Myeloid cell expansion elicited by the progression of spontaneous mammary carcinomas in c-erbB-2 transgenic BALB/c mice suppresses immune reactivity

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Transgenic female mice expressing the transforming rat oncogene c-erbB-2 (HER-2/neu) under the mouse mammary tumor virus (MMTV) promoter (BALB-neuT) spontaneously develop mammary carcinomas with a progression resembling that of human breast cancer. In these mice, activating antitumor immunotherapy fails to induce T cell–mediated cytotoxicity, suggesting a suppression of the immune response. We found a direct correlation between tumor multiplicity and an increased proportion of Gr-1$^+$ (Ly6G)/Mac-1$^+$(CD11b)/ER-MP12$^+$(CD31) immature myeloid cells in the peripheral blood (PB) and spleen, suggesting that tumor load profoundly affects overall BALB-neuT hematopoiesis. In fact, myeloid colony formation was increased in bone marrow (BM) and spleen. The immature myeloid cells displayed suppressive activity on host T lymphocytes, which progressively failed to respond to alloantigens and CD3 triggering, while maintaining the ability to proliferate in response to nonspecific mitogens. Transplantation of normal BM into BALB-neuT mice readily resulted in hypertrophic hematopoiesis with myeloid cell expansion. This persistent influence of the tumor was mediated through the release of vascular endothelial growth factor (VEGF) but not granulocyte-macrophage colony-stimulating factor (GM-CSF), and was down-modulated when tumor load was reduced but not when BM was transplanted. Together, the data obtained in the BALB-neuT model of naturally occurring carcinogenesis show that tumor-associated immune suppression is secondary to a more general alteration of host hematopoiesis, conditioned by tumor-secreted soluble factors. (Blood. 2003;102:2138-2145)

Introduction

Alterations in hematologic parameters have been described in patients with tumors of various histotypes.[1-4] Leukocytosis, granulocytosis, thrombocytosis, and erythrocytosis have been considered among the so-called "paraneoplastic syndromes," which include signs and symptoms affecting sites distant from the tumor or its metastases. Such syndromes are not caused by invasion, obstruction, or bulk mass of the tumor but are instead due to inappropriate secretion of cytokines, growth factors, and hormones.[5] Tumors secreting vascular endothelial growth factor (VEGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been shown to affect differentiation of multiple hematopoietic lineages[6,7] and to alter CD34$^+$ cell homing[8] and dendritic cell (DC) maturation.[9-12] An expansion of immature myeloid cells with immune suppressive capacity is evident in the peripheral blood (PB) of patients bearing tumors of various types.[13,14] Because a defective immune response allows tumors to evade host immune control, these issues assume importance in the immunotherapy of cancer.[15]

Increased numbers of myeloid cells expressing the Gr-1 (Ly6G) and Mac-1 (CD11b) markers have been detected in the spleen and bone marrow (BM) of mice bearing transplantable tumors[12,16-20] and in many conditions associated with impaired immune reactivity, such as cyclophosphamide treatment,[21] severe *Trypanosoma* infections,[22] lymphoid irradiation, and intensive immunization protocols.[23] The Gr-1$^+$/Mac-1$^+$ cell population is phenotypically heterogeneous, including mature and immature myeloid cells and cells expressing immature DC markers,[12] but share the ability to suppress the T-cell immune response.[12,18,20,24] Immature myeloid cells isolated from human or murine BM or PB can be induced to differentiate in vitro into mature antigen-presenting cells (APCs)/DCs by means of various cytokine combinations or inducers such as 1,25-dihydroxyvitamin D3 or all-*trans* retinoic acid, thus restoring their immune-stimulating function in vitro.[12,20,25] However, in vivo, both defective DCs and immature suppressive myeloid cells cooperate in inhibiting the immune response.[13,26] Thus, interventions aimed at activating an antitumor immune response should take these cells into account together with other tumor immune escape mechanisms, since the effectiveness of cancer vaccine strategies will be impaired by deficient endogenous DC function, while T-cell activation strategies, such as adjuvant cytokines or adoptive lymphocyte transfer, could fail owing to interaction with suppressive immature myeloid cells. In addressing the mechanisms eliciting and supporting the generation of these cells, and in defining their association to tumor progression, a murine model of spontaneous tumor development provides an advantage over models involving injection of a transplantable tumor into healthy mice, because the former can recapitulate aspects of human tumor progression and allow monitoring of the steps leading to a progressive alteration in hematopoiesis and an impaired immune response.

Transgenic female mice expressing the transforming rat oncogene c-erbB-2 (HER-2/neu) under the mouse mammary tumor

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virus (MMTV) promoter (BALB-neuT) spontaneously develop mammary carcinomas with a well-defined progression from dysplasia to in situ carcinoma to overt invasive carcinoma.[27] In these mice, immunotherapeutic interventions aimed at inducing an immune response against the c-erbB-2 oncogene expressed in breast tissue have failed to activate a cytotoxic T-cell response, while a B-cell response has been demonstrated,[28,29] suggesting that immunosuppression is a likely alternative to unbroken tolerance to c-erbB-2, which is a self-antigen in BALB-neuT.

We used this model to study the mechanisms by which the tumor drives the proliferation and myeloid differentiation of hematopoietic stem cells in the host, resulting in expansion of an immunosuppressive Gr-1$^+$/Mac-1$^+$/ER-MP12$^+$ (CD31) subpopulation. The progressively altered hematopoiesis of BALB-neuT mice does not depend on a genetic abnormality of transgenic mice or on their aging, but rests on soluble factors secreted by mammary tumors. The immune suppression associated with progression of c-erbB-2–induced carcinogenesis reflects a more general and lasting conditioning of host hematopoiesis. Our results underscore the importance of models of spontaneous tumor development for designing protocols of immunotherapeutic intervention and for studying their efficacy in the context of an immunosuppressed host.

Materials and methods

Animals and cell preparation

BALB-neuT mice were bred and maintained in the animal facility at the Istituto Nazionale Tumori (Milan, Italy) according to institutional guidelines. Hemizygous transgenic mice were screened at 3 weeks of age by polymerase chain reaction (PCR).[30] Positive females were monitored weekly for mammary tumor development and progression, whereas PCR$^-$ littermates served as age-matched controls. Normal 8- to 10-week-old BALB/c, C57Bl/6, FVB, and CB6F1 female mice were purchased from Charles River (Calco, Italy). For analysis of circulating cells, 200 μL PB was collected from retro-orbital sinus and mixed with an equal volume of 5 mM EDTA (ethylenediaminetetraacetic acid); erythrocytes were lysed by hypotonic shock in NH_4Cl lysis buffer, and leukocytes were counted.

Spleen cells were prepared by mechanical disruption of the organ, followed by lysis of erythrocytes and passage through a 0.7-μM diameter cell strainer (Falcon; BD Labware Europe, Le Port de Claix, France) to obtain a single-cell suspension. Gr-1$^+$/Mac-1$^+$ cells were isolated from spleen cells by means of CD11b-conjugated microbeads (Miltenyi Biotec, Gladbach, Germany), according to manufacturer's instructions.

T lymphocytes were purified by means of a nylon wool fiber column (Polysciences, Warrington, PA).

BM cells were obtained by flushing femurs and tibiae with 1 mL Iscove modified Dulbecco medium (IMDM) (GIBCO Invitrogen, Paisley, Scotland) supplemented with 2% heat-inactivated fetal calf serum (FCS), with the use of a 22-gauge needle attached to a 1-mL syringe.

Stem cell antigen–1 positive (Sca-1$^+$) cells were isolated from BM cell suspensions by means of the Sca-1 Multisort Kit (Miltenyi Biotec), according to the manufacturer's instructions.

DCs were generated from BM according to Inaba et al,[31] with minor modifications. The BM cell suspension was plated at 2×10^6/mL in Petri dishes with 10 mL RPMI medium supplemented with 5% FCS, 10 ng/mL recombinant murine interleukin 4 (IL-4) (PeproTech EC, London, United Kingdom), and 10% conditioned medium from cell line F1/GMCSF, which produces 100 ng/mL GM-CSF upon infection with retroviral vector LmGMSN.[32] Cells were fed on days 3 and 5; on day 6, loosely adherent cells, including DCs, were harvested and analyzed by cytofluorometry.

Spontaneous mammary tumors were removed from BALB-neuT females, minced, and plated in 10 mL complete Dulbecco modified Eagle medium (DMEM; GIBCO), supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 150 U/mL streptomycin, and 200 U/mL penicillin. After 24 or 48 hours, the medium was collected, filtered (0.2 μM) to remove debris, and frozen for further use. Adherent tumor cells were supplemented with complete DMEM and established as cell lines. The c-erbB-2$^+$ mammary carcinoma cell line N2C was further characterized.

Cytofluorometric analysis

The following fluorescein isothiocyanate (FITC)–labeled antibodies (Abs) were used: rat antimouse Ly-6G (Gr-1 clone RB6-8C5) from Caltag Laboratories (Burlingame, CA), rat antimouse CD86 (clone GL1), rat antimouse CD40 (clone 3/23), rat antimouse CD4 (clone H129.19), mouse antimouse H-2 K^b (clone AF6-88.5), rat immunoglobulin G2b (IgG2b) anti-KHL (clone A110-2), and mouse IgG2a antitrinitrophenyl (anti-TNP) (clone G155-178) as isotypic controls, all from BD Pharmingen (San Diego, CA). Phycoerythrin (PE)–labeled Abs used were rat antimouse CD11b (Mac-1a clone M1/70.15) from Caltag Laboratories, and hamster antimouse CD11c (clone HL3), rat antimouse CD8 (clone 53-6.7), rat antimouse CD45/B220 (clone RA3-6B2), rat antimouse I-A/I-E (clone M5/114.15.2), mouse antimouse H-2 K^d (clone SF1-1.1), hamster IgG (clone G235-2356), rat IgG2a (clone R35-95), rat IgG2b (clone A95-1), and mouse IgG2a (clone G155-178) as isotypic controls, all from BD Pharmingen. Biotin-conjugated rat antimouse ER-MP12 (BMA, Augst, Switzerland) and rat antimouse IgG2a (clone R35-95) isotypic control (BD Pharmingen) were revealed by peridinin chlorophyll-a (PerCP)–conjugated streptavidin (BD Pharmingen). Cells were blocked with 10 μg/mL rat antimouse CD16/CD32 Ab (clone 2.4G2; BD Pharmingen) before staining with the desired combination of FITC- and PE-labeled Abs at 5 to 10 μg/mL (2 to 5 μg/10^6 cells) in 2% bovine serum albumin (BSA) and 0.05% azide in phosphate-buffered saline (PBS) for 40 minutes in ice. For triple fluorescence, biotin-conjugated primary Abs were added at 5 to 10 μg/mL (2 to 5 μg/10^6 cells) and incubated for 40 minutes on ice. Cells were washed and then incubated with the desired mixture of FITC- and PE-conjugated Abs and PerCP-labeled streptavidin (20 μL/10^6 cells). Analysis was performed on a FACSCalibur E2551 cytometer (Becton Dickinson, San Jose, CA). Data were collected on 10^4 viable cells and analyzed by means of CellQuest software (Becton Dickinson).

Proliferation assays

Lymphocyte proliferation assays were carried out in 6 replicates, with splenocytes or purified T lymphocytes cultured in complete RPMI medium for 72 hours or 5 days at 37°C in a 5% CO_2 atmosphere in the presence of the specific stimulation, with the addition of [^{3}H]-thymidine (1 μCi [0.037 MBq] per well) (Amersham Pharmacia Biotech, Piscataway, NJ) for the last 18 hours of incubation. Cells were harvested with a Tomtec cell harvester and [^{3}H]-thymidine incorporation (counts per minute) was measured on a Wallac 1205 β-counter.

CFU (colony-forming unit) assays

BM cell suspensions were diluted to a cell count of 2×10^5/mL in IMDM medium (GIBCO) supplemented with 2% heat-inactivated FCS (BioWhittaker Europe, Verviers, Belgium), and 0.3 mL cells was mixed with 3 mL MethoCult GF M3434 complete methylcellulose medium (StemCell Technologies, Vancouver, BC, Canada). Cells were plated in duplicate in 35-mm dishes, at 2 to 2.5×10^4 cells per dish, and incubated for 13 days at 37°C in a 5% CO_2 humidified incubator. Colonies were scored at days 7, 10, and 13.

Assay for tumor-mediated conditioning of BM cells

Sca-1$^+$ BM cells, enriched in hematopoietic stem cells (HSCs)/hematopoietic precursor cells (HPCs), were plated at 10^4/mL per well in 24-well plates coated with irradiated (2000 cGy) AFT024 stromal cells as feeder layer,[33] kindly provided by Dr C. Carlostella (University of Milan, Italy), and incubated in the presence of different stimuli. After 5 or 7 days of liquid

culture, BM cells were recovered and assayed in duplicate for CFU scores as described above.

BM transplantation

Groups of 12- and 22-week-old BALB-neuT mice were lethally irradiated with a total dose of 900 cGy given in 2 equal doses with a 3-hour interval. Mice were injected intravenously with BM cells (10^7 cells per mouse) recovered from CB6F1 mice. Some irradiated mice did not receive transplants and were used as controls for irradiation; they died within 2 weeks. In mice with transplants, BM uptake was monitored by immunofluorescence for major histocompatibility complex (MHC) H-$2K^b$ expression on PB. Similarly, 8- to 9-week-old CB6F1 mice were lethally irradiated with a total dose of 950 cGy (450 plus 500 cGy). BM cells were prepared from 23- to 28-week-old BALB-neuT females, bearing 4 to 10 tumors and showing 12% to 22% of Gr-1/Mac-1 cells in their spleen. Irradiated recipients received 10^7 BM cells intravenously and were monitored for BM uptake and Gr-1/Mac-1 in PB as described. BM uptake was monitored for disappearance of H-$2K^b$ and expression of H-$2K^d$ in PB lymphocytes (PBLs). Control mice, irradiated and without transplants, died within 2 to 3 weeks.

Statistical analysis

Data were expressed as mean plus standard deviation (SD). Differences between groups were analyzed for statistical significance by means of the Student *t* test, with $P < .05$ as the significant cutoff.

Results

Gr-1/Mac-1 double-positive cell expansion in PB of BALB-neuT mice

We analyzed 47 BALB-neuT female mice at different time points between 7 and 34 weeks of age. PB samples were repeatedly obtained before and after the onset of mammary tumors and analyzed for the double expression of markers Gr-1 and Mac-1. Control samples were from sibling females not carrying the c-erbB-2 oncogene, BALB/c females of different ages (8 to 44 weeks), and BALB-neuT males.

The percentage of Gr-1^+/Mac-1^+ cells increased in the PB of BALB-neuT females with age (Figure 1A) in direct correlation with the progression of multiple mammary carcinomas. At tumor onset, Gr-1^+/Mac-1^+ cells represented, on average, more than 16% of total blood cells, increasing progressively to 30% when half of the mammary glands presented palpable tumors and reaching more than 50% in mice bearing 9 to 10 carcinomas. In control BALB/c females at all ages examined, double-positive cells in PB never represented more than 16% (mean, 13.1% ± 2.56%) (not shown). In BALB-neuT males, which stochastically develop tumors in the salivary or Harderian glands when older,[34] the mean percentage of Gr-1^+/Mac-1^+ cells in PB of mice with tumors was higher than in tumor-free littermates (mean, 37.7% ± 7.3% versus 17.8% ± 5.1%, respectively) (not shown), indicating the direct effect of tumor on myeloid cell expansion.

Gr-1/Mac-1 double-positive cell expansion in spleen of BALB-neuT mice

Since the increase of Gr-1/Mac-1 double-positive cells has been described mainly in the spleen of mice bearing transplantable tumors,[16-20] we analyzed the spleen of 34 BALB-neuT female mice at 7 to 34 weeks of age and bearing different numbers of tumors; the percentage of double-positive cells increased with tumor multiplicity, reaching 30% of total spleen cells in mice with 10 tumors (not shown). Tumor-bearing BALB-neuT females always displayed splenomegaly, and increased spleen cellularity included an increased number of immature hematopoietic precursors such as proerythroblasts, promyelocytes, and

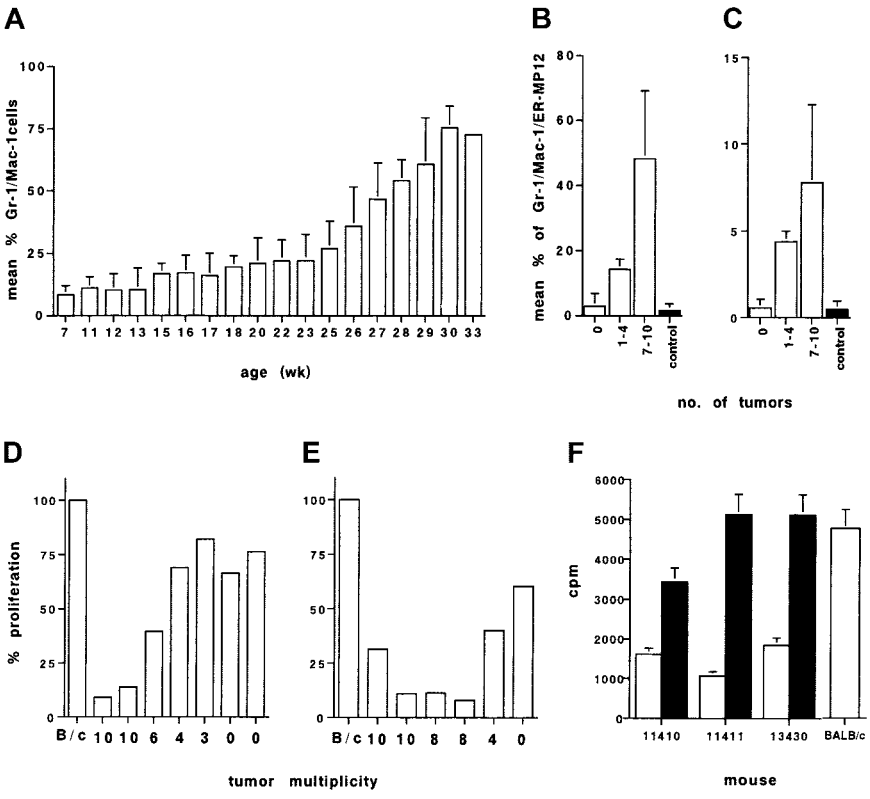

Figure 1. Expansion of myeloid immunosuppressive cells in the PB and spleen of BALB-neuT mice. (A) Correlation between age and increased percentages of Gr-1/Mac-1 double-positive cells in the PB of BALB-neuT females. Results are from 47 independent evaluations. (B-C) Correlation between tumor multiplicity and percentage of Gr-1/Mac-1/ER-MP12 triple-positive cells in the PB (B) and spleen (C) of tumor-bearing BALB-neuT females (□) and sibling BALB/c females (controls, ■). Error bars indicate SD. (D-E) Proliferative response of splenocytes from individual BALB-neuT females with distinct tumor multiplicities to immobilized anti-CD3 (10 μg/mL) plus IL-2 (50 U/mL) (D) and allogeneic C57/Bl6 irradiated spleen cells (E). Results are expressed as percentage of control BALB/c proliferation on day 3 in panel D and day 5 in panel E. (F) Increased proliferative alloresponse of splenocytes from individual BALB-neuT females (□) upon removal of Gr-1/Mac-1 cells (■), expressed as counts per minute ([cpm]; mean of 6 replicates ± SD).

Table 1. Phenotype of spleen cells from individual normal BALB/c and tumor-bearing BALB-neuT females

Mouse strain (no. tumors)	Spleen cells, no. × 10^6	CD4, % (no. × 10^6)	CD8, % (no. × 10^6)	B220, % (no. × 10^6)	CD11c, % (no. × 10^6)	CD11c/B220, % (no. × 10^6)	Gr-1/Mac-1, % (no. × 10^6)	ER-MP12/Gr-1/Mac-1, % (no. × 10^6)
BALB/c (0)	149.2	20.5 (30.6)	9.12 (13.6)	53.08 (79.2)	1.17 (1.74)	3.1 (4.62)	3.77 (5.6)	31.1 (1.74)
BALB/c (0)	148.4	20.7 (30.7)	8.51 (12.6)	52.3 (77.6)	1.46 (2.16)	2.59 (3.8)	5.6 (6.8)	32.3 (2.2)
BALB-neuT (10)	361.5	10.3 (37.3)	2.7 (20)	33 (119.4)	1.6 (5.8)	2.06 (7.4)	21.8 (78.8)	28.9 (22.7)
BALB-neuT (10)	401.6	11.8 (47.7)	3.3 (13.25)	33.9 (136.1)	1.01 (5.05)	1.15 (4.6)	16.7 (66.9)	41.05 (27.5)
BALB-neuT (10)	279.3	6.7 (18.7)	4.2 (11.7)	ND	ND	ND	21.4 (59.7)	ND
BALB-neuT (7)	195.7	7.9 (15.46)	4.5 (8.8)	ND	ND	ND	9.5 (18.6)	ND
BALB-neuT (4)	266	12.2 (32.4)	3.8 (10.1)	28 (74.5)	1.13 (3.0)	0.8 (2.13)	5.9 (15.7)	9.7 (5.8)

ND indicates not done.

myelocytes. In BALB-neuT spleen, the percentage of T and B lymphocytes was reduced as compared with normal BALB/c spleens, whereas the absolute number of $CD4^+$, $CD8^+$, $CD11c^+$, and $B220^+$ cells was normal or even increased owing to increased cellularity (Table 1), suggesting an overall increase of hematopoiesis in these mice and a retention of cells in the spleen.

The ER-MP12 antigen is a general marker expressed on a major population of myeloid precursor cells and early murine macrophage precursors from BM.[35] In freshly isolated PB and spleen cells, the percentage of $Gr\text{-}1^+/Mac\text{-}1^+$ cells also expressing ER-MP12 correlated with tumor multiplicity; in mice bearing 7 to 10 tumors, about half of the circulating $Gr\text{-}1^+/Mac\text{-}1^+$ cells and 7.5% of the splenic $Gr\text{-}1^+/Mac\text{-}1^+$ cells were triple positive (Figure 1B-C), suggesting that a relevant number of hematopoietic precursors was mobilized by the presence of tumors.

$Gr\text{-}1^+/Mac\text{-}1^+$ cells in tumor-bearing mice have been described as immature APCs.[12,16] $Gr\text{-}1^+/Mac\text{-}1^+$ cells from BALB-neuT female spleens expressed both MHC class I and II molecules, and stained positive for CD80 (15% to 30%) and for CD86 (30% to 50%), but negative for CD11c (not shown). After 6-day culture in the presence of GM-CSF, expression of Gr-1 was dramatically reduced, whereas Mac-1 expression was conserved, and cells acquired a macrophagic morphology (not shown).

Immunosuppressive activity of immature myeloid cells

Analysis to determine whether $Gr\text{-}1^+/Mac\text{-}1^+$ cells inhibit T-cell function revealed that proliferation of splenocytes in response to concanavalin A was unaffected in BALB-neuT females of different ages and with distinct tumor multiplicities (not shown), whereas proliferation of splenocytes in response to anti-CD3 Ab and IL-2 was reduced as a function of the percentage of $Gr\text{-}1^+/Mac\text{-}1^+$ cells and tumor burden (Figure 1D).

To evaluate allogeneic reactivity, splenocytes from tumor-bearing BALB-neuT females and from BALB/c females of the same age were cocultured with irradiated allogeneic spleen cells (C57BL/6), and proliferation was tested after 3 and 5 days. The proliferative response of BALB-neuT splenocytes to allogeneic antigen was reduced, ranging from 7% to 30% of that by normal BALB/c splenocytes (Figure 1E). Purification of T lymphocytes or removal of CD11b cells by magnetic bead sorting restored proliferation in response to alloantigens (Figure 1F). Accordingly, addition of purified $Gr\text{-}1^+/Mac\text{-}1^+$ cells to T lymphocytes from normal BALB/c mice partially inhibited the primary alloresponse (not shown). The extent of suppression correlated with the number of $Gr\text{-}1^+/Mac\text{-}1^+$ cells.

Origin of myeloid cell expansion

To test whether myeloid cell expansion was due to increased activity of the entire hematopoietic compartment, we measured the clonogenic activity of BM and spleen cells derived from BALB-neuT females with different tumor multiplicities. The total number of hematopoietic colonies from BM and spleen of BALB-neuT was significantly higher ($P < .003$ and $P < .009$ for BM and spleen, respectively) than that of control BALB/c of similar age (Figure 2A). The increase in the number of hematopoietic precursors in spleen correlated directly with tumor multiplicity (not shown), suggesting a progressive colonization of spleen by hematopoietic progenitors derived from hyperplastic BM or a preferential increase in spleen hematopoiesis. The main expansion occurred in the myeloid compartment,

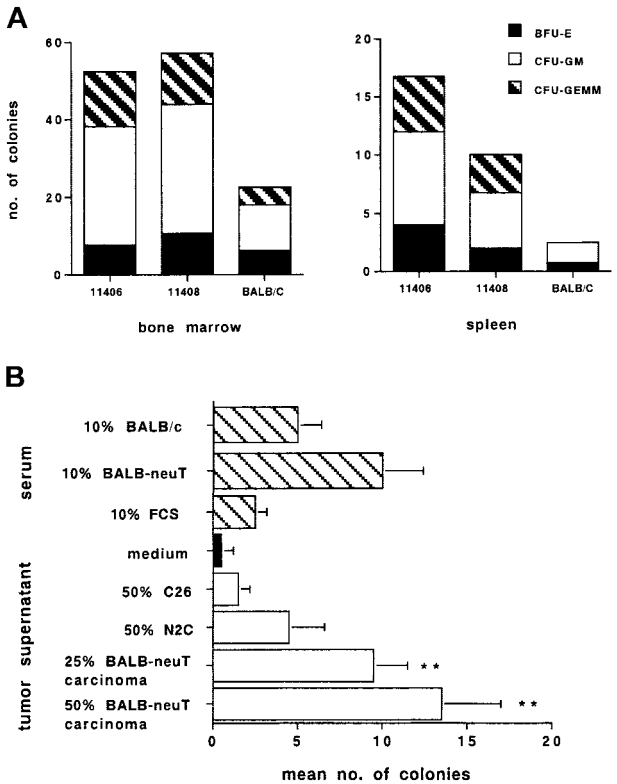

Figure 2. Effect of mammary tumors on hematopoiesis and clonogenicity. Mammary tumors alter BALB-neuT hematopoiesis and affect normal BALB/c BM cells' clonogenicity. (A) Phenotype of colonies from BM and spleen of individual BALB-neuT and BALB/c. (B) Effects of BALB-neuT serum and tumor supernatant on CFUs of normal BALB/c BM cells. $Sca\text{-}1^+$ hematopoietic stem/progenitor–enriched BM cells seeded on irradiated AFT024 stromal cells were conditioned for 5 days with 10% serum from tumor-bearing BALB-neuT or normal BALB/c mice (▧); with supernatant from tumor cell lines (C26 colon carcinoma and N2C mammary carcinoma) or from primary cultures of mammary BALB-neuT carcinomas (□), at the indicated concentrations; or with medium alone (■). Cells were recovered and scored for CFUs in methylcellulose at day 10 (mean of 4 replicates ± SD). $^{**}P < .05$ indicates statistically significant differences from control.

with higher percentages of CFU-GMs and granuloctye, erythrocyte, macrophage, mixed CFUs (CFU-GEMMs), particularly in spleen (Figure 2A).

To test whether the hematopoietic hyperplasia was due to soluble factors secreted by the tumor, purified Sca-1$^+$–enriched BM cells from BALB/c in liquid culture were conditioned with various tumor supernatants or serum from BALB-neuT or BALB/c mice and scored after 5 or 7 days for CFUs in methylcellulose. The mean CFU number was significantly higher when hematopoietic stem cells were conditioned with tumor supernatants or serum from BALB-neuT mice, as compared with BALB/c serum (Figure 2B). Because irradiated AFT024 stromal cells were used as feeder layers for Sca-1$^+$–enriched cells in liquid culture, we tested whether tumor-released soluble factors acted directly on partially enriched HSC/HSP cells or indirectly through activation of stromal cells to release hematopoietic growth factors. In cultures of feeder cells incubated for 24 hours with the same stimulating supernatants or sera, washed, and then cocultured with Sca-1$^+$ cells for 5 or 7 days, very few colonies developed from the HSC/HSP–enriched cells plated in methylcellulose (Figure 2B), confirming a direct effect of soluble factors secreted by tumors on hematopoietic stem cells.

Tumor-driven host hematopoiesis

To assess the role of tumors in hematopoiesis in vivo, we analyzed the expansion of Gr-1/Mac-1 double-positive cells in 22- or 12-week-old BALB-neuT females, showing a few or no tumors, respectively, and receiving transplants of BM from CB6F1 normal donors. The BM uptake was tested by immunofluorescence for expression of MHC class I H-2 determinants. In mice without tumors, more than 80% of PB lymphocytes expressed H-2K^b within 18 days, while the percentage of Gr-1$^+$/Mac-1$^+$ cells was within the normal range. Mice bearing 1 to 2 tumors at the time of transplantation revealed an increased percentage of Gr-1$^+$/Mac-1$^+$ cells at day 18. In both groups, the percentage of Gr-1$^+$/Mac-1$^+$ derived from donor BM progressively increased according to the tumor multiplicity (Figure 3A). Both spleen and BM cells displayed increased clonogenic activity when assayed in methylcellulose at 10 weeks after transplantation (Figure 3B).

Analysis of the immune response to allogeneic antigens using spleen cells from BALB-neuT mice with transplants at 33 weeks of age indicated reduced proliferation in response to FVB (H-2K^q) allogeneic cells due to the presence of Gr-1$^+$/Mac-1$^+$ cells; removal of the double-positive cells restored the alloresponse; however, when Gr-1$^+$/Mac-1$^+$ cells from mice with transplants were added to normal CB6F1 splenocytes, their proliferation to FVB alloantigens was reduced (Figure 3C). These results directly demonstrate that the tumor drives host hematopoiesis toward progressive expansion of immunosuppressive myeloid cells.

To determine whether tumor conditioning induces a preleukemic transformation of multipotent BM cells or, instead, a reversible deregulation of their differentiation, we assessed MHC class I and Gr-1/Mac-1 expression in PB of lethally irradiated 9-week-old CB6F1 mice that had received transplants of BM from 23- to 28-week-old BALB-neuT females bearing 4 to 10 tumors and having a high percentage of double-positive cells in their PB and spleen. At 2 weeks after transplantation, all lymphocytes and myeloid cells from recipients lost the MHC class I K^b determinants and expressed the donor K^d antigen. Gr-1$^+$/Mac-1$^+$ cells represented 5% to 20% of PB cells 14 days after transplantation, progressively decreasing to normal values within 5 weeks (Figure 4A).

A

mouse	7147				7148				7773			
weeks after BMT	2.5	4	7	10	2.5	4	7	10	2.5	4	7	10
K^q/K^b % of positivity	94.9	83.1	81	95.3	92.8	86	84	86	85.9	91.4	90.3	94.3
no. of tumors	0	1	6	10	2	3	9	10	1	2	3	9
% of Gr-1/Mac-1	3.67	14.5	50.3	75.3	28.6	39.4	57.5	63.8	8.23	25	46	56

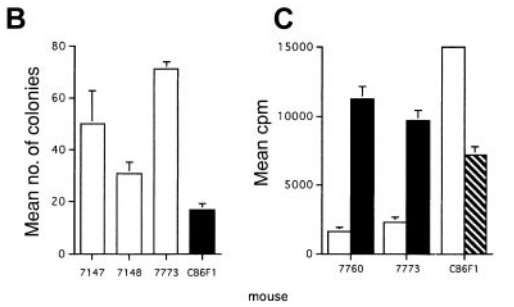

Figure 3. Conditioning of donor BM by host tumor. Lethally irradiated 22-week-old BALB-neuT females received transplants of BM from CB6F1 mice. (A) Kinetics of BM uptake, tumor development, and myeloid expansion in the PB of 3 representative mice that had received transplants. (B) CFU assay with spleen cells of 33-week-old BALB-neuT mice that had received transplants (10 to 11 weeks after the transplantation) and of CB6F1 control mice. (C) Proliferation of total splenocytes from BALB-neuT mice with transplants in response to alloantigens in mixed leukocyte reaction (□), increased upon removal of Gr-1$^+$/Mac-1$^+$ cells (■); alloproliferation of normal donor CB6F1 splenocytes declined upon addition of 10% of Gr-1$^+$/Mac-1$^+$ cells (▨) from BALB-neuT mice receiving transplants. Results are from 3 of 10 mice tested. Mean of 3 replicates ± SD.

Clonogenic activity of BM and spleen cells tested at 12 weeks after transplantation was within the normal range (Figure 4B), and the immune response to allogeneic antigens, tested with FVB-stimulating cells, was also normal (Figure 4C). These data indicate that tumor conditioning of host hematopoiesis is a reversible phenomenon.

Mechanism of tumor conditioning of host hematopoiesis

Tumor-secreted GM-CSF has been shown to influence myeloid differentiation in mice bearing subcutaneous tumors,[12] and VEGF acts to severely impair DC function and differentiation from BM precursors.[7,10] To explore the mechanisms by which mammary tumor development modifies host hematopoiesis, we focused on these 2 soluble factors and measured levels in the supernatant from primary culture and from cell lines established from mammary tumors as well as in sera of BALB-neuT mice by enzyme-linked immunosorbent assay (ELISA). While GM-CSF was undetectable in all samples, VEGF was secreted by primary tumor cell explants in culture and by cell lines established from mammary tumors (Figure 5A) and was detected in sera of tumor-bearing mice. The level of VEGF in BALB-neuT sera correlated directly with tumor multiplicity and percentage of Gr-1$^+$/Mac-1$^+$ cells in their PB, as indicated by sequential serum samples (Figure 5B). Similarly, levels of VEGF were detectable in serum of BALB-neuT males bearing salivary gland tumors and in BALB/c bearing subcutaneous tumors of mammary carcinoma cell lines derived from BALB-neuT (not shown). Together, the results suggest that c-erbB-2$^+$ tumors spontaneously arising in BALB-neuT mice can directly drive host hematopoiesis toward expansion of a myeloid compartment with immunosuppressive ability through the release of VEGF and not GM-CSF. In fact, Sca-1$^+$–enriched BM cells derived from normal BALB/c mice and conditioned with supernatant

A

mouse	1			2			10		
week after BMT	2	5	12	2	5	12	2	5	12
K^d % of positivity	95.2	91.1	92	94.7	88.8	92.3	79.5	75	89
% of GR1/Mac1	16.2	6.3	0.94	13.4	6.6	0.67	5.8	3.2	0.68

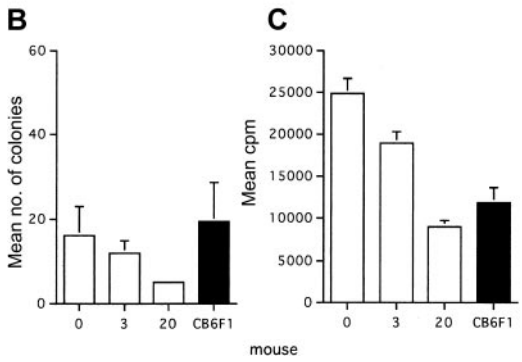

Figure 4. Reversion of BALB-neuT BM hyperplasia after transplantation into normal mice. BM from 23- to 28-week-old BALB-neuT females bearing 4 to 10 tumors was transplanted into 9-week-old CB6F1 mice. (A) Kinetics of BM uptake and reversion of myeloid hyperplasia in PB of mice with transplants. (B) CFU assay in spleen cells of 21-week-old CB6F1 mice with transplants (12 weeks after transplantation). (C) Proliferation of total splenocytes from CB6F1 (□) and control (■) mice with transplants in response to alloantigens in mixed leukocyte reaction. Results shown are from 3 of 6 mice tested. Mean of 3 replicates ± SD.

from BALB-neuT tumors or BALB-neuT serum in the presence of anti-VEGF neutralizing Ab, showed significantly reduced CFU numbers in methylcellulose, similar to those of nonconditioned Sca-1$^+$–enriched BM cells (Figure 5C).

Discussion

We investigated the relationship between tumor progression and host hematopoiesis in the model of transgenic BALB-neuT female mice. The multistep progression of breast carcinoma in these mice[27] was accompanied by increased splenomegaly and erythrocytosis. A progressive increase in circulating leukocytes with an expansion of Gr-1/Mac-1 double-positive cells and a reduced percentage of lymphocytes was found in the PB obtained from the retro-orbital sinus of BALB-neuT females. Within the expanded myeloid population, a high percentage of Gr-1$^+$/Mac-1$^+$ cells expressed ER-MP12, the vascular adhesion molecule platelet endothelial cell adhesion molecule 1 (PECAM-1) (CD31). This marker of immature hematopoietic precursors[36] regulates the transmigration of CD34$^+$ cells across the endothelium,[37] allowing an increase in circulating hematopoietic progenitors. The dilution effect caused by the increased spleen cellularity was responsible for the apparent reduction of lymphocytes and DCs. These findings show that the entire hematopoietic system of BALB-neuT mice was altered as a function of tumor progression. Indeed, a progressive increase in the colony-forming ability of the BM and spleen cells from BALB-neuT females correlated directly with tumor multiplicity. The strict relationship between tumor progression and host hematopoiesis was confirmed by the conditioning effect of BALB-neuT serum and the supernatant from explanted mammary carcinomas on normal BM progenitors in vitro. More directly, BM transplantation experiments showed that the tumor was responsible for driving normal BM to hypertrophic hematopoiesis, resulting in expansion of an immunosuppressive myeloid population within a few weeks. On the other hand, BALB-neuT hyperplastic BM reverts to normal when transferred to a healthy host, and thus reversion was associated with the restoration of immune functions. Thus, tumor load appears to be the crucial factor in the outcome of any immunotherapeutic approach.

Expansion of Gr-1/Mac-1 double-positive cells has been described in mice bearing transplantable tumors[12,16-20] or treated with cyclophosphamide[21] and has been associated with immune suppression. In BALB-neuT females, various immunotherapy protocols aimed at breaking tolerance to the c-erbB-2 oncogene and activating an immune response have been successful if started very early.[28-30] However, even in successfully treated mice, a T-cell–mediated cytotoxic response has not been detected, while an antibody response appears to be involved in tumor protection.[28,29] Such an immune suppression might be due to impaired antigen presentation. In fact, the number of DCs from mice bearing transplantable tumors is frequently reduced.[11,17,38] Our analysis of the phenotype of splenic DCs and the generation of DCs from BM in tumor-bearing BALB-neuT mice revealed an increase in the absolute number of CD11c$^+$ cells in spleen owing to the increased cellularity, and an expression profile of MHC class II, CD86, and CD40 antigens similar to that of normal splenic DCs from BALB/c mice; generation of DCs from BM of BALB-neuT females bearing 1 to 4 tumors, as well as the expression pattern of MHC class II, CD 86, and CD40, was similar to that of age-matched BALB/c mice (not shown). The finding of a normal number and differentiation pattern of splenic DCs in tumor-bearing BALB-neuT mice contrasts with the studies by Gabrilovich et al[7,17] and Ishida et al,[11] who described the reduced number and altered maturation of DCs in nodes and spleen of tumor-bearing and normal mice

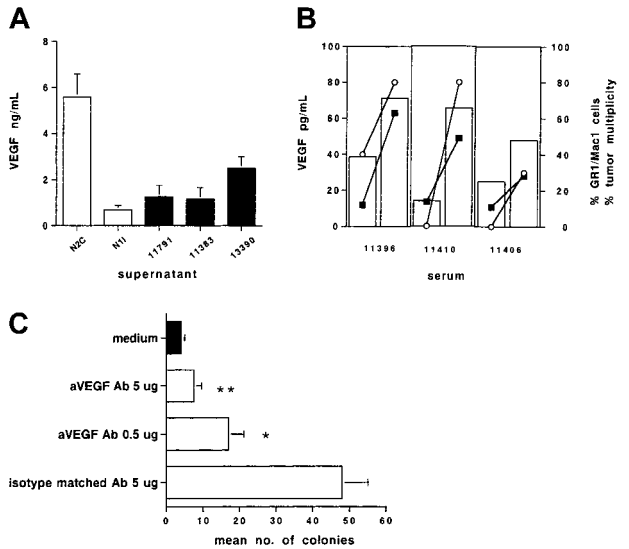

Figure 5. Effect of tumor-produced VEGF on hematopoiesis. Tumor-produced VEGF underlies altered hematopoiesis. (A) Levels of VEGF in the supernatant of established mammary carcinoma cell lines (□) and in the supernatant of 48-hour primary culture of spontaneous mammary tumors (■), measured by ELISA. (B) Levels of VEGF in serum from 3 representative BALB-neuT females at 22 to 26 weeks of age (□) in correlation with the rising percentage of Gr-1/Mac-1 cells in the PB (■) and tumor multiplicity (○). (C) VEGF-neutralizing Ab-mediated inhibition of the effects of BALB-neuT tumor supernatant on normal BALB/c hematopoietic stem/progenitor–enriched cells. Sca-1$^+$–enriched BM cells were conditioned for 5 days with medium containing 50% tumor supernatant (□), in the presence of anti-VEGF Ab (Ab JH121), isotype-matched control Ab, or medium alone (■) before CFU assay in methylcellulose. Statistically significant differences of anti-VEGF treatment from control: *$P < .03$; ** $P < .01$ (mean of 3 replicates ± SD).

chronically treated with recombinant VEGF (as described later in this section). Since we found no difference between BALB/c and tumor-bearing BALB-neuT mice in the generation of DCs from BM, we hypothesize that the immune suppression observed in tumor-bearing BALB-neuT mice is due to immature myeloid cell expansion rather than defective antigen presentation. Consistent with this hypothesis, Gr-1$^+$/Mac-1$^+$ cells were responsible for the reduced proliferative response of T lymphocytes to allogeneic antigens, and the BALB/c alloresponse was suppressed upon addition of Gr-1$^+$/Mac-1$^+$ cells from BALB-neuT mice. The increasing immature myeloid cell population first impairs the T-cell response to allogeneic antigens and the response to CD3 triggering and IL-2, which appears to correlate inversely with the percentage of Gr-1$^+$/Mac-1$^+$ cells, while the nonspecific proliferative response to the mitogen concanavalin A (ConA) is conserved. This is in accord with previous observations that Gr-1$^+$/Mac-1$^+$ cells from mice bearing transplantable tumors inhibit an MHC class I–restricted CD8-specific response (ie, interferon-γ release), but not ConA-induced proliferation.[20]

Contact hypersensitivity tests in tumor-bearing or tumor-free BALB-neuT females and in BALB/c mice, to assess overall T-cell function, revealed similar kinetics of the delayed immune response in all 3 groups (data not shown). The normal reaction of tumor-bearing BALB-neuT mice to haptens in contact hypersensitivity assays, consistent with data from other models,[12,20,23] suggests that immune suppression occurs when immature myeloid cells interact directly with lymphocytes. This finding raises the possibility that delayed-type hypersensitivity and cutaneous recall responses are not appropriate indicators of patient's immune responsiveness or eligibility for immunotherapy vaccination. The impairment of the alloresponse and the proliferative response to anti-CD3 and IL-2 increases with the increase in circulating Gr-1$^+$/Mac-1$^+$ cell proportions and tumor multiplicity, thus explaining the successful response to immunotherapy in young animals without tumors.

Several soluble factors might underlie tumor-driven host hematopoiesis, including VEGF, which is produced in large amounts by most tumors and whose production has been associated with poor prognosis.[39,40] VEGF plays a role in the differentiation of CD34$^+$ progenitors,[8] in the expansion of immature myeloid cells, in blocking DC maturation, in modifying hematopoiesis, and in increasing the number of committed myeloid progenitors and colonies.[20,41,42] In several mouse models,[7,17,20] continuous but not short-term treatment with recombinant VEGF has been shown to reduce the number of DCs in nodes and spleen, inhibit splenic DC function, and block DC maturation, thereby increasing antigen uptake. Conversely, treatment with anti-VEGF antibodies has been shown to restore DC maturation and function.[17] In BALB-neuT serum, increasing levels of VEGF secreted by mammary tumors accounted for the hematopoietic conditioning, as shown by the effect of neutralizing VEGF on CFUs obtained from normal BM cells cultured with tumor supernatant, but did not impair DC differentiation or number. The use of different mouse models and of different amounts of VEGF may account for this discrepancy. The VEGF serum level detected by Gabrilovich et al[17] in their mouse models, either tumor bearing or receiving recombinant VEGF, were much higher than those measured in BALB-neuT mice, which never exceeded 80 to 100 pg/mL.

In another study, an imbalance between tumor-released GM-CSF and IL-4 was responsible for Gr-1$^+$/Mac-1$^+$ cell expansion and impaired DC maturation in tumor-bearing mice.[12] We did not detect GM-CSF in BALB-neuT sera or supernatant from BALB-neuT mammary tumor explants, suggesting that multiple mechanisms might underlie the altered myeloid differentiation. It has been shown that the matrix metalloproteinase-9 (MMP-9) mediates the release of growth factor, such as VEGF, stromal cell–derived factor 1 (SDF-1), c-Kit ligand (c-Kit-L),[43] and that VEGF stimulates MMP-9 secretion by hematopoietic stem cells to enhance recovery of hematopoiesis after chemotherapy.[42] Our own search for MMP-9 production by c-erbB-2 mammary tumors revealed the presence of 2 bands, 75 kDa and larger than 200 kDa, corresponding to the mature form of MMP-9 and perhaps to a complex MMP-9/tissue inhibitor of metalloproteinase (TIMP), in Western blot analysis of supernatant from primary tumor explants but not in the supernatant from established mammary tumor cell lines (data not shown), suggesting that MMP-9 production occurs in the context of an organized tumor mass, functioning in the growth and metastasis of the tumor cells. However, ELISA analysis revealed higher levels of pro–MMP-9 in sera of BALB-neuT than in normal BALB/c sera, and tumor multiplicity correlated directly with increased MMP-9 levels (data not shown). These results are consistent with the use of different soluble factors by mammary tumors to condition host hematopoiesis directly and indirectly. The release of such factors as VEGF and MMP-9 further benefits the tumor by directly activating neoangiogenesis in situ, and by digesting the extracellular matrix to accommodate growth and metastasis, in addition to their effect on the BM to drive host hematopoiesis toward progenitor cell expansion and eventual immune suppression. With continuing growth, the tumor progressively improves its own vascularization and spread, as well as its escape from host immune recognition. In fact, early immunotherapy treatments that prevent tumor growth are associated with reduced VEGF serum levels and normal BM and spleen cellularity and CFU scores (not shown), while VEGF serum levels, CFU capability, and immune response return to normal in healthy recipients of transplants of tumor-conditioned BM, confirming that hematopoiesis is controlled in the absence of tumor.

Our data indicate that tumor-induced immune suppression is but one aspect of altered host hematopoiesis. Factors involved in the conditioning of host BM might serve as new targets for therapies aimed at reducing myeloid expansion, at delaying the associated immune suppression, and perhaps at improving the effects of immunotherapy. In addition, characterization and quantification of immature myeloid cells in the PB as well as assessment of VEGF or MMP-9 serum levels might provide surrogate markers to monitor response to therapies or progression of certain malignancies.

The BALB-neuT mouse has proven to be a useful model to study the effects of tumor-released factors on host functions in a more physiologic way than in healthy mice injected with such factors. In fact, BALB-neuT mice provide a model of a progressively "sick" patient where therapy protocols can be adjusted to account for compromised host functions and systems due to tumor progression.

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