL-1β-secreting cells as mediators of suppression of in vitro Con A-induced T cell activation

To verify that CD11b$^+$/Gr-1$^+$ immature myeloid cells indeed play a role in tumor-mediated suppression, we tested whether spleen cells from tumor-bearing mice, enriched in CD11b$^+$/Gr-1$^+$ immature myeloid cells, would suppress Con A-induced responses of normal spleen cells. For this aim, we added graded numbers of spleen cells from tumor-bearing mice to normal spleen cells and assessed T cell proliferation and IFN-γ secretion in response to Con A. As can be seen in Fig. 5*A*, spleen cells from mice bearing tumors of IL-1β-secreting cells suppressed the proliferative response of normal splenocytes in a dose-dependent manner. Maximal suppression was observed at a 1:1 cell ratio. No suppression was induced by spleen cells from mice bearing tumors of mock-transfected cells. Similar patterns of suppression were observed for IFN-γ secretion in cocultures of normal spleen cells and spleens

Table I. *Hemological alterations in mice bearing similar ssIL-1β- or mock-transfected tumor burdens*[a]

	Normal	Mock	ssIL-1β
Tumor			
Days after injection	—	22–23	13–14
Diameter	—	14.9 ± 0.4	14.5 ± 0.3
Peripheral blood			
RBC($\times 10^6/\mu$l)	7.01 ± 0.43	5.93 ± 2.78	3.84 ± 0.51[b]
WBC($\times 10^3/\mu$l)	6.18 ± 0.27	5.75 ± 0.53	49.67 ± 2.75[b,c]
CD11b$^+$/Gr-1$^+$ (%)	6.20 ± 1.23	9.30 ± 2.43	44.30 ± 3.34[b,c]
Spleen			
Weight (g)	0.12 ± 0.01	0.2 ± 0.01	0.38 ± 0.02[b,c]
CD11b$^+$/Gr-1$^+$ (%)	2.71 ± 0.60	3.16 ± 0.53	26.80 ± 2.31[b,c]
CFU-Cs			
Blood (1×10^6)	15.0 ± 3.5	11.7 ± 4.4	64.3 ± 2.8[b]
Spleen (1×10^6)	126.0 ± 3.5	119.0 ± 3.0	279.0 ± 15.1[b]
Bone marrow (5×10^4)	92.0 ± 4.0	99.3 ± 14.3	39 ± 6.9[b]
Serum IL-1β (ng/ml)	—	—	1.8 ± 0.3
T cell proliferation (%)	100	105 ± 1.5	15 ± 2.5[b,c]

[a] Mock-transfected tumor cells were injected i.f.p. 10 days before the injection of ssIL-1β-transfected cells (2×10^5/mouse). Twenty-two to 23 days and 13–14 days after injection of mice with control tumor cells and ssIL-1β-transfected cells, respectively, the tumor size in both groups of tumor-bearing mice was similar (around 15 mm), indicating a similar tumor burden. In these mice, hemological parameters, numbers of granulocyte/macrophage CFU-Cs in the blood, spleen and bone marrow, systemic IL-1β levels and Con A-induced T cell proliferation were assessed as described in *Materials and Methods.*

[b] A value of $p < 0.01$, vs the group of normal mice.

[c] A value of $p < 0.01$, vs the group of mice bearing tumors induced by mock-transfected cells.

from tumor-bearing mice (Fig. 5*B*). In this figure, shown are patterns of IFN-γ secretion by spleen cells from tumor-bearing mice and normal mice as well as mixtures of normal splenocytes from control mice and splenocytes from tumor-bearing mice. Similar patterns of suppression of Con A responses were also observed with lymph node cells from mice bearing tumors of IL-1β-secreting cells (results not shown).

Separation of the normal spleen cells and splenocytes from tumor-bearing mice in Transwell cocultures, which allow diffusion of soluble materials, but prevent cell-to-cell interactions, significantly abrogated suppression (Fig. 5*C*). This indicates that cell-to-cell contact between the suppressor cells and the target T cells is required for the induction of suppression. However, this does not exclude the possibility that soluble factors generated by the suppressor cells synergize with cell-to-cell contact signals that are obligatory for inducing suppression.

To directly show that the suppressive effects in splenocytes from mice with tumors of IL-1β-secreting cells are due to CD11b$^+$/Gr-1$^+$ immature myeloid cells, we depleted splenocytes from tumor-bearing mice, with anti-Gr-1 Abs and complement, before assessing their suppressive capacity. Most CD11b$^+$/Gr-1$^+$ immature myeloid cells (>90%) were depleted by this treatment protocol. After depletion, splenocytes of mice bearing tumors of IL-1β-secreting cells lost their ability to suppress the Con A-induced T cell proliferative responses of normal splenocytes (Fig. 5*D*). These results demonstrate that indeed CD11b$^+$/Gr-1$^+$ immature myeloid cells are responsible for mediating suppression in mice bearing tumors of IL-1β-secreting cells.

Table II. *IL-1Ra attenuates IL-1β-induced tumor invasiveness and hemological alterations in the spleen*[a]

	ssIL-1β	ssIL-1β + IL-1Ra
Tumor size (mm)	14 ± 1.03	8 ± 0.7**
Spleen weight (g)	0.35 ± 0.07	0.24 ± 0.036**
CD11b$^+$/Gr-1$^+$ (%) in the spleen	28.4 ± 1.12	20.4 ± 0.48**

[a] Mice were injected i.f.p. with 5×10^5 tumor cells. Part of the mice were treated with IL-1Ra, as indicated in *Materials and Methods.* Mice were opened on day 15. **$p < 0.01$, vs the group of nontreated mice.

In mice bearing tumors that secrete IL-1β, an immune memory develops, despite tumor-mediated suppression

Mice were initially injected with tumor cells secreting IL-1β (two cell lines), the wild-type parental cells and mock-transfected cells. Primary tumors were resected on day 18, and mice were left to recover for 3 mo and then challenged with the wild-type parental cells. Tumor development was assessed after 45 days. As a negative control, mice were injected with a cell line transfected with the precursor of IL-1α, the expression of which was shown to increase the immunogenicity of the malignant cells and to induce tumor regression, accompanied by an immune memory, which protects mice from a challenge with the wild-type parental cells (1, 7–13). As a positive control, a group of naive mice was freshly injected with the wild-type cells on the day of challenge. As can be seen in Fig. 6, all naive mice injected with the wild-type cells developed tumors. Most mice that had been injected with the wild-type tumor cells or mock-transfected cells, before tumor amputation, were not protected and developed progressive tumors upon challenge (Fig. 6). In mice that were injected with IL-1α-expressing cells, before challenge in the protocol indicated above, protection was observed, in accordance to our previous results (13). To our surprise, mice, which had been injected with IL-1β-secreting cells, and had developed progressive tumors, before tumor resection, were significantly protected when challenged with wild-type invasive parental cells. Thus, in mice injected with tumor cells secreting IL-1β, tumor-mediated suppression does not impair the development of immune memory; it is possibly the function of antitumor effector mechanisms that is inhibited.

Discussion

In the present study, we have demonstrated that fibrosarcoma cells that actively secrete IL-1β induce generalized suppression that is mediated by CD11b$^+$/Gr-1$^+$ immature myeloid cells. Immature CD11b$^+$/Gr-1$^+$ myeloid cells are comprised of heterogeneous subpopulations that include precursor cells of granulocytes and macrophages, myeloid BM-derived dendritic cells, as well as other

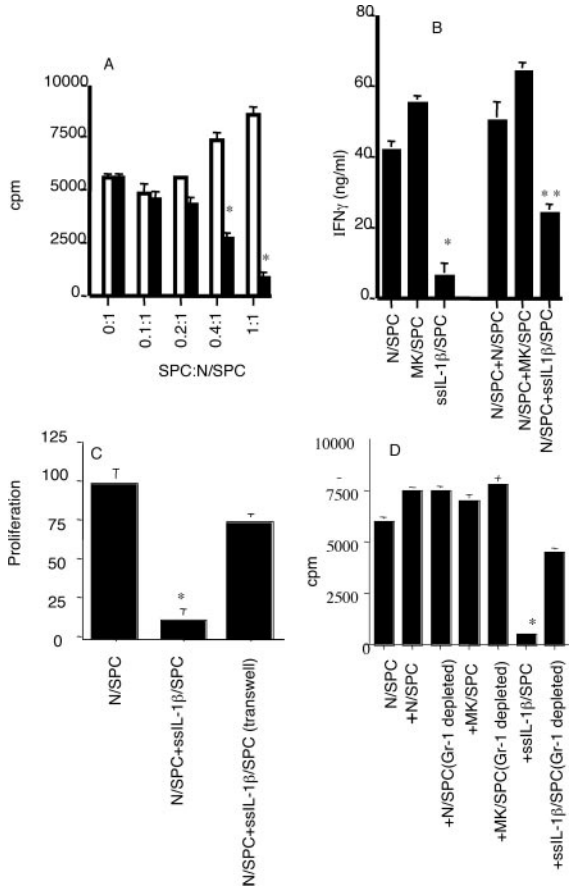

FIGURE 5. Suppressive effects of spleen cells from mice bearing tumors of IL-1β-secreting cells on normal spleen cells. Dose-response kinetics of inhibition of Con A-induced proliferation by spleen cells from mice bearing tumors secreting IL-1β (*A*). Spleen cells from mice 15–18 days after inoculation of ssIL-1β (■) or mock-transfected (□) cells were added to cultures of normal spleen cells (N/SPC) (1.0×10^6/ml) at different ratios, as indicated, for 24-h coculture. Thereafter, Con A (2.5 μg/ml) was added to culture, and mitogenic responses (48 h) and IFN-γ production (24 h) were assessed, as indicated in *Materials and Methods*. Inhibition of Con A-induced IFN-γ secretion by spleen cells from mice bearing tumors secreting IL-1β (*B*). Spleen cells from tumor-bearing mice (ssIL-1β/SPC, MK/SPC) were incubated alone or together with normal spleen cells at a 1:1 ratio, under conditions indicated in *A*. Cell-to-cell interactions are essential for suppression of Con A-induced responses of normal spleen cells by spleen cells from tumor-bearing mice (*C*). Experimental conditions were as described above; cells were cultured in transwell plates or in conventional tissue culture plates. Abrogation of suppression of Con A-induced mitogenic responses of normal spleen cells by depletion of Gr-1^+ cells from spleen cells from tumor-bearing mice (ssIL-1β/SPC, MK/SPC) (*D*). Experimental conditions were as described in *Materials and Methods*. The plus (+) indicates the subpopulation that was added to normal spleen cells at a 1:1 ratio. Results are presented as the mean ± SD of three experiments performed. Statistical significance: *, $p < 0.01$ and **, $p < 0.05$ vs the appropriate controls in each panel.

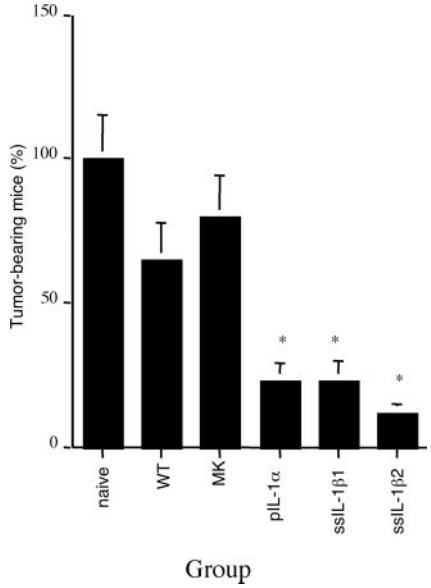

FIGURE 6. Resistance to rechallenge with wild-type malignant cells in tumor-bearing mice after resection of primary tumors. Mice were inoculated i.f.p. with the various IL-1 transfectants, wild-type or mock-transfected cells, as described in legend to Fig. 1. Primary tumors were surgically resected on day 18. Three months later, the mice were rechallenged i.f.p. with wild-type tumor cells (1×10^5/mouse), and tumor growth was measured after 40 days. Presented are results from three experiments performed. *, Statistical significance: $p < 0.001$ vs the appropriate controls.

early myeloid precursors {reviewed in Refs. 23–25). In cancer patients (breast, lung, head, and neck cancers) and also in rodents, advanced tumors are frequently accompanied by splenomegaly, abnormal differentiation of myeloid cells, and accumulation of immature myeloid cells in lymphoid organs and possibly also at tumor sites. Under such conditions, different hematological alterations, such as leukocytosis, granulocytosis, and thrombocytosis, and suppression of cell-mediated immune responses are also seen (reviewed in Refs. 23–25 and 28). Aberrant secretion of hemopoietic cytokines by the malignant cells mediates these syndromes, as resection of experimental tumors results in a reduction in the number of immature CD11b$^+$/Gr-1^+ cells, normalization of hematological alterations, and an increase in the number and function of T cells in the spleen (reviewed in Refs. 23–25 and 29). Cytokines, shown to be secreted by malignant cells in culture, such as vascular endothelial growth factor, GM-CSF, M-CSF, IL-6, and IL-10, have been implicated in increased myelopoiesis in tumor-bearing hosts (reviewed in Refs. 23–25). In most of these studies, cytokine secretion by the malignant cells was demonstrated in culture rather than in vivo.

In this study, we show that overexpression and secretion of IL-1β by malignant cells correlate with immune suppression and manifestation of hematological alterations, such as splenomegaly, leukocytosis, anemia, and the accumulation of increased numbers of immature CD11b$^+$/Gr-1^+ cells in the peripheral blood and spleen. Hematological alterations and CD11b$^+$/Gr-1^+ cell-mediated immune suppression developed with time and correlated with high systemic levels of IL-1β that were secreted by the malignant cells, rather than with an increased tumor burden per se. In mice bearing tumors of the parental fibrosarcoma cell line or mock-transfected cells, no hematological alterations or immune suppression could be detected. Also, all types of fibrosarcoma cell lines used in this study secreted similar levels of vascular endothelial growth factor and colony-stimulating factors in vitro; however, IL-1β is the only cytokine that could be detected in the serum. The correlation between systemic IL-1β and hematological alterations was previously not described in cancer patients or experimental tumor systems; however, it was reported in healthy volunteers injected with IL-1 and in patients suffering from diverse autoimmune and inflammatory diseases (2, 30). In patients, upon systemic blocking of IL-1β with the IL-1Ra, the neutrophilia and the number of immature cells in the blood fall within a few days.

The hematological alterations and immune suppression were completely normalized ~1 wk after tumor resection and elimination of the stimulus for the myelopoietic response, as already described (29). In addition, the hematological alterations were attenuated after in vivo treatment of tumor-bearing mice with the IL-1Ra, which is a physiological inhibitor of preformed IL-1, mainly IL-1β. Following resection of the tumor, the immature CD11b$^+$/Gr-1$^+$ cells undergo apoptosis, as a result of abrupt deprivation of growth factors (reviewed in Refs. 23–25). In accordance, addition of IL-1Ra to cultures of acute myeloid leukemia blasts resulted in cell apoptosis (31, 32). These cells use an autocrine loop for their proliferation; secretion of IL-1β by the malignant cells induces GM-CSF production, which is a growth factor for the cells.

The major target organ for the hemopoietic actions of IL-1β is the bone marrow, in which IL-1β stimulates myelopoiesis and the release of myeloid cells into the blood. Immature myeloid cells then migrate into secondary lymphoid organs, i.e., the spleen and lymph nodes, where they may further proliferate. Multiple hemopoietic functions have been attributed to IL-1, especially to the secretable form IL-1β (reviewed in Ref. 2). IL-1 was characterized as hemopoietin-1, a factor that is essential for hemopoiesis by inducing the expression of receptors for CSFs on primitive precursor cells (2, 33), and it also stimulates the expression and secretion of CSF and other hemopoietic factors in marrow stromal cells, leukocytes, and other cells (34). The in vivo importance of IL-1 in stimulating hemopoiesis is best shown by its ability to rescue mice after lethal irradiation, mainly through induction of recovery of the myeloid compartment (35). Some of the effects of IL-1β on hemopoiesis are indirect and are mediated by the activation of stromal cells in the marrow. Thus, in stromal cells, IL-1β stimulates the production of myelopoietic cytokines and modulates expression of adhesion molecules, which result in the accelerated release of myeloid cells from the marrow and their migration to lymphoid tissues (reviewed in Refs. 1 and 2). The decrease in the number of immature CD11b$^+$/Gr-1$^+$ cells in the BM, concomitantly to their massive accumulation in the blood and spleen, may indicate their increased release from the marrow. Anemia is probably due to a shift in hemopoietic factors that stimulate granulopoiesis rather than erythropoiesis. The induction of these hemopoietic alterations in mice bearing tumors of cells secreting IL-1β is part of a systemic inflammatory response induced by tumor cell-derived IL-1β, which includes leukocytosis, cachexia, liver necrosis, and interstitial pneumonia (X. Song, E. Voronov, and R. N. Apte, unpublished results).

In most studies on IL-1β-mediated tumor invasiveness, local, rather than systemic, expression of IL-1β was demonstrated (1, 2). Indeed, we have shown that the invasive potential of tumor cells secreting IL-1β appears early and is evident even when small doses of the malignant cells were injected into mice (13). We think that IL-1β-mediated suppression contributes to invasiveness, as we could not detect antitumor cell immunity in tumor-bearing mice, even at early times after injection of malignant cells. Suppression is initially Ag specific (13), and only later, after the accumulation of critical systemic levels of IL-1β, suppression becomes generalized. This is, to our knowledge, the first report on tumor-mediated suppression induced by IL-1β.

Mice that had been injected with IL-1β-secreting malignant cells, and which developed immune suppression and large overt tumors that were resected on day 18, were resistant to a challenge with the parental wild-type cells. This indicates that an immune memory against tumor-specific Ags was established, possibly due to the adjuvant-like features of IL-1β (1–4, 36). This is despite the manifestations of immune suppression. Suppressive cells were observed in the spleen and also in the draining lymph nodes, indicating that there is no favorable site in which efficient antitumor memory cells are generated without the interference of suppressive cells. Thus, immune suppression induced by IL-1β-secreting tumor cells is not due to the deletion of specific lymphocytes, but rather, it involves masking the function of effector cells that had been generated, as was already suggested (29). In contrast, mice previously injected with cells of the parental wild-type cell line or mock-transfected cells, in a comparable protocol, were not protected and developed tumors upon challenge, similar to naive mice injected with the malignant cells. This indicates that these cells are either nonimmunogenic or they induce other mechanisms of suppression than those mediated by CD11b$^+$/Gr-1$^+$ immature myeloid cells.

Although it is well established that CD11b$^+$/Gr-1$^+$ suppress T cell-mediated immune phenomena in tumor-bearing subjects, the range of target cells that are affected by them and the mechanisms of suppression are largely unknown (reviewed in Refs. 23–25). In some studies, only specific immune responses were shown to be impaired, while in others general suppression was reported. In all studies, it was demonstrated that in vitro, direct cell-to-cell interactions between CD11b$^+$/Gr-1$^+$ cells and their target T cells are essential. In some cases, IFN-γ secretion was shown to be essential for the induction of suppression that is mediated by NO (37, 38) and reactive oxygen species (39). These suppressive interactions down-regulate the expression of CD3/TCRζ, p56^{lck}, and p59^{fyn} on T cells, resulting in defective signaling (40–42), which could give rise to phenomena that are reversible, such as anergy (43), or irreversible, such as apoptosis (44, 45). The T cell targets of CD11b$^+$/Gr-1$^+$-mediated suppression range from naive to activated CD8$^+$ (reviewed in Refs. 23–25) and CD4$^+$ T cells (46, 47). In our experimental system, suppression of Con A-induced T cell responses in naive T cells requires cell-to-cell interactions between spleen cells from tumor-bearing mice, which are enriched in CD11b$^+$/Gr-1$^+$ cells, and is best achieved when the naive cells are preincubated with the suppressor cells for 24 h and then Con A is added to culture. Thus, in our in vitro experimental system, suppressor immature CD11b$^+$/Gr-1$^+$ myeloid cells have to first accumulate/activate the cell-associated and secreted suppressor molecules that render naive T cells hyporesponsive to Con A. However, it is possible that, in vivo, more complex suppressor circuits dependent on CD11b$^+$/Gr-1$^+$ myeloid cells operate. Thus, in tumor-bearing mice, complex suppressive circuits are activated; the nature and extent of suppression possibly depend on the number of suppressor cells, the composition of this heterogeneous population, the phase in the immune response when they develop, the duration of their existence in lymphoid organs, and their pattern of interaction with immune/suppressor populations in the host.

In summary, we have described the potential of IL-1, mainly IL-1β, which is secretable, as an important element in inducing tumor-mediated suppression by CD11b$^+$/Gr-1$^+$ immature myeloid cells. The immunosuppressive potential of secretable IL-1β is in addition to its ability to potentiate tumor angiogenesis and the invasiveness and metastasis of malignant cells. Neutralization of secretable IL-1, by the IL-1Ra, may thus represent a feasible treatment in tumor-debulked patients to prevent tumor recurrence and metastasis. The IL-1Ra is currently used to treat patients suffering from rheumatoid arthritis, and the initial proof of concept for its antitumor potential has been shown in our previous studies (2–4, 14, 48).

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Disclosures

The authors have no financial conflict of interest.

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