**EblacZ Tumor Dormancy in Bone Marrow and Lymph Nodes: Active Control of Proliferating Tumor Cells by CD8+ Immune T Cells**

Markus Müller, Fotini Gounari, Sophia Prifti, Hans Jörg Hacker, Volker Schirrmacher, and Khashayarsha Khazaie

**ABSTRACT**

A well-defined lacZ gene tagged DBA/2 lymphoma (EblacZ) was used to examine the role of host immune responses in controlling tumor dissemination and persistence, as well as metastasis. In s.c. and intra-ear pinna-inoculated mice, low numbers of EblacZ cells homed to the bone marrow and lymph nodes. The frequency of bone marrow residing tumor cells did not change with the growth of primary tumor or with multiple inoculations of tumor cells. The bone marrow residing tumor cells expressed the proliferation-associated Ki67 antigen and expanded upon CD8+ depletion. In contrast, inoculation of nu/nu or severe combined immunodeficiency mice or of immune-suppressed DBA/2 mice led to the rapid outgrowth of EblacZ cells in the bone marrow and their metastasis to other organs.

Transfer of bone marrow from EblacZ immunized MHC congenic or syngeneic DBA/2 donors, but not from naïve donors, protected s.c.-inoculated DBA/2 mice. Protection was abrogated by in vitro depletion of CD8+ T cells prior to transfer of bone marrow. These experiments show that bone marrow and lymph nodes are privileged sites where potentially lethal tumor cells are controlled in a dormant state by the immune system. Metastasis may be a consequence of the breakdown of this immune control.

**INTRODUCTION**

A well-characterized animal model for tumor metastasis is represented by the methylcholanthrene-induced lymphoma L5178Y (Eb), induced originally in a female DBA-2 (H-2b) mouse. The Eb tumor line expresses characteristic MHC I-restricted CTL epitopes and is nonmetastatic in syngeneic hosts; however, several metastatic variants, such as ESb and ESb-MP, have been further established from this original tumor line (1, 2). The transition from the nonmetastatic to the metastatic phenotype required successive i.p. transplantations and in situ selection (3, 4). The Eb and derived cell lines express characteristic class I major histocompatibility Kd-associated tumor antigens and elicit specific CTL responses in immunized syngeneic hosts (5).

L5178 (Eb) cells were also among the first tumor cell lines used in tumor dormancy studies. To establish dormancy, DBA/2 mice were first inoculated s.c. with a lethal dose of Eb cells, and the resulting tumor mass was then surgically excised. Such mice were challenged i.p. with Eb cells at a dose that produced ascitic tumors and death in 100% of naïve mice. The vaccinated mice were initially protected but eventually succumbed to rapid tumor cell outgrowth and ascites after several weeks, some as late as 340 days after the challenge (6-8). This delay in tumor outgrowth was shown to be due to cytotoxic and cytostatic effects mediated by interleukin 2, IFN-γ and tumor necrosis factor-α secreted by lymphocytes and macrophages (9, 10).

We have shown that immunization through s.c. inoculation of DBA/2 mice with Eb tumor cells genetically modified to express the bacterial lacZ gene (EblacZ) and rendered nontumorigenic, either through irradiation or genetic modification, resulted in the persistence of these cells in the host bone marrow. This long-term persistence correlated with the duration of antitumor immunity (11). Similarly, potentially lethal live Eb-lacZ cells did not grow as a tumor when injected i.p. but rather disseminated to the bone marrow and established a state of tumor dormancy. Because EblacZ cells were lethal when injected s.c., the persistence of these cells after iep inoculation in part satisfied the definition for tumor dormancy (12) as a state in which potentially lethal tumor cells persist for a prolonged period of time in a clinically normal host with little or no increase in the tumor cell population.

Here, we have exploited our earlier observations on the dissemination and persistence of EblacZ cells to the bone marrow of vaccinated mice to define the factors responsible for the dormancy of tumorigenic EblacZ cells. We report that the rapid dissemination and persistence of low numbers of Eb-lacZ tumor cells in the bone marrow is found in mice with or without lethal primary Eb-lacZ tumors. Our observations suggest that in tumor-resistant hosts, the bone marrow and lymph nodes can be privileged sites for the persistence of tumor cells in an apparent dormant state. The maintenance of low numbers of Eb cells in these organs was not caused by the tumor cells resting in the G0 state of the cell cycle but by active T-cell immunity, resulting in low numbers of proliferating tumor cells. Transplantation of bone marrow from Eb dormant mice to naive mice yielded both tumor cells and protective immune effector cells. The bone marrow thus appears to be a privileged site where protective immune T cells keep tumor cells in a dormant state.

**MATERIALS AND METHODS**

Recombinant Retroviruses, Transduction, Characterization, and Detection of Modified Tumor Cells. Tumor cells were genetically modified by retroviral gene transduction using the M48 retroviral vectors (13) encoding a single bacterial lacZ gene fused to the nuclear localization signal of the SV40 large T, driven by the phosphoglucomutase gene promoter. The vector was packaged using the ecotropic CRE packaging cell line (14). Several independent modified clones were isolated and tested. The most aggressive of these clones was used for additional experiments. To visualize tumor cells, whole organs or dispersed cells were fixed in 2% formaldehyde/0.2% glutaraldehyde and stained overnight with 5-bromo-4-chloro-3-indolyl-β-D-galactosidase. Dispersed cells from bone marrow, lymph node, or spleen were cytospun (106 cells) onto poly-L-lysine-treated glass slides before staining for β-galactosidase (11). This method allowed confident scoring of single tumor cells among 106 nontumor cells, as determined by mixing lacZ-positive and -negative cells. This was necessary because dispersed cells were cytospun (106 cells) onto poly-L-lysine-treated glass slides before staining for β-galactosidase (11). This method allowed confident scoring of single tumor cells among 106 nontumor cells, as determined by mixing of lacZ-positive and -negative cells. The Ki67 (anti-mouse rabbit polyclonal) was a kind gift from Prof. J. Gerdes (Forschungsinstitut Borstel, Kiel, Germany). For double staining, cells were fixed with 3.7% formaldehyde and stained with Ki67 rabbit anti-mouse serum (1:100 dilution) and secondary Cy3 conjugated α-rabbit, followed with X-gal staining overnight and incubation with DAPI.

**Vaccination and Irradiation.** Mice were between 8 and 12 weeks of age at the beginning of the experiments. Tumor cells (2 × 106 live EblacZ cells) were washed with PBS and resuspended in 50 μl of PBS for injection iep or i.p.

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100 μl s.c. Mice were irradiated at 5 Gy (500 rads) with a 137Cs source (Gammacell 1000; Atomic Energy, Ottawa, Canada).

**Immunostaining.** Staining of bone marrow cells was done with biotin/streptavidin directly coupled α-CD8-FITC and α-CD4-PE (Life Technologies, Inc.) and pan-β-TCR-Red670 (15).

**T-Cell Depletion in Vivo and in Vitro.** For in vivo depletion of CD8+ cells, the monoclonal antibody YTS-169.4.2 (purified IgG; Ref. 16) was injected once or several times (as indicated) i.p. in a pretreated concentration of 0.5 mg in 200 μl of PBS. Three-color fluorescence-activated cell sorter analysis (10^6 events) with α-CD8, and α-CD4 and pan-β-TCR-T revealed 0.26% mature bone marrow T-cells before depletion and 0.01% after depletion. In vitro depletion was performed using α-CD8 IgM mAb and treatment with complement and DNase.

**Bone Marrow Transplantation.** In bone marrow transplantation experiments, donor mice were injected iep or s.c. with PBS or 5 × 10^6 to 2 × 10^6 EblacZ cells in a total volume of 50 μl. At indicated time points, 2 × 10^6 bone marrow cells were prepared and injected i.v. into recipient mice. One day later, the recipient mice were challenged s.c. with 5 × 10^5 or 2 × 10^6 EblacZ cells, and their survival was followed.

**Quantitation of CTL Activity.** Quantitations of in vitro and in situ activated CTLs were performed as described earlier (5, 11). Briefly, for in vitro restimulation, 9 days after inoculation with EblacZ cells (2 × 10^6), spleen cells (2 × 10^5) were removed and restimulated in vitro for 5 days with EblacZ cells (2 × 10^5 cells, 100 Gy irradiated). For in situ restimulation, after 7 days the mice were restimulated i.p. with EblacZ cells (2 × 10^5 cells, 100 Gy irradiated), and 3 days later, peri toneal exudate cells (PEC) were isolated. ^51Cr release assays were performed after 4 h of incubation of the CTLs with ^51Cr-labeled target.

**ELISPOT.** ELISPOT assays were performed by culturing 2 × 10^5 immune spleen cells (9 days after bone marrow transfer) with 2 × 10^5 irradiated EblacZ cells for 5 days. The cells were then plated in 96-well cell-based microtiter plates (MAHA N45; Millipore) coated with rat-α mouse IgM-μ mAb; after 20 h, the wells were washed with PBS/Tween/1% BSA and incubated with biotin-conjugated rat-α mouse mAb for 2 h. After additional washes, treatment with avidin peroxidase (Pharmingen; 1:1000) for 3–10 min relieved positive spots, which were scored visually using a light microscope.

**ELISA.** For ELISA, β-galactosidase-coated (Sigma Chemical Co.; 2.5 μg/ml in 0.05 m NaCO₃/0.05 m NaHCO₃, pH 9.6) Falcon-ELISA plates were washed three times with PBS/Tween, blocked with 0.2% gelatin/PBS (1 h, 37°C), incubated with test sera for 1 h at room temperature, washed, and incubated with goat-α mouse IgG-Peroxidase Ab-900 (Dianova, Hamburg, Germany; dilution; 1:5000). After wash with PBS/Tween, OPD/HzO₂ was added for 5–30 s in the dark, the reaction was stopped with 1 N H₂SO₄, and measurements were performed at 492 nm.

**RESULTS**

**Vaccination and Tumor Development.** When 2 × 10^6 EblacZ cells were s.c. inoculated into syngeneic DBA/2 mice, growth of primary tumors and death was observed within 1 month of inoculation (Fig. 1A, a and b). When the same number of live tumor cells was injected iep at 14 or 56 days prior to the s.c. inoculation, the mice were protected against Eb tumor cell challenge (11). In agreement with earlier results, iep injection of the mice did not result in any tumors, and mice vaccinated by this protocol exhibited long-term protection against further challenge with tumor cells (Fig. 1A, a and b).

When s.c.-injected mice received 2 weeks after the first inoculation an equal number of EblacZ cells in the opposite flank, they did not exhibit any significant change in survival (Fig. 1Ba), but at the second site, only regressive small tumors were found (Fig. 1Bb). This observation indicated existence of concomitant immunity in the EblacZ-inoculated mice.

**Active and Specific Host T- and B-Cell Responses Correlate with Immunity against EblacZ Cells.** CTL assays were performed to analyze the status of tumor-specific T-cell responses in tumor-bearing mice. Iep-vaccinated or s.c.-inoculated tumor-bearing mice were used as source of immune spleen cells, which were restimulated with EblacZ in vitro. The activated spleen cells were used as effector cells in CTL assays against EblacZ cells. Alternatively, some inoculated mice were restimulated i.p. with irradiated tumor cells to examine the ability of the mice to elicit a specific T-cell response in vivo. Peritoneal exudate cells were used as source of effector T cells. Significant tumor-specific CTL activities were detected both in the vaccinated and the tumor-bearing DBA/2 mice, using either method (Fig. 2A). Anti-β-galactosidase ELISA tests of sera from EblacZ-transplanted mice revealed comparable humoral immune responses in both instances (Fig. 2B).

**Fig. 2C shows the production of IFN-γ by spleen cells from individual, immune bone marrow-reconstituted DBA/2 mice upon in vitro restimulation with either the same (EblacZ) or different (ESb) tumor-associated antigen(s).** In these experiments, donor B10.D2 mice were inoculated i.v. with EblacZ cells. After 2 weeks, 2 × 10^7 bone marrow cells from these mice were transplanted to naive recipient DBA/2 mice. Nine days later, spleen cells were obtained from the recipient mice, stimulated in vitro with EblacZ or with the closely related ESb cells, and analyzed for production of INF-γ using an ELISPOT assay. An antigen-dependent increase in the number of cytokine-releasing cells was observed in all recipient mice but not in naive mice. Thus, specific cellular (Fig. 2a and C) and humoral (Fig. 2B) immune responses against EblacZ cells were detected in tumor-bearing mice as well as immune cell-reconstituted mice.

**T-Cell Immunity Is Required for the Control of EblacZ Tumor Metastasis.** Staining for lacZ readily allowed detection of single tumor cells in organs, frozen tissue sections, or in cytopsins of
In late-stage disease, the bone marrow and spleen were heavily infiltrated by tumor cells (Fig. 4A, a and c), and the draining lymph nodes were overgrown by tumors (Fig. 4Ab). A similar metastatic spread was seen in SCID mice and in sublethally irradiated DBA/2 mice (data not shown). These observations underline the importance of T-cell immunocompetence for the control of EblacZ tumor metastasis.

Active Immune Responses of Tumor-bearing Mice Prevent Metastases but Fail to Control the Dissemination and Persistence of Tumor Cells in Privileged Sites. In DBA/2 mice the iep-inoculated EblacZ cells disseminated rapidly into the lymphatic system, appearing in the draining lymph nodes within 30 min and in nondraining lymph nodes, bone marrow, and spleen by 24 h. Over several months of observation, only residual numbers (1–100/109) normal cells) of tumor cells were detected in the bone marrow and lymph nodes (Fig. 4A, a and b), whereas the tumor cells were rapidly cleared from the spleen (Fig. 4Ac). The bone marrow of tumor-bearing DBA/2 mice resembled closely that of iep-vaccinated mice. Thus, in contrast to immune-compromised, tumor-bearing mice, immune-competent mice presented only small numbers of EblacZ cells in the bone marrow that did not increase with time (Fig. 4B). A second inoculation with tumor cells did not significantly affect the number of EblacZ cells in the bone marrow (Fig. 4B). These observations show a close correlation of immunity and control of EblacZ tumor metastasis. In this particular animal model, the host immune response effectively cleared all examined tissues from tumor cells, with the exception of two privileged sites: the bone marrow and the lymph nodes.

A Significant Fraction of the Residual Tumor Cells Detected in the Bone Marrow Express an S-Phase Proliferation Marker. To analyze the tumorigenic potential of bone marrow-residing EblacZ cells, bone marrow from iep-inoculated DBA/2 mice was placed in growth medium with 5% FCS, conditions under which only tumor cells were expected to survive. Staining for lacZ indicated that the expanded nonadherent cells indeed represented EblacZ cells. s.c. inoculation of varying numbers of these cells into DBA/2 mice clearly showed that the bone marrow-derived EblacZ cells had maintained their tumorigenic potential (Fig. 5A).

To investigate the proliferation potential of the tumor cells in the bone marrow of DBA/2 mice directly ex vivo, cytospin preparations from bone marrow of mice inoculated with EblacZ were triple stained for lacZ (to visualize tumor cells; Fig. 5Bb), DAPI (to visualize DNA; Fig. 5Bb), and Ki67 (to identify potentially dividing cells; Fig. 5Bc). In contrast to the broad staining for lacZ that marked the shape of intact nuclei in cytospins and histological sections (see Fig. 3), nuclear lacZ appeared as a collapsed aggregate when cells were fixed for staining with the Ki67 antibody (Fig. 5Ba). In the iep-inoculated, disease-free, immune-competent mice, 21.3 ± 4.0% of the bone marrow-derived tumor cells were positive for the S-phase-specific Ki67 proliferation marker (Fig. 5Bc). The fraction of Ki67-positive tumor cells in the diseased bone marrow of immune-compromised DBA/2 mice was 40 ± 12.2%, whereas tissue culture-derived EblacZ cells contained 87 ± 3.5% Ki67-positive cells. Thus, a fraction of bone marrow-residing EblacZ cells were proliferating in both s.c.-inoculated, tumor-bearing mice and iep-inoculated, tumor-free mice, but the absolute number of cells did not increase, indicating that the cell number was controlled by the on-going immune response.

CD8+ Immune T Cells Control Tumor Cell Numbers and Transfer Protective Immunity. To address the potential role of CD8+ immune T-cells in the control of tumor dissemination and metastasis, DBA/2 mice were either injected with depleting monoclonal CD8 antibodies or irradiated with 5 Gy to prevent primary antitumor responses. In both instances, iep inoculation of immune-suppressed mice with EblacZ cells led to rapid dissemination and continuous growth of the tumor cells in the host bone marrow, resulting in the deaths of the animals (Fig. 6, a and b). In contrast, similarly inoculated, immune-competent DBA/2 mice did not develop any metastases.
Tumor dormancy in bone marrow

Fig. 3. T-cell immunocompetence is a critical requirement for the control of EblacZ tumor metastasis. X-gal and periodic acid-Schiff staining of sections of DBA/2 and nu/nu mice inoculated with EblacZ cells. A, syngeneic DBA/2 (a) and nu/nu (b) mice were injected with $2 \times 10^6$ EblacZ cells iep and sacrificed 24 days later. Organs were removed, fixed in 2% formaldehyde and 0.2% glutaraldehyde, X-gal stained for 24 h, and cut on a cryostat. A, intact organs (top, liver; middle, lungs; bottom, kidneys) of immune DBA/2 or nu/nu mice, stained for lacZ. B, histological analysis of the same organs. a, liver of nu/nu mouse with focal metastasis; b, unstained liver of DBA/2 mouse; c, lung of nu/nu mouse with sparse distributed, single tumor cells; d, unstained lung/heart of DBA/2 mouse; e, unspecific (not cellular) staining of nu/nu mouse kidney; f, unspecific (not cellular) staining of DBA/2 mouse kidney.

but rather established tumor dormancy in the bone marrow and lymph nodes as described before.

To investigate the influence of immune suppression on the frequency of bone marrow-residing dormant tumor cells, DBA/2 mice were vaccinated iep with EblacZ tumor cells. After 3 weeks, bone marrow of three mice was checked for establishment of tumor dor-
TUMOR DORMANCY IN BONE MARROW

Fig. 4. Active immune response of tumor-bearing mice prevents metastases but fails to control the dissemination and persistence of tumor cells in privileged sites. A, groups of DBA/2 or nu/nu mice were injected iep with 2 x 10^6 EblacZ cells. At the indicated time points, organs were removed from 20 animals per group, and single-cell suspensions were prepared from bone marrow (a), lymph nodes (b), or spleen (c). Two cytospins with 10^6 cells each for each individual mouse were fixed and stained overnight for lacZ. Stained cells were scored visually under a light microscope. Results were averaged; bars, SD. B, groups of 10 immunocompetent (C), sublethally irradiated DBA/2 (△) mice were injected iep with 2 x 10^6 EblacZ cells once in the right flank (○) or in both flanks (●). The frequency of EblacZ cells in the bone marrow was scored as before as a function of days after the first challenge.

DISCUSSION

Our data show that potentially metastatic EblacZ cells can reside as dormant tumor cells in lymph nodes and bone marrow as long as CD8 + T cells can mediate protective immunity that prevents the at least partially cycling cell population from expanding and disseminating. Although the persistence of dormant tumor cells has been described previously in tumor cell-vaccinated mice (11, 19), our results here show that they also exist in tumor-bearing, s.c.-injected hosts, and in both [Fig. 7, groups VII and VIII] mice also transferred CD8-dependent protective immunity against challenge with live EblacZ cells, but the effects were not as significant and long-lasting as those seen with BMT from B10.D2 donors.

These observations provide evidence for the coexistence of tumor cells and immune CD8⁺ T-cells with the potential for protective immunity in the bone marrow of mice inoculated with EblacZ tumor cells.
Fig. 5. Tumorigenic potential and proliferation of residual tumor cells detected in the marrow. A, tumorigenic potential of bone marrow-derived EblacZ cells. To test the tumorigenic potential of bone marrow-derived tumor cells, $2 \times 10^5$ (●) or $2 \times 10^6$ (○) EblacZ cells expanded in vitro from the bone marrow of iep-inoculated DBA/2 mice were injected s.c. into each of at least 10 naive DBA/2 mice. As control, $2 \times 10^5$ (□) normal, in vitro-passaged EblacZ cells were inoculated. B, proliferation potential of bone marrow-residing EblacZ cells. Bone marrow cytospins from three independent experiments each using groups of three mice were stained with anti-Ki67, anti-β-galactosidase, and DAPI. Each panel shows three representative fields from independent cytospins at ×1000. At least 100 bone marrow-residing, β-galactosidase-stained cells were scored to quantitate the frequency of Ki67-positive tumor cells. a, β-galactosidase-stained tumor cells, showing precipitation of the blue staining in nuclei of tumor cells under the fixing conditions used. b, DAPI staining for DNA showing general outline of nuclei in the same field; note the uppermost EblacZ cell with condensed chromosomes, undergoing cell division. c, Ki-67 staining in the same field. Arrows, tumor cells. See main text for quantitations.

Fig. 6. Control of EblacZ tumor cell number in the bone marrow requires CD8 immune T cells. DBA/2 mice were injected iep (a) 1 day after depletion with anti-CD8 monoclonal antibody (zebra arrow), 1 day after irradiation (closed arrow; b), 3 weeks before the start of a course of depletion (zebra arrows; c), or 3 weeks before irradiation (closed arrow; d). At each indicated time point, three mice (α-CD8-treated) or six mice (irradiated) were sacrificed, and the frequency of lacZ-positive cells in the bone marrow was determined in duplicate cytospins for untreated (●), CD8⁺-depleted (□), or irradiated (▲) mice.
strongly supports the notion of postoperative persistence of tumor cells in a dormancy state. Several clinical observations point to the bone marrow as a major site of persistence of tumor cells in curatively operated or treated cancer patients. A significant fraction of patients with non-small-cell lung carcinomas (25), prostate cancer (26, 27), colon cancer (28, 29), squamous cell carcinomas of the head and neck region (30), gastric cancer (31, 32), and breast cancer (33, 34) harbored low numbers of tumor cells in the bone marrow at the time of and after operation of the primary tumor. Similarly, tumor cells were found in regional lymph nodes in metastasis-free cancer patients (35).

The detection of bone marrow-residing tumor cells has been considered to be a significant and independent predictor for a later clinical relapse in distant organs (25, 28, 31).

The microenvironment of the tumor cells is likely to play a determining role in the maintenance of tumor dormancy state as well as the transition to tumor metastasis (12, 20, 36, 37). Although neovascularization may be a critical factor for the eventual outbreak of metastasis (38), it is highly unlikely to be limiting the number of tumor cells in the bone marrow or lymphatic system. Bone marrow and lymph nodes are two prominent organs involved in antigen presentation and immune response (39, 40). The bone marrow of immunized animals is a rich source of protective immune cells (41, 42). Our observations are consistent with these organs offering favorable conditions for homing of tumor cells and their survival, in spite of the presence of protective immune cells at the same sites.

### References


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