T lymphocytes restrain spontaneous metastases in permanent dormancy

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Abstract

Tumor dormancy is a clinical phenomenon related to immune equilibrium during cancer immunoediting. The mechanisms involved in dormant metastases are poorly understood due to the lack of preclinical models. Here, we present a non-transgenic mouse model in which spontaneous metastases remain in permanent immune-mediated dormancy with no additional antitumor treatment. After the injection of a GR9-B11 mouse fibrosarcoma clone into syngeneic BALB/c mice, all animals remained free of spontaneous metastases at the experimental endpoints (3-8 months) but also as long 24 months after tumor cell injection. Strikingly, when tumor-bearing mice were immunodepleted of T lymphocytes or asialo GM1-positive cells, the restraint on dormant disseminated metastatic cells was relieved and lung metastases progressed. Immunostimulation was documented at both local and systemic levels, with results supporting the evidence that the immune system was able to restrain spontaneous metastases in permanent dormancy. The GR9-B11 tumor clone doesn’t presented surface expression of MHC class I molecules, and all metastases in immunodepleted mice were MHC class I-positive. This model system may be valuable for more in-depth analyses of metastatic dormancy, offering new opportunities for immunotherapeutic management of metastatic disease.
Introduction

The initiation and progression of cancer in an immunocompetent host involve numerous interactions between tumor cells and the immune system. The immune response exercises selective pressure against tumor cells, eliminating the more immunogenic phenotypes. This constant interaction between the immune system and cancer cells may ultimately result in the selection of less immunogenic “cancer-escape” variants that are able to survive and progress in the host (1, 2). The diverse escape mechanisms developed by cancer cells to evade the immune response (3-6) include the loss of surface expression of MHC class I molecules (7-9). This loss may make the tumor cells invisible to T lymphocytes, allowing them to enter an “immunoblindness” stage (10).

It is feasible that some cancer cells neither progress nor are destroyed by immune system during this selective process, remaining in a dormant stage and reaching equilibrium with the host tumor microenvironment (11-13). Cancer dormancy has been observed in humans (14, 15), and several experimental studies have reported an immune-mediated control of primary tumor cells in dormancy (16-20). There is considerable evidence of metastasis relapse in human cancer patients after long periods of remission, when disseminated metastatic cells can persist for years or even decades as minimal residual disease (21, 22). Other clinical examples related to cancer immune control include tumors that arise after immunosuppressive treatments (23, 24) and cases of transplanted organs carrying an undetectable tumor that grows after immunosuppressive treatment of the patient (25-27). These clinical phenomena support the existence of a state of equilibrium between the host and the cancer
cells. The fact that immunosuppression can disturb this equilibrium and activate dormant cancer cells strongly suggests the existence of an immune-mediated state of dormancy in these cases.

The mechanisms involved in cancer dormancy remain largely unknown, due to difficulties in isolating dormant human metastatic cells and constructing preclinical models of dormant metastases. Here, we presented a novel non-transgenic preclinical mouse model of permanent immune-mediated metastatic dormancy. We have used an extensively studied fibrosarcoma mouse model (GR9) developed in our laboratory and composed of several tumor clones with different MHC class I expression patterns and spontaneous metastatic capacities (28). Thus, the metastatic capacity is elevated in the clones with high MHC class I expression and reduced in those with low MHC class I expression (29). We show that an MHC-I negative GR9-B11 tumor cell clone did not generate spontaneous lung metastasis in immunocompetent mice, which remained free of metastasis until their euthanasia at the end of the assays (3-24 months). Strikingly, immunodepletion of T or asialo GM1-positive cells in the mice awoke the disseminated metastatic cells from dormancy, generating lung metastases.

Materials and Methods

Cell lines and IFN-γ treatment

GR9 cell line is derived from a mouse fibrosarcoma induced by methylcholanthrene in BALB/c mice and has been extensively characterized in
our laboratory. It is composed of cell clones with distinct H-2 class I expression patterns and metastatic capacities (29). Spontaneous metastasis assays were performed with different GR9 cell clones, and one of these, the GR9-B11 clone, was selected for this study. GR9-B11 and GR9-A7 are clones obtained by a limited dilution method from the GR9 cell line. GR9-B11 and GR9-A7 cell lines were recloned by picking up individual cells under phase contrast microscopy. All cell lines were characterized by PCR assay using short tandem repeat, and they were also regularly tested for MHC-I genotype and surface expression.

Cell lines were maintained in Dulbecco’s medium (Sigma–Aldrich, Madrid, Spain) supplemented with 10% fetal bovine serum (Life Technologies, Milan, Italy), 2 mM glutamine (Sigma–Aldrich), and antibiotics. In some experiments, cell lines were treated with 100 U/ml IFN-γ for 48 h (Sigma–Aldrich).

**Mice**

Eight-week-old male BALB/c and athymic nu/nu BALB/c (Charles River Laboratories, Barcelona, Spain) mice were used in the experiments. The breeding and care of animals were undertaken in compliance with European Community Directive 86/609/CEE and Spanish law (Real Decreto 1201/2005) for the use of laboratory animals. Housing and all experimental procedures involving animals were performed according to protocols approved by the hospital Animal Care Committee and in compliance with the animal welfare guidelines of the National Committee for Animal Experiments.

**Spontaneous metastasis assay**

Different cell doses (50 × 10^5, 25 × 10^5, 12.5 × 10^5, and 6.25 × 10^5) of GR9-B11 were subcutaneously injected into the footpad of groups of syngeneic
immunocompetent and nude BALB/c mice. The growth of local tumors was recorded 3 times/week in all animals, measuring the largest diameter of each tumor with electronic calipers. Tumors were excised when the largest diameter reached 10 mm. The model resembles metastatic development in humans after surgical removal of the primary tumor. Mice were anesthetized with 0.04 ml diazepam (Valium, Roche, Madrid, Spain) and 0.1 ml ketamine (Ketolar, Pfizer, Spain) before removal of the primary tumors with sterilized instruments, using electrocautery to minimize bleeding and closing the wounds with surgical clips and adhesive. After the surgery, each animal was housed alone until recovery from anesthesia. At the end of the assays, animals were anesthetized and euthanized by cervical dislocation. A complete necropsy was performed, and the number of spontaneous metastases was counted. Local tumors and macroscopically visible metastatic nodules were excised, disaggregated, and adapted to tissue culture. Then, the lungs were fixed in Bouin’s solution (Sigma-Aldrich) and the micrometastases were counted.

**Preparation of splenic and lung leukocytes**

Spleens and lungs were excised and gently homogenized in a stomacher in cold PBS (Sigma-Aldrich). A tissue fragment was removed, and a sterile falcon cell strainer (BD Bioscience, Madrid, Spain) was used to create a single cell suspension. Red blood cells were lysed with ACK lysing buffer (Gibco, Paisley, UK) for 5 min and then washed twice in PBS. Viable cells were counted and used for the antibody staining reaction.

**Flow cytometry analysis of immune-cell subsets**
For direct immunofluorescence, the following labeled antibodies (Miltenyi-Biotec, GmbH, Germany) were used: CD3-APC, CD4-FITC, CD8-PE, CD25-PE, FoxP3-APC, CD19-FITC, CD49b-FITC, anti-MHC class II-APC, anti-CD11c-PE, and anti-CD11b-FITC. Isotype-matched non-immune mouse IgGs conjugated with FITC, PE, or APC served as controls. FcR Blocking Reagent was used to block unwanted binding of antibodies to mouse cells expressing Fc receptors. Immunofluorescence was done according to the manufacturer’s instructions (Miltenyi Biotec), using FoxP3 staining buffer to obtain optimal FoxP3 immunofluorescent staining. Cells were analyzed on a FACSCanto cytometer (BD Bioscience). Each sample consisted of a minimum of 5 × 10⁴ cells and was analyzed with CellQuest-Pro software.

**MHC class I surface expression**

MHC class I surface expression was analyzed by indirect immunofluorescence using FACS (FACScan; Becton Dickinson, Mountain View, CA, USA) according to a standard protocol. In brief, 5 × 10⁵ cells were washed twice with PBS and incubated for 30 min at 4ºC with the primary antibodies anti-H-2 K<sup>d</sup> (K9-18), anti-H-2 D<sup>d</sup> (34-5-8), and anti H-2 L<sup>d</sup> (28.14.8 and 30.5.7), all obtained from the ATCC (Rockville, MD, USA). The secondary fluorescein isothiocyanate (FITC)-conjugate antibody (anti-mouse FITC IgG/Fab, Sigma-Aldrich) was used in 1:120 dilution for 30 min at 4ºC in the dark. Isotype-matched non-immune mouse IgG and cells labeled with the fluorescein-conjugated antibody alone served as controls. A minimum of 1 × 10⁴ cells were analyzed with CellQuest-Pro software. All cell lines were studied in baseline conditions and after IFN-γ treatment.
**Real-time RT-PCR analysis**

An mRNA isolation kit (Miltenyi-Biotech) was used to extract mRNA from tumor cell lines. First-strand cDNA was synthesized with 100 ng of mRNA using a High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 µl. These cDNAs were diluted to a final volume of 100 µl. Real-time quantitative PCR analyses were carried out in the 7500 Fast System (Applied Biosystems), performing PCR reactions in quadruplicate and expressing the values obtained as means ± standard deviation (SD). Quantitative PCR was performed with the Power SYBR Green Master mix (Applied Biosystems); the primers and amplicon size for each gene were previously reported (30). GADPH and β-actin genes were used as housekeeping genes. PCR conditions were 40 cycles of 15 sec of denaturation at 95°C and 60 sec at 60°C.

**Immunodepletion protocols in spontaneous metastasis assay**

12.5 × 10⁵ GR9-B11 cells were injected into the footpad of syngeneic BALB/c mice. The growth of the tumors was measured three times per week. At around 20-22 days, the tumors reached 10 mm and were excised as described above. At 151 days, one group of mice was euthanized and the remaining mice were randomly divided among six groups. Four of these groups received immunodepletion treatments with monoclonal antibodies. The following protocols were used: i) 100 µg anti-CD4 mAb (clone YTS191) + 100 µg anti-CD8 mAb (clone YTS 169), ii) 100 µg anti-CD4 mAb, iii) 100 µg anti-CD8 mAb, and iv) 50 µg anti-asialo GM1 (Wako chemicals USA Inc., Virginia). A fifth group was treated with 100 µg control immunoglobulin (Policlonal rat IgG, Sigma-
Aldrich), while the remaining group received no treatment (untreated mice). The agents were administered twice a week for 90 days from day 152 after the tumor cell injection. Depletion of the corresponding immune subpopulations in the mice was confirmed by flow cytometry. Mice from each group were euthanized at the end of the treatments (day 242 post-cell injection). A complete necropsy was performed, isolating and analyzing the lungs as described above.

**Statistical analysis**

Data were expressed as means ± SD. The paired Student's t test was used to compare mean values. A significance level of \( p < 0.05 \) was assumed for all statistical tests. SPSS 16.0.2 (IBM, Chicago IL) was used for the data analyses. All statistical tests were two-sided.

**Results**

**H-2 class I phenotype of GR9-B11 fibrosarcoma clone cell line and local primary tumors**

GR9 is a methylcholanthrene-induced fibrosarcoma generated in BALB/c mice and is composed of several tumor clones with different H-2 class I surface expressions (28). GR9-B11 fibrosarcoma cell line was derived from the GR9 tumor by limited dilution cloning. The GR9-B11 clone was recloned by picking up individual cells under phase contrast microscopy. Under baseline conditions, this tumor clone cell line has a negative expression of H-2 K\(^d\), D\(^d\) and L\(^d\) molecules (Fig. 1a). IFN-\( \gamma \) treatment upregulated all the three molecules (Fig.
1a). Possible mechanisms underlying the loss of surface MHC-I expression of GR9-B11 were investigated by analyzing the transcriptional gene expression of antigen processing machinery (APM), H-2 class I heavy chains, and β2-microglobulin genes. The GR9-B11 fibrosarcoma clone was compared with a positive MHC-I GR9-A7 clone cell line, normalizing the data to the expressions of GAPDH and β-actin housekeeping genes. Figure 1b depicts the results, using the values for GR9-A7 cells as reference (assigned a relative value of 1). GR9-B11 showed a downregulation of H-2 Ld, calreticulin, LMP2, TAP-1, and tapasin (Fig. 1b). These results demonstrate that the molecular mechanism underlying the loss of MHC-I surface expression involves the coordinated transcriptional downregulation of several APM components and MHC-I heavy chains.

In tumor-initiating capacity (T-IC) assays, four cell doses (50.0 × 10^5, 25.0 × 10^5, 12.5 × 10^5 and 6.25 × 10^5 cells) were locally injected into the footpad in groups of ten mice. The local tumors were removed from the animals when the largest tumor diameter reached 10 mm. The tumors were then adapted to tissue culture to analyze their H-2 class I surface expression in comparison to that of the GR9-B11 clone. The surface expression of H-2 class I molecules in vitro was higher in the local tumor cell lines than in the original clone. The tumors showed positive surface expression of H-2 Kd and Dd molecules and negative for H-2 Ld molecule in baseline conditions (Fig. 1c). The H-2 Kd and Dd molecules were strongly upregulated after in vitro treatment with IFN-γ and showed an even higher expression than was observed on the original tumor clone. However, two populations were observed with different patterns of expression of H-2 Ld molecule: in one population, H-2 Ld molecule was clearly
upregulated, whereas in the other, H-2 L$^d$ molecule was negative (Fig. 1c).

These results were observed in all local tumors analyzed regardless of the cell dose injected.

**Spontaneous metastasis assays from GR9-B11 clone in immunocompetent and nu/nu BALB/c mice**

To determine the *in vivo* metastatic capacity of the GR9-B11 tumor clone, the mice were monitored weekly for spontaneous metastatic spread after removal of primary tumors. The mice showed no signs of disease and were eventually euthanized at 90 days after the tumor excision. The autopsy revealed that all mice were metastasis-free, regardless of the cell dose used. We then repeated these assays and found that the mice remained metastasis-free at 24 months after primary tumor removal, when the mice were euthanized.

Two possible explanations for these findings were then explored: the disseminated metastatic cells might not be capable of migrating or invading, or GR9-B11 metastatic cells might be eliminated by the immune system. These possibilities were examined by performing spontaneous metastasis assays with GR9-B11 cells in immunodeficient nu/nu BALB/c mice, injecting $6.25 \times 10^5$ cells into two groups of 10 nude mice. The local growth rate of tumors in these mice was similar to that in immunocompetent mice, reaching a largest diameter of 10 mm in 32 *versus* 29 days, respectively. The local tumors were removed when this diameter was reached and were adapted to tissue culture. Analysis of their H-2 class I surface expression revealed the same H-2 class I phenotype as expressed by the primary tumors generated in immunocompetent mice (Fig. 1c). The mice were monitored for the appearance of spontaneous metastases.
and were euthanized when signs of disease were observed. Unexpectedly, lung metastases (range, 1-8) were found in 80% of these mice (Fig. 2a). The immunocompetent mice served as controls in this assay, and all remained metastasis-free (Fig. 2a). These results indicate that GR9-B11 cells have intrinsic migratory and invasive capacities. All macroscopically visible metastatic nodules were adapted to tissue culture, and H-2 class I phenotype analysis revealed that all of the metastases had the same H-2 class I phenotype, characterized by the high baseline expression of H-2 K^d and D^d molecules, which were induced by IFN-\(\gamma\) treatment, and the absence of surface expression of H-2 L^d molecule under baseline conditions and after IFN-\(\gamma\) treatment (Fig. 2b). This phenotype represents a new tumor variant that is not present in the original clone but is observed in the primary tumors.

To summarize, the GR9-B11 fibrosarcoma clone generated spontaneous metastases in T-cell immunodeficient nu/nu BALB/c mice but not in immunocompetent BALB/c mice, and all metastases evidenced total loss of H-2 L^d molecule surface expression.

The immunogenicity of the metastases derived from nude mice was evaluated by injecting these metastatic cell lines into the footpad of immunocompetent BALB/c mice, administering different cell doses (12.5 \(\times\) 10^5 and 6.25 \(\times\) 10^5 cells) in two groups of five mice each. The metastatic cells demonstrated an elevated immunogenicity, being rejected in 67% of the mice injected with the higher cell dose and in 40% of those injected with the lower dose. Moreover, these mice did not develop spontaneous metastases.


Changes in immune-cell subpopulations promoted by GR9-B11 tumor cells

We carried out assays to evaluate individual immune-cell subpopulations at local and systemic level in GR9-B11 tumor-bearing immunocompetent mice. Two groups of thirty mice were euthanized on days 25 and 50 after removal of the local tumors, and spleen leukocyte populations were analyzed by flow cytometry (Fig. 3a and Table 1). Tumor-bearing mice showed statistically significant changes in the lymphocyte subpopulations on days 25 (25d) and 50 (50d) in comparison to the non-tumor-injected animals (NT) \( (p < 0.05) \), with an increase in CD3\(^+\) (44.4 and 53.7 vs. 33.1\%, respectively), CD3\(^+\)CD4\(^+\) (33.3 and 40.4 vs. 26.1\%), and CD3\(^+\)CD8\(^+\) (11.1 and 13.0 vs. 6.9\%) lymphocytes, a slight increase in NKT cells (1.0 and 1.4 vs. 0.4\%), and an increase in dendritic cells (8.7 and 8.0 vs. 3.0\%) and macrophages (8.6 and 7.1 vs. 3.8\%) (Fig. 3b and Table 1).

We also analyzed the changes in lymphocyte subpopulations in the lungs of mice on day 25 and 50 after removal of the local tumor (Fig. 4a and Table 1), finding a major rise in the percentage of CD3\(^+\) lymphocytes, which had increased to 64\% on both days, versus 51.9\% in non-tumor-injected mice (Fig. 4b); this expansion corresponded to increases in T-helper lymphocytes (46.2\% and 50.5\% vs. 40.6\%, respectively) and T-cytotoxic lymphocytes (17.4 and 12.1 vs. 9.7\%, respectively (Fig. 4b and Table 1). We highlight that the percentage of CD8\(^+\) T lymphocytes had increased by 180\% on day 25.

T or asialo GM1-positive cells maintain dormant spontaneous metastases in a state of equilibrium
According to the above experiments, the GR9-B11 clone produced spontaneous pulmonary metastatic nodules in nude mice but not in immunocompetent mice, which remained metastasis-free and developed an immune response. We therefore hypothesized that disseminated metastatic cells would be eliminated by the immune system in immunocompetent mice or, alternatively, would be kept in a dormant state. These possibilities were tested in a new experiment (Fig. 5a), in which $12.5 \times 10^5$ GR9-B11 cells were injected into the footpad in seven groups of immunocompetent BALB/c mice. The local tumors were removed at 20-22 days, and one of the groups was euthanized at 151 days; the necropsy revealed that no metastases were present in any mice in this group. From day 152, the mice in another four groups were treated weekly with anti-CD4 + anti-CD8 mAbs, anti-CD4 mAb, anti-CD8 mAb, or immunoglobulins (control Ig group) (Fig. 5a). A sixth group of mice was treated with the anti-asialo GM1 antibody (Fig. 5a). After three months of treatment, the mice were euthanized on day 242 (Fig. 5a), and the depletion of each subpopulation was confirmed by flow cytometry. In the mice treated with control immunoglobulin, no metastatic nodules were detected in the necropsy (Table 2). In the groups treated with anti-CD4 + anti-CD8 mAbs or with anti-CD8 mAb alone, 100% of the mice developed spontaneous pulmonary metastases. Similar results were found in the group treated with anti-asialo GM1 antibody, in which 87% of the mice developed metastases (Table 2). However, in the group in which CD4+ T cells alone were depleted, only 23% of the mice developed metastases. The number of metastases per mouse differed among the groups (Table 2): it ranged from 4-62 micrometastases in the mice treated with anti-CD4 + anti-CD8 mAbs and from 3-17 in 50% of the group with CD8+ T
lymphocyte depleted-mice but reached 100 micrometastases in the other 50% of this group; it ranged from 2-35 micrometastases in 62% of asialo GM1 depleted-mice but reached more than 100 micrometastases in 25% of this group; it ranged from 1-2 micrometastases in the group treated with anti-CD4 mAb. Macrometastases were only detected in the mice treated with anti-CD4 mAb (13%) or anti-asialo-GM1 (30%) in a range of 1-2 per mouse. Mice from a 7th group that received no treatment were maintained up to 24 months after local tumor removal and remained metastasis-free (Table 2).

To summarize, we found a higher number of mice with metastases and higher number of metastases in the lungs in CD8- or asialo GM1-depleted mice than in CD4-depleted mice. The metastases remained in dormancy for 5 months and awoke when T or asialo GM1-positive cells were depleted.

After necropsy, all macroscopically visible spontaneous pulmonary metastases from immune-depleted mouse groups were adapted to tissue culture, and H-2 class I surface expression was analyzed. All metastases were characterized by a positive expression of Kd and Dd molecules and a total absence of Ld molecule expression under baseline conditions, while all three molecules were induced after IFN-γ treatment (Fig. 5b).

Discussion

This study describes for the first time a novel non-transgenic mouse tumor model of permanent immune-mediated metastatic dormancy. In this model, spontaneous metastases are completely controlled and maintained in a
dormant state by the wild-type mice immune system, with no application of any anti-cancer treatment. The local primary tumors grew rapidly, and the mice remained metastasis-free after tumor removal at the end of the assays (3-8 months) and for more than 24 months. Interestingly, immunodepletion of host T or asialo GM1-positive cells promoted the awakening of dormant disseminated spontaneous metastatic cells, which invaded the lungs of the mice. We highlight that the spontaneous metastases in our model are dormant, remaining in latency throughout the life of the animals; furthermore, this dormant metastatic state is maintained by the murine immune system through T and asialo GM1-positive cells, with no previous immunization or treatment of the hosts. This metastatic tumor murine model faithfully resembles the progression of human cancers in which a long latency period with minimal residual disease can follow the primary tumor resection, with the metastatic cells clinically manifesting years or even decades later (31-33). The GR9-B11 metastatic tumor model is a unique and reproducible experimental system for detailed analysis of the phenomenon of immune-mediated metastatic dormancy.

Various authors recently demonstrated that the immune response can delay cancer progression. Koebel et al. studied a MCA-induced tumor mouse model and reported that the immune response kept occult primary tumors in a state of equilibrium and that the cancer progressed after the joint depletion of CD4/CD8 cells and the depletion of IFN-γ or IL-12 (34). Unlike the present study, they investigated dormant preneoplastic or neoplastic tumor cells but not metastatic tumor cells. Another group found that the IFN-γ produced by lung NK cells played a major role against the development of pulmonary metastases (35), although these were experimental and not spontaneous metastases and
they were not dormant. It was also reported that cancer progression was accelerated by depletion of CD8+ T cells in a RET.ADD melanoma transgenic mice model, although the non-depleted mice also eventually developed cancer (36). In our assays, the mice remained metastasis-free until euthanized.

In the GR9 fibrosarcoma mouse model, MHC-I expression on different tumor clones has been indirectly correlated with in vivo T-IC (28). In the present study, tumor cells grew rapidly immediately after the injection of GR9-B11, possibly escaping from the immune system due to their altered MHC-I phenotype with low antigen presentation capacity. An inverse behavior was reported in spontaneous metastasis assays, which showed a higher metastatic capacity in the MHC-positive GR9 tumor clones than in the MHC-negative clones. A very high spontaneous metastatic capacity was previously reported for an MHC-I positive clone of the GR9 tumor model, GR9-A7, whose metastases were also MHC-I positive (37). However, unlike findings for the GR9-B11 clone, GR9-A7 tumor growth and metastatic dissemination produced an immunosuppressive effect in the hosts. Restoration of the host immune response by immunotherapy treatments completely eradicated the spontaneous metastases, and the animals remained metastasis-free (37). In the present study, immune stimulation was observed during the period of GR9-B11 primary tumor growth and dissemination of spontaneous metastatic cells, which was characterized by an increase in immune cells, especially T lymphocytes. In both cases, the spread of metastases was dependent on the immune effect promoted by the primary tumor cells. Our group also previously reported that GR9-B9, another tumor clone of GR9 primary fibrosarcoma, produced a larger number of spontaneous pulmonary metastases in nu/nu BALB/c mice than in
immunocompetent BALB/c mice (38, 39). These results and the present findings suggest that immunosurveillance plays a key role against metastatic progression, and that GR9-B11 tumor cells promote an immune response able to completely control disseminated metastatic cells and maintain them in a state of dormancy. This proposition is further supported by the finding that spontaneous metastases were generated in nu/nu and immunodepleted BALB/c mice injected with GR9-B11 tumor cells.

Baseline MHC-I surface expression was higher on all spontaneous metastases originated from the GR9-B11 tumor clone in immunodeficient and immunodepleted mice in comparison to GR9-B11 tumor cells. A similar phenomenon was previously reported for the GR9-B9 tumor clone, on which MHC-I expression is absent, finding that all metastases in immunodeficient mice were MHC-I positive under baseline conditions (38, 39). Hence, the MHC-I alterations were reversible (i.e., “soft lesions”) in both clones (39, 40). These results strongly suggest that MHC-I loss is not a requirement for escape in the absence of a T cell-mediated immune response. Recent investigations in chemoresistance human models of hormone-refractory prostate cancer (HRPC) identified an HLA-I negative cell subpopulation of the bulk population of primary and metastatic prostate cancer tissue (41). These HLA-I negative tumor cells exhibited resistance to chemotherapy, and their number correlated with the stage of the disease and its recurrence. In agreement with the present data, these HLA-I negative tumor cells displayed a higher T-IC in immunodeficient murine hosts in vivo, with the primary tumors again reproducing the initial HLA-I phenotypic heterogeneity of the original tumor (41). In this context, relapse after adoptive cell transfer therapies (ACTs) in patients with melanoma has been
related to the reversible downregulation of antigen expression (42). In brief, MHC-I positive and MHC-I negative cells may exist in a dynamic and interchangeable state of equilibrium that adapts in response to signals from the microenvironment.

The depletion of T or asialo GM1-positive cells was sufficient to awaken disseminated spontaneous metastatic cells from their dormant state. These results demonstrate the capacity of T or asialo GM1-positive cells in wild-type mice to maintain spontaneous micrometastases permanently occult in a state of equilibrium. In BALB/c mice, NK cells and some other minority subpopulations express asialo GM1. We hypothesize that NK cells did not exert a direct cytotoxic effect in our model, because no increase in NK cells was observed during the systemic or local immune response generated by GR9-B11 tumor cells. Furthermore, the depletion of CD8+ T lymphocytes produced spontaneous metastases in 100% of immunocompetent mice, and GR9-B11-bearing nude mice developed a considerable number of spontaneous metastases, despite the large amount of NK cells in these hosts. It was previously reported that NK cells may facilitate the development of an anti-tumor protective CTL response independent of CD4+ T lymphocytes (43-45). Another possibility is that an asialo GM1+ activated CD8 T subpopulation might be involved on the restraint the dormant metastases in this model (46, 47). However, another subpopulations of T cells should also be implicated, because the results found in CD8- versus asialo GM1-depleted tumor-bearing mice were different. In addition, we would highlight that MHC-I molecules can act directly as tumor suppressor genes, arresting cancer cell proliferation (48), and the FHIT tumor suppressor gene is directly implicated in MHC-I cell surface
expression (30). Taken together, these results suggest that the expression of MHC-I molecules on these metastatic cells may promote the dormant state via immune-mediated and oncogenic suppression mechanisms. Future investigations will be designed to clearly decipher the role of MHC-I molecules in this dormant state and the molecular mechanisms that may be involved.

In summary, we present a novel murine metastatic tumor model in which the host immune response per se can fully control spontaneous pulmonary metastases, maintaining them in a state of dormancy. Dormant micrometastases were awoken after the immunodepletion of T or asialo GM1-positive cells, revealing the major role of these immune cells in maintaining the metastases in a dormant state. This tumor model resembles the metastatic dormancy observed in some human cancer patients. This preclinical metastatic tumor model offers the possibility of in-depth investigation of the intrinsic characteristics of the premetastatic niche (49) and of the mechanisms underlying metastatic dormancy (50), offering new opportunities for immunotherapeutic management of metastatic disease. These data may help us understand how cancer might become a chronic disease that persists in non-fatal form in a clinically healthy individual.

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## Tables

### Table 1. Changes in splenic and lung leukocyte populations

#### Splenic leukocyte populations

<table>
<thead>
<tr>
<th></th>
<th><em>CD3</em></th>
<th><em>CD4</em></th>
<th><em>CD8</em></th>
<th>CD3 CD4 CD25 FoxP3</th>
<th><em>CD3</em></th>
<th>CD3 CD4 CD49b</th>
<th>CD3 CD11b</th>
<th><em>MHCII</em> CD11c</th>
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<td>NT</td>
<td>33.1 ± 4.3</td>
<td>26.1 ± 3.1</td>
<td>6.9 ± 1.8</td>
<td>4.4 ± 0.8</td>
<td>61.0 ± 4.2</td>
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<td>0.4 ± 0.3</td>
<td>3.8 ± 0.1</td>
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<td>25d</td>
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<td>33.3 ± 5.1</td>
<td>11.1 ± 1.9</td>
<td>4.3 ± 1.0</td>
<td>48.9 ± 6.8</td>
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</tr>
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<td>40.4 ± 4.1</td>
<td>13.0 ± 2.9</td>
<td>3.6 ± 1.7</td>
<td>41.0 ± 4.7</td>
<td>3.7 ± 1.0</td>
<td>1.4 ± 0.6</td>
<td>7.1 ± 1.4</td>
</tr>
</tbody>
</table>

#### Lung lymphocyte populations

<table>
<thead>
<tr>
<th></th>
<th><em>CD3</em></th>
<th><em>CD4</em></th>
<th><em>CD8</em></th>
<th>CD3 CD4 CD25 FoxP3</th>
<th><em>CD3</em></th>
<th>CD3 CD4 CD49b</th>
<th>CD3 CD11b</th>
<th><em>MHCII</em> CD11c</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>51.9 ± 6.3</td>
<td>40.6 ± 5.5</td>
<td>9.7 ± 1.5</td>
<td>1.9 ± 0.7</td>
<td>35.2 ± 5.9</td>
<td>12.2 ± 0.9</td>
<td>1.8 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>25d</td>
<td>64.5 ± 8.2</td>
<td>46.2 ± 7.0</td>
<td>17.4 ± 1.4</td>
<td>3.3 ± 0.8</td>
<td>24.2 ± 7.9</td>
<td>11.1 ± 4.4</td>
<td>1.5 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>50d</td>
<td>64.1 ± 5.2</td>
<td>50.5 ± 6.9</td>
<td>12.1 ± 2.2</td>
<td>3.7 ± 0.6</td>
<td>26.2 ± 2.1</td>
<td>9.6 ± 4.0</td>
<td>1.6 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD of 30 mice of each group

* Percentage among CD4+ cells
* Percentage among MHC-II+ cells
* *p* < 0.05 compared with NT group
Table 2. Number of metastases generated in control and depletion groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Metastasis incidence</th>
<th>Range</th>
<th></th>
<th>*micro-PMs</th>
<th>*macro-PMs</th>
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</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0/30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Control Ig</td>
<td>0/30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-CD4+CD8</td>
<td>30/30</td>
<td>4-62</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD8</td>
<td>30/30</td>
<td>3-17 (50%)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;100 (50%)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-asialo-GM1</td>
<td>26/30 (87%)</td>
<td>2-35 (62%)</td>
<td>1</td>
<td>0 (32%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;100 (25%)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>7/30 (23%)</td>
<td>1-2 (23%)</td>
<td>2</td>
<td>0 (10%)</td>
<td></td>
</tr>
</tbody>
</table>

*micro-PMs: pulmonary micrometastases; macro-PMs: pulmonary macrometastases
Figure legends

Figure 1. H-2 class I surface expression on GR9-B11 clone and in local primary tumors. A, H-2 class I expression of the GR9-B11 fibrosarcoma cell line under baseline conditions and after treatment with IFN-γ (100 U/ml) for 48 h: H-2 K (gray line), H-2 D (dotted line), and H-2 L (black line). GR9-B11 is H-2 negative and all three molecules are induced after IFN-γ treatment. B, Transcription levels of H-2 class I heavy chains, β2m, and several APM components detected by real-time RT–PCR. Expression level of the genes of interest was determined with respect to levels of β-actin and GAPDH housekeeping genes. Data for GR9-A7 are set to 1. Values are depicted as mean ± SD of three independent experiments performed in quadruplicate; *p < 0.01 for comparisons of GR9-A7 with GR9-B11. C, H-2 class I expression of local GR9-B11 tumor under baseline conditions and after IFN-γ treatment. All local tumors exhibited a practically identical pattern of H-2 class I expression: positive expression of H-2 K and D molecules in baseline conditions; after IFN-γ treatment, two populations were observed, one induced all three molecules and the other showed no induction of H-2 L molecule. A representative example is depicted.

Figure 2. Spontaneous metastasis assays in immunocompetent and nu/nu BALB/c mice. A, The graph depicts the mice with metastases and the number of lung metastases per mouse. A dot represents the number of lung metastases identified in each mouse. These results were reproducible in another two independent experiments. B, H-2 class I phenotype of spontaneous pulmonary metastases in nude mice. All metastases showed a single phenotype under baseline conditions and after IFN-γ treatment, with positive expression of K and
D molecules alone. H-2 K (gray line), H-2 D (dotted line), and H-2 L (black line).

A representative example is depicted.

**Figure 3.** Changes in splenic leukocyte populations (lymphocytes, macrophage and dendritic cells [DCs]). The different leukocyte populations were analyzed at days 25 (25d) and 50 (50d) after local tumor removal in comparison to non-tumor-injected mice (NT). A, A representative experiment showing B (CD3−CD19+) and T (CD3+CD4+ and CD3+CD8+) lymphocyte subpopulations, macrophages (MHC-II+CD11b+), and DCs (MHC-II+CD11c+). B, The graphs depict the percentages (mean ± SD) of CD3+/CD19+, CD4+/CD8+ and CD11b+/CD11c+ cells. *p < 0.05.

**Figure 4.** Lung lymphocytes were quantified at different time points. At days 25 (25d) and 50 (50d) after removing the local tumor, the lymphocyte subpopulations were measured and compared with non-tumor-injected mice (NT). A, A representative experiment shows B (CD3−CD19+) and T (CD3+CD4+ and CD3+CD8+) lymphocyte subpopulations. B, The graphs depict the (mean ± SD) percentages of CD3+/CD19+ and CD4+/CD8+ lymphocytes. *p < 0.05.

**Figure 5.** Spontaneous metastasis assays in immunocompetent and immunodepleted BALB/c mice. A, Time schedules of the immunodepletion protocols in spontaneous metastasis assays. The mice were injected with GR9-B11 cells on day 0. The local tumors were removed between days 20 and 22. One of the groups was euthanized at 151 days. Five groups of mice were treated, twice a week for 90 days, by intraperitoneal injection of monoclonal antibodies (anti-CD4 + anti-CD8, or anti-CD4, or anti-CD8, or anti-asialo GM1) or lgs (control Ig), beginning on day 152, while another group remained
untreated. All mice were euthanized on day 242. B, H-2 class I surface expression of metastases awoken from dormancy. H-2 K (gray line), H-2 D (dotted line), and H-2 L (black line). All macrometastases showed positive expression of H-2 K and D molecules under baseline conditions, and all three molecules were induced after IFN-γ treatment. A representative example of three independent experiments is depicted.
Figure 1
Figure 2
Figure 4
Figure 5
T lymphocytes restrain spontaneous metastases in permanent dormancy

Irene Romero, Cristina Garrido, Ignacio Algarra, et al.

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