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Tumor-derived cytokines induce bone marrow suppressor cells that mediate immunosuppression through transforming growth factor β*

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Summary. Normal bone marrow cells become immunosuppressive when cultured with supernatants of metastatic Lewis lung carcinoma (LLC-LN7) cells. The suppressor-inducing activities in the LLC-LN7 supernatants are interleukin-3 and granulocyte/macrophage-colony-stimulating factor. In the present study, the mechanisms by which these induced suppressor cells (LLCsup-BM) mediate their immunosuppression were investigated. The suppression by LLCsup-BM of splenic concanavalin CA blastogenesis was not dependent on cell contact since immunosuppression occurred regardless of whether the LLCsup-BM were separated from the responder spleen cells by a permeable membrane or if the LLCsup-BM were cocultured with the spleen cells. Culture supernatants of LLCsup-BM also inhibited T cell blastogenesis, being more suppressive than were supernatants of control bone marrow cells, which had been precultured with medium. The suppression by the soluble inhibitors elaborated from the LLCsup-BM was not restricted to the inhibition of T cell function as the supernatants also inhibited the natural killer activity of normal spleen cells. Studies to determine the identity of the suppressive activity produced by the LLCsup-BM showed increased levels of transforming growth factor β (TGFβ) in their supernatants. Immunosuppressive bone marrow and spleen cells obtained from mice bearing metastatic LLC-LN7 tumors also secreted more TGFβ than did the cells obtained from normal mice. When anti-TGFβ antibodies were added to the LLCsup-BM supernatants, the suppressive activity was diminished. These results suggest that the LLCsup-BM mediate at least part of their immunosuppression through production of TGFβ.

Key words: Lewis lung carcinoma – TGFβ – Bone marrow – Immunosuppressor

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Introduction

Tumor cell destruction by immuno-effector cells can be diminished by tumor-induced immunosuppressor cells [1, 3, 15, 18, 24]. These immunosuppressor cells have classically been characterized to be Thy1+ T lymphocytes or adherent macrophages. More recently, tumors have been shown to secrete colony-stimulating factors (CSFs), including granulocyte/macrophage(GM)-CSF and interleukin-3 (IL-3), and consequently induce the appearance of immunosuppressor cells [6, 22, 29]. In the Lewis lung carcinoma (LLC) tumor model, we have shown that progressive growth of metastatic LLC variant tumors, such as LLC-LN7, results in myelopoietic stimulation, which coincides with a progressive decline in T cell competence and with the appearance of immunosuppressor cells, first in the bone marrow and then in the spleen [25, 26]. Both the myelopoietic stimulation and the induction of immunosuppressor cells are caused by the GM-CSF and IL-3 secreted by the LLC-LN7 tumor cells [26, 27, 29]. The bone-marrow-derived suppressor cells are distinct from lymphoid cells or mature macrophages, and resemble immature cells of the monocyte lineage [26]. Similar immunosuppressor cells can also be induced by culture of normal bone marrow cells with LLC-secreted GM-CSF and IL-3, or by culture with recombinant GM-CSF and IL-3 [26, 27, 29].

Several studies in non-tumor systems where myelopoiesis is stimulated have suggested that bone-marrow-derived suppressor cells might mediate their immunosuppression through the production of cytokines, including interferons, tumor growth factor β (TGFβ), and other previously uncharacterized soluble factors [4, 5, 14, 16, 23]. However, studies have not been conducted in tumor systems to delineate the mechanisms of immunosuppression for the bone marrow suppressor cells, which are induced by tumor-derived CSFs. The objective of the present study was to determine whether such bone marrow suppressor cells mediate their immunosuppression through the elaboration of a soluble suppressive cytokine and, if so, to determine its identity. Our results show that the immunosuppressor cells

induced by tumor-derived CSFs mediate immunosuppression at least in part through their secretion of TGFβ.

Materials and methods

Medium and tumor cells. The culture medium used for all studies was RPMI-1640 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, 0.02 M HEPES buffer solution, 50 μM 2-mercaptoethanol, 2 mM L-glutamine and 10% low-endotoxin fetal bovine serum (Sigma Chemical Co., St. Louis, Mo). Cloned metastatic LLC-LN7 variants, which had been isolated from lung nodules of mice bearing subcutaneously implanted parental LLC tumors, were used as the source of tumor supernatant to induce suppressive bone marrow cells [29]. Supernatants of the LLC-LN7 cells were prepared by culturing 1×10^6 cells/ml for 24 h. Cultures were then centrifuged and supernatants were collected and filtered.

Mice. Male C57Bl/6 mice, 6–8 weeks old, were used for all studies. The mice were obtained from Harlan Sprague Dawley (Indianapolis, Ind.) and then housed at the Hines V. A. animal research facility. In selected experiments, tumor cells were inoculated into mice by dorsal s. c. injection of 5×10^5 LLC-LN7 cells. Bone marrow and spleen cells were collected from these tumor-bearing mice during the 4th week after tumor implantation. After s. c. injection, the LLC-LN7 tumor cells readily metastasize to the lungs. However, metastatic spread to other sites, such as bone marrow or spleen, has never been detected either histologically or after in vitro culture of these tissues.

Immunosuppressive bone marrow cells. Bone marrow cells obtained from normal mice were cultured for 3 days at a concentration of 4×10^6 cells/ml in medium alone (med-BM) or in a 25% concentration of supernatant from LLC-LN7 cells (LLCsup-BM). Bone marrow cells were then washed and either used in immunosuppressive assays or cultured for an additional 24 h, after which their culture supernatants were used in suppressor assays. In some studies, the bone marrow supernatants were mixed with dilutions of either 20 μg/ml rabbit anti-TGFβ antiserum (R & D systems, Minneapolis, Minn.) or control serum prior to analysis for immunosuppressive activity.

Assay for TGFβ bioactivity [13]. The level of active TGFβ in cell culture supernatants was measured by the capacity to inhibit [^{3}H]thymidine incorporation by Mv1Lu mink lung epithelial cells (CCL-64; American Type Culture Collection, Bethesda, Md.) Samples of 3×10^3 Mv1Lu cells were plated into microtiter wells with 0.1 ml twofold dilutions of TGFβ sample or control diluent. Positive controls were dilutions of porcine TGFβ ranging from 10 ng/ml to 0.001 ng/ml. After 2 days, cells were pulsed with 1 μCi [^{3}H]thymidine and harvested 18 h later. Incorporated radioactivity was counted in a liquid scintillation counter.

Assay for immunosuppressive activity. Immunosuppressive activity was measured by the capacity to inhibit normal splenic T cell blastogenesis to the mitogen concanavalin A (ConA) or to inhibit NK cytolysis of normal spleen cells to the YAC-1 target cells [27, 29]. The assay to measure suppressive effects on T lymphocyte blastogenesis was conducted by incubating 2×10^5 normal spleen cells with 4 μg/ml ConA and various concentrations of bone marrow supernatants for 3 days in flat-bottom microtiter plates. For the last 18 h of culture, 1 μCi [^{3}H]thymidine was added per well. Spleen cells were then sedimented and the incorporated [^{3}H]thymidine was counted in a Beckman liquid scintillation counter. One series of studies was instead performed in TransWell chambers in which normal spleen cells and ConA were added to the lower compartment of the chamber while irradiated bone marrow cells (25 Gy) were added either to the insert, so as to be separated from the spleen cells by a permeable membrane, or to the lower compartment for coculture with the spleen cells. For the NK cytotoxicity assay, 5×10^5 normal spleen cells were incubated in round-bottom microtiter wells with various dilutions of bone marrow supernatants and 1×10^4 ^{51}Cr-labeled JAC-1 target cells. After 4 h, the amount of ^{51}Cr released into the supernatant was counted

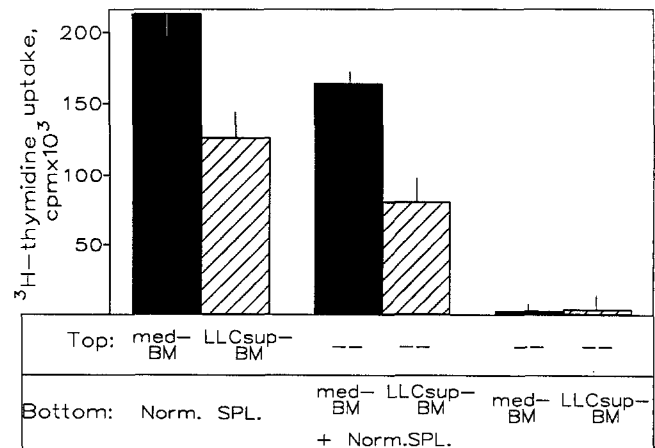

Fig. 1. Inhibition of T-cell blastogenesis by immunosuppressive bone marrow cells induced by Lewis lung carcinoma (*LLCsup-BM*), which are separated from the T-cells by a permeable membrane. Samples of 2.5×10^4 irradiated LLCsup-BM or bone marrow cells precultured with medium (*med-BM*) were cultured in TransWell chambers either together with 2×10^5 normal spleen cells (*Norm. SPL*) and 4 μg/ml concanavalin A (ConA), or in a compartment that was separated from the spleen cells by a permeable membrane. In the absence of added bone marrow cells, the T cell blastogenic response of normal spleen cells was 226053 ± 15854 cpm. Values are means ± SEM of three experiments

in a Packard gamma counter and the percentage cytotoxicity calculated as follows:

$$\frac{[\text{experimental release (cpm)–spontaneous release (cpm)}]}{\text{total release (cpm)–spontaneous release (cpm)}} \times 100$$

Analysis of data. Student's *t*-test was used to determine the significance of the differences between values. All data were expressed as means of at least three experiments ± SEM.

Results

Lack of contact dependence for the suppression of T cell blastogenesis by LLCsup-BM

LLCsup-BM have previously been shown to be immunosuppressive to T cell and natural killer (NK) function [27, 29]. To determine whether the suppression of T cell activity requires contact between the LLCsup-BM and the T cells, the cells were cultured in TransWell chambers either together or in compartments which were separated by a permeable membrane. Shown in Fig. 1 are the means of three experiments in which bone marrow and spleen cells were cultured at a ratio of 1 : 8. Regardless of whether LLCsup-BM were admixed with the spleen cells or were separated from the spleen cells by a permeable filter, they were suppressive to normal T cell blastogenesis ($P < 0.01$). In contrast, med-BM were less suppressive. Thus, the suppression of T cell activity by LLCsup-BM did not require cell contact.

Next, supernatants were collected from LLCsup-BM and assessed for immunosuppressive activity. The addition of increasing concentrations of supernatants from LLCsup-BM resulted in a dose-dependent inhibition of T cell blastogenesis (Fig. 2). By contrast, the supernatants of

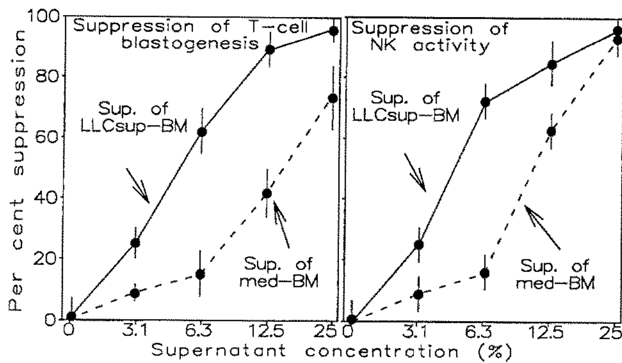

Fig. 2. Inhibition of T-cell blastogenesis and of natural killer (*NK*) cytolysis by supernatants of LLCsup-BM. Various dilutions of 24-h culture supernatants from 4×10^6 LLCsup-BM or med-BM were added either to spleen cells plus ConA (*left panel*), or to spleen cells plus ^{51}Cr-labeled YAC-1 target cells (*right panel*). In the absence of added supernatant, the T cell blastogenic response was $230\,378 \pm 13\,484$ cpm, and the NK cytotoxic activity was $37 \pm 1\%$. Values shown are mean percentage inhibition of blastogenesis or NK cytotoxicity $\pm$ SEM of three experiments

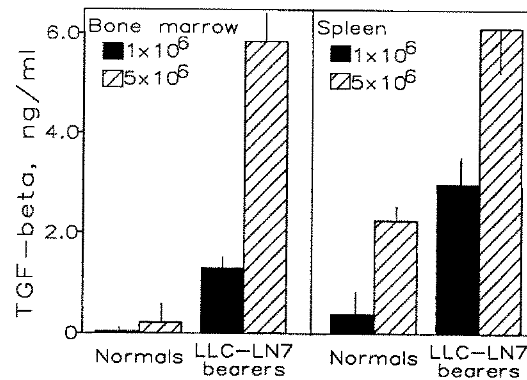

Fig. 4. TGFβ activity in supernatants of bone marrow and spleen cells of LLC-LN7-bearing mice. 24-h culture supernatants from 1×10^6 or 5×10^6 bone marrow or spleen cells were added to Mv1Lu cells for assessment of TGFβ activity. The positive controls, dilutions of porcine TGFβ1, were used as a reference for quantification of TGFβ levels. Values shown are means $\pm$ SEM of three experiments

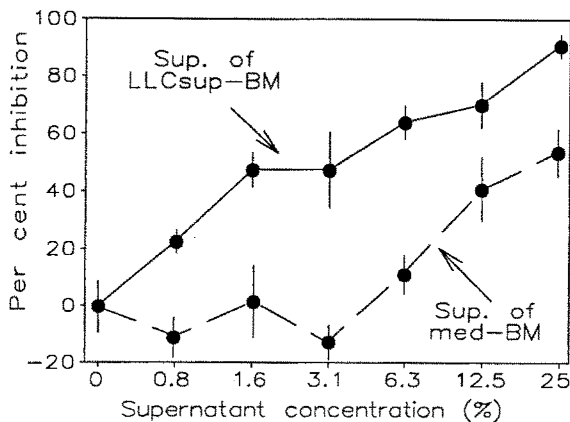

Fig. 3. Transforming growth factor β (TGFβ) activity in supernatants of LLCsup-BM cells. Various dilutions of 24-h culture supernatants from 4×10^6 LLCsup-BM or med-BM cells were added to Mv1Lu cells. After 2 days, cells were pulsed with [^{3}H]thymidine and harvested 18 h later. In the absence of added supernatants, the [^{3}H]thymidine incorporation by the Mv1Lu cells was $81\,082 \pm 5522$ cpm. Values shown are mean percentage inhibition by the supernatants of [^{3}H]thymidine incorporation $\pm$ SEM of four experiments

med-BM were less suppressive. The suppressive effects of the LLCsup-BM supernatants were not restricted to T cells since NK-mediated cytolysis of YAC-1 targets was also suppressed to a similar extent. The bone marrow supernatants were not directly toxic to the YAC-1 target cells (data not shown). The results of the studies with the Trans-Well chambers and with the bone marrow culture supernatants showed that the LLCsup-BM mediated their immunosuppression through the elaboration of a soluble immunosuppressive product (s).

TGFβ activity in supernatants of LLCsup-BM

Prior studies in various tumor and non-tumor models have shown immune suppression being mediated through secretion of TGFβ [4, 7, 11, 21]. Because TGFβ is known to suppress a variety of immune parameters [2, 11, 17, 21] and to have biological function in the bone marrow [8, 10, 12, 19], we considered the possibility that the LLCsup-BM might mediate their immune suppression through TGFβ. Thus, the levels of active TGFβ produced by the immune suppressive LLCsup-BM cells and by the less suppressive med-BM were compared by the capacity of the bone marrow supernatants to inhibit [^{3}H]thymidine incorporation by Mv1Lu cells (Fig. 3). More TGFβ activity was present in the supernatants of LLCsup-BM than in those of med-BM cells ($P < 0.01$). That the activity was TGFβ was verified by its neutralization with anti-TGFβ antiserum (data not shown). Using porcine TGFβ1 as a positive control, the LLCsup-BM cells were estimated to produce 11.7 ng/ml TGFβ/10^6 cells while the med-BM produced 2.7 ng/ml TGFβ/10^6 cells.

The level of TGFβ produced by immunosuppressive bone marrow and spleen cells from tumor-bearing mice was also compared to that produced by cells from normal mice. Both bone marrow and spleen cells from mice bearing LLC-LN7 tumors secreted increased levels of TGFβ compared to the levels secreted by cells of normal mice ($P < 0.001$) (Fig. 4). Thus, the production of increased levels of TGFβ was apparent for suppressor cells induced both in vitro and in vivo.

Effect of anti-TGFβ antiserum on suppressive activities produced by LLCsup-BM

Since the supernatants of suppressor cells induced both in vitro and in vivo contained increased levels of active TGFβ, the possibility was considered that their immune suppression might be attributable to TGFβ. This possibility was tested by determining whether antibodies to TGFβ would neutralize the suppressive activity produced by LLCsup-BM. When anti-TGFβ antiserum was added to the supernatant of LLCsup-BM, immunosuppression by the supernatant was diminished (Fig. 5; $P < 0.01$). The suppressive activities of supernatants from bone marrow and

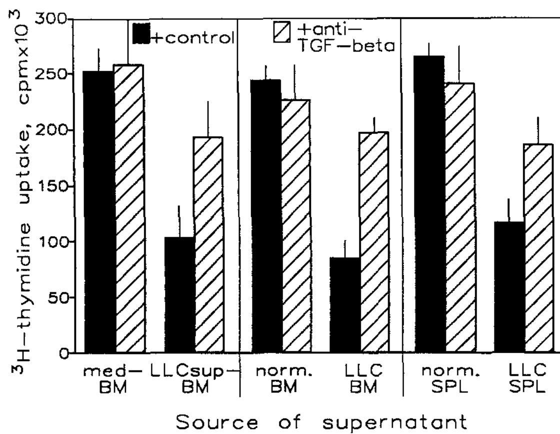

Fig. 5. Effects of anti-TGFβ antiserum on suppression by supernatants from immunosuppressive bone marrow or spleen cells. Supernatants were mixed with 20 μg/ml antiserum to TGFβ, and then added at a 1/4 dilution to a blastogenesis culture containing normal spleen cells plus ConA. The supernatants used were from 4×10^6 LLCsup-BM, med-BM, bone marrow (*BM*) or spleen (*SPL*) cells from either normal mice or mice bearing LLC-LN7 tumors. In the absence of supernatants, the T cell blastogenic response was 230488 ± 14497 cpm without antiserum, and 211937 ± 11052 cpm in the presence of anti-TGFβ antiserum. Values shown are means ± SEM of three experiments

spleen cells isolated from tumor-bearing mice were likewise diminished by the anti-TGFβ antiserum ($P<0.05$). Increasing the concentration of antibody above the 20 μg/ml used in the studies of Fig. 5 did not further diminish suppressive activity produced by the LLCsup-BM or by the freshly isolated bone marrow and spleen cells of tumor bearers (data not shown). Thus, the suppression by LLCsup-BM appears to be mediated, at least in part, by the increased production of TGFβ.

Discussion

Metastatic LLC-LN7 variant cells have previously been shown to induce the appearance of immunosuppressive bone marrow cells through the tumor cell secretion of GM-CSF and IL-3 [27, 29]. The present study has shown that the immunosuppression induced by the LLCsup-BM cells is not dependent on cell contact, but is instead mediated through elaborated immunosuppressive cytokine(s). The suppressive activity produced by LLCsup-BM was inhibitory to both T cell blastogenesis and to NK-mediated tumor lysis. This is in agreement with our previous demonstration that bone marrow cells of mice with metastatic LLC tumor can inhibit both T cell and NK functions [28]. The inhibitory effects on NK cells were clearly induced rapidly as inhibition occurred within the 4-h duration of the assay. Whether inhibition of T cell blastogenesis occurred at the same rate as for NK cytotoxicity is unknown.

The immunosuppression of LLCsup-BM was identified to be mediated, at least in part, by TGFβ. This conclusion was supported by the demonstration that the immunosuppressive supernatants of the suppressor cells contained increased levels of TGFβ, and that suppression was diminished by neutralization of TGFβ with antibodies. The results of antibody neutralization studies described in this report are currently being confirmed by Northern blot hybridization analyses to determine whether the LLCsup-BM cells express increased levels of mRNA encoding TGFβ.

TGFβ has previously been shown to inhibit a broad spectrum of immunological parameters such as macrophage-mediated cytotoxicity [17], the generation of lymphokine-activated killer (LAK) activity [2, 7], cytolysis by LAK effector cells [20], and the accessory function of $CD4^+$ T helper cells resulting in reduced generation of H-2-restricted cytolytic effector cells [21]. TGFβ has also been shown to mediate immunosuppression of multiple suppressor systems, such as of $CD4^+$ suppressor cells [11], tumors [21], or allopregnant mouse decidua [4]. In addition to being immunosuppressive, TGFβ also functions in the bone marrow to regulate hematopoiesis negatively and to down-regulate CSF receptor expression on myeloid progenitor cells [9, 10]. Therefore, it seems contradictory to have tumor stimulation of myelopoiesis occurring in the presence of TGFβ-producing immune suppressor cells in bone marrow [25, 26]. However, in some studies, the inhibitory effects of TGFβ on myelopoiesis were shown to be selective and did not occur when the colony formation was in response to GM-CSF or to M-CSF [12]. Since production of GM-CSF by LLC-LN7 tumor cells leads to myelopoietic stimulation and the appearance of associated immunosuppressor cells, it appears plausible for myelopoiesis to be stimulated in the presence of increased production of TGFβ by bone marrow suppressor cells.

The combined results of this study and of our prior studies [27–29] have shown that through the production of GM-CSF and IL-3, metastatic LLC cells induce the appearance of immune suppressor cells, which mediate at least part of their immune suppression through production of TGFβ. Ongoing studies in our laboratory are aimed at utilizing various therapeutic strategies to induce differentiation of the bone-marrow-derived immunosuppressor cells into nonsuppressive mature macrophages. We anticipate that these efforts will also result in a concomitant reduction in the production of TGFβ by bone marrow and spleen cells of the tumor bearers.

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