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# Interferon-γ-dependent Phagocytic Cells Are a Critical Component of Innate Immunity against Metastatic Mammary Carcinoma[1]

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## ABSTRACT

**IFN-γ is a pleiotropic cytokine that plays an important role in regulating the growth of primary tumors. Although numerous studies of the effects of IFN-γ on primary-solid-tumor growth have been performed and several potential mechanisms for its efficacy have been proposed, it remains unclear how IFN-γ modulates tumor progression and whether it exerts its effects indirectly via host cells or directly by interacting with tumor cells. Using the well-characterized mouse metastatic mammary carcinoma 4T1 in a postsurgery setting, IFN-γ-deficient mice were found to have significantly shorter survival time relative to wild-type mice, demonstrating that IFN-γ is also a critical component in regulating innate immunity to metastatic disease. Experiments quantifying lung and liver metastasis indicate that decreased survival of IFN-γ-deficient mice is attributable to increased metastatic disease. To determine whether IFN-γ is acting directly on the tumor cells, IFN-γ-nonresponsive 4T1 cells were generated by transfection (4t1/IRt). Metastasis experiments with 4T1/IRt demonstrated that IFN-γ mediates its effects via host-derived cells, rather than by directly affecting tumor growth. To identify the population of cells responsible for IFN-γ efficacy, perforin-deficient, T-cell subset-depleted, natural killer cell-depleted, or carrageenan-treated phagocytic cell-depleted mice were inoculated with 4T1 and assessed for primary tumor growth and metastatic disease. None of the conditions altered primary tumor growth; however, the carrageenan treatment significantly increased metastatic disease in the liver and lungs. Survival experiments in 4T1-inoculated, carrageenan-treated mice confirmed that the elimination of phagocytic cells significantly reduces survival time and yields a survival phenotype comparable with IFN-γ deficiency. Therefore, IFN-γ is a critical component of innate immunity to metastatic mammary carcinoma that probably mediates its effects via host-derived phagocytic cells.**

## INTRODUCTION

IFN-γ is a pleiotrophic cytokine that regulates hundreds of diverse genes. Many of these genes are involved in responses to pathogens, and their effects are manifested via the immune system (1–3). For example, IFN-γ regulates immune functions such as immunoglobulin heavy-chain class switching, cell-mediated ($T_{h1}$ *versus* $T_{h2}$) and NK[3] cell immune responses, phagocytic cell clearance of bacteria, antigen processing and presentation by MHC class I and class II molecules, and leukocyte-endothelial interactions. In addition to its role in combating pathogens, IFN-γ has also been implicated in immune responses to tumors, and recent studies demonstrated that the induction of IFN-γ underlies the therapeutic efficacy of IL-12-based tumor immunotherapy (4). Although it is appreciated that IFN-γ plays an important role in regulating tumor growth, the mechanism(s) by which it exerts its effect is unclear.

On the basis of *in vivo* and *in vitro* observations, several antitumor mechanisms have been ascribed to IFN-γ. For example, it induces tumor cell production of antiangiogenic factors, which promote antitumor effects through starvation of tumor cells (5). Similarly, Fas/FasL interactions which are necessary for IFN-γ up-regulation of antiangiogenic factors, promote vascular endothelial apoptosis in the tumor microenvironment, thereby limiting tumor growth (6). Other studies show that tumor-induced antiangiogenesis requires expression of IFN-γ receptor (CD119) on nonhematopoietic cells, which suggests that IFN-γ-dependent host factors are involved (7). Other studies show the effects of IFN-γ on the host's immune system that suggest that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression by IFN-γ-activated NK cells (8) and IgG2a and IgG2b antibody production by B cells (9) are important for the generation of IFN-γ-dependent antitumor responses. Clearly IFN-γ potentially acts through multiple mechanisms and may alter tumor growth either by its direct effects on tumor cells or via its action on host cells and/or factors. Not only is it unclear which of these mechanisms is (are) relevant for tumor rejection, but it is also uncertain whether these mechanisms work cooperatively or synergistically. Likewise, the relative contribution of each mechanism to overall tumor rejection is unknown.

We have undertaken the present study to identify the predominant IFN-γ-mediated mechanism(s) responsible for regulating tumor growth and to clarify whether host and/or tumor cells are the target for IFN-γ activity *in vivo*. Because metastatic cancer is the major cause of death for patients with solid tumors, our studies focus on the role of IFN-γ in metastatic disease. Several types of animal models are available for these studies. Transgenic mice carrying transforming genes under the control of viral promoters provide animals with spontaneously developing primary tumors (9, 10). However, these animals typically die from massive, multifocal primary tumors rather than disseminated metastatic disease and, hence, are not optimal models for the study of metastatic cancer. Transplantable tumors include both immunogenic and nonimmunogenic tumors. Because human tumors are poorly immunogenic, nonimmunogenic or poorly immunogenic mouse tumors are the better models. We have used the poorly immunogenic 4T1 mouse mammary carcinoma. This BALB/c-derived transplantable tumor shares many characteristics with human breast tumors and is an established model for studies of metastatic cancer (11–14). After inoculation of small quantities of 4T1 tumor cells in the abdominal mammary gland, primary tumor grows progressively and spontaneously metastasizes to the lungs, liver, blood, lymph nodes, brain, and bone marrow (11, 12, 14, 15). Analogous to human mammary carcinoma, metastatic cells proliferate at distant sites while the primary tumor is in place, and continue to proliferate when the primary tumor is surgically removed (13). The 4T1 system allows us to focus on the development of metastatic disease after the surgical removal of primary tumor. This scenario has not been previously explored in animal models, and may be very relevant for human cancers such as breast cancer, in which metastatic

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[3] The abbreviations used are: NK, natural killer; Mig, monokine induced by IFN-γ; IP-10, IFN-γ-inducible protein; TD, tumor diameter; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent; IL, interleukin; mAb, monoclonal antibody; LPS, lipopolysaccharide; DC, dendritic cell; iNOS, inducible nitric oxide synthetase.

disease after the excision of primary tumor is the principal cause of death.

Our studies indicate that although several tumor- and host-derived IFN-γ-dependent factors regulate the growth of distant metastasis, host phagocytic cells play a major role. Furthermore, IFN-γ acts via host cells and/or factors and does not regulate metastatic growth by directly acting on tumor cells.

## MATERIALS AND METHODS

**Animals, Cell Lines, cDNA Expression Vectors, and Transfectants.** Female BALB/c, BALB/c.IFNγ$^{-/-}$, and BALB/c.IL4$^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). BALB/c.Pfp$^{-/-}$ mice were generated as described previously (16). All of the mice were maintained and bred in the University of Maryland-Baltimore County (UMBC) Biology Department animal facility and were used at 8–16 weeks of age. All of the animal procedures were reviewed and approved by the UMBC Institutional Animal Care and Use Committee and are in compliance with the NIH guidelines for the humane treatment of laboratory animals. 4T1, a 6-thioguanine-resistant, BALB/c-derived spontaneous mammary carcinoma (11), was cultured in Iscove's modified Dulbecco's medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine product (Hyclone, Logan, UT) and 1× Antibiotic-Antimycotic (Life Technologies, Inc.; Ref. 12). Wild-type tumor cells were transfected with the expression vector pEF2.mugR (5), containing the cDNA for a truncated IFN-γR$_\alpha$, by using Pfx-5 lipid (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were selected with 400 μg/ml G-418 (Life Technologies, Inc.), stained for surface antigen expression, and analyzed by flow cytometry (12). mAbs 34-5-8 (H-2D$^d$) and MKD6 (I-A$^d$) were affinity purified and used as previously described (12). mAb CD119 (IFN-γRα) was purchased from BD BioScience (San Diego, CA).

***In Vitro* Proliferation Assay.** 4T1 and 4T1/IRt transfectants at $1 \times 10^4$/ml were cultured with recombinant IFN-γ (Pierce Endogen, Rockford, IL) for 3 days, and proliferation was measured using MTT (13). The percentage of growth inhibition = $[(A_{0U/ml} - A_{nU/ml})/(A_{0U/ml})] \times 100\%$. 4T1/IRt transfectants that expressed low levels of CD119 remained responsive to IFN-γ.

***In Vitro* Induction of Mig.** 4T1 and 4T1/IRt cells were induced *in vitro* with 100 units/ml IFN-γ for 2 h. RNA was isolated, and Mig was detected using RT PCR (17).

**Tumor Inoculation, Clonogenic Metastasis Assay, *in Vivo* Depletion, and Surgery.** Mice were challenged s.c. in the abdominal mammary gland with $7 \times 10^3$ 4T1 or transfected 4T1 tumor cells. Primary tumor growth and spontaneous metastasis in the lungs and liver using the clonogenic assay were measured as described previously (12). Mice were depleted of CD4$^+$ T, CD8$^+$ T, and NK cells and were monitored to ascertain depletion as described previously (17). Phagocytic cells were depleted by injecting mice i.p. with 2 mg carrageenan iota type (Ref. 18; Sigma, St. Louis, MO) on days −3 and −1 before tumor inoculation, followed by 1 injection every 14 days throughout the experiment. Depletion of macrophages by carrageenan treatment was evaluated by measuring reduced susceptibility to LPS-induced toxic shock syndrome. Primary tumors were surgically removed on days 21–24 after 4T1 inoculation as described (13).

**Statistical Analyses.** Data were analyzed using Student's *t* test for unequal variances (Microsoft Excel v5.0).

## RESULTS

**IFN-γ-deficient Mice Have a Significantly Reduced Survival Time after Inoculation of 4T1 Mammary Carcinoma Cells, Relative to IFN-γ-Competent Mice.** We previously established that syngeneic BALB/c mice inoculated with $7 \times 10^3$ 4T1 mammary carcinoma cells in the abdominal mammary gland develop disseminated metastasis within 10–21 days of 4T1 inoculation and will die from metastatic cancer even if the primary tumor is surgically removed (13). If IFN-γ reduces metastatic tumor growth, then IFN-γ-deficient (BALB/c.IFNγ$^{-/-}$) mice might have a decreased survival time relative to wild-type BALB/c mice. To test this hypothesis, BALB/c and BALB/c.IFNγ$^{-/-}$ mice were inoculated with 4T1 cells, had primary tumors surgically removed on days 21–24, and were followed for survival. The average survival time for BALB/c and BALB/c.IFNγ$^{-/-}$ mice was 44 (±6) and 36 (±2) days, respectively ($P < 0.001$). To determine whether the shortened survival of the BALB/c.IFNγ$^{-/-}$ mice was caused by more metastases arising from larger primary tumors, survival time was plotted as a function of TD at the time of surgery. The average sizes of the primary tumors in BALB/c and BALB/c.IFNγ$^{-/-}$ mice were similar at their time of removal (Fig. 1*A*). Therefore, BALB/c.IFNγ$^{-/-}$ mice are more susceptible to the 4T1 tumor, which suggests that metastatic disease progresses more rapidly in IFN-γ-deficient hosts.

**BALB/c.IFNγ$^{-/-}$ Mice Develop Spontaneous Lung and Liver Metastases Earlier Than Wild-Type BALB/c Mice.** To determine whether the deletion of IFN-γ results in earlier deaths because of increased metastatic disease, the number of metastatic tumor cells in 4T1-inoculated BALB/c and BALB/c.IFNγ$^{-/-}$ mice was measured. Mice were inoculated s.c. in the abdominal mammary gland with $7 \times 10^3$ 4T1 tumor cells and killed on day 35, after which their lungs and livers were removed. The number of metastatic 4T1 tumor cells in these organs was quantified using the clonogenic assay. Primary tumors were not surgically removed for this experiment because

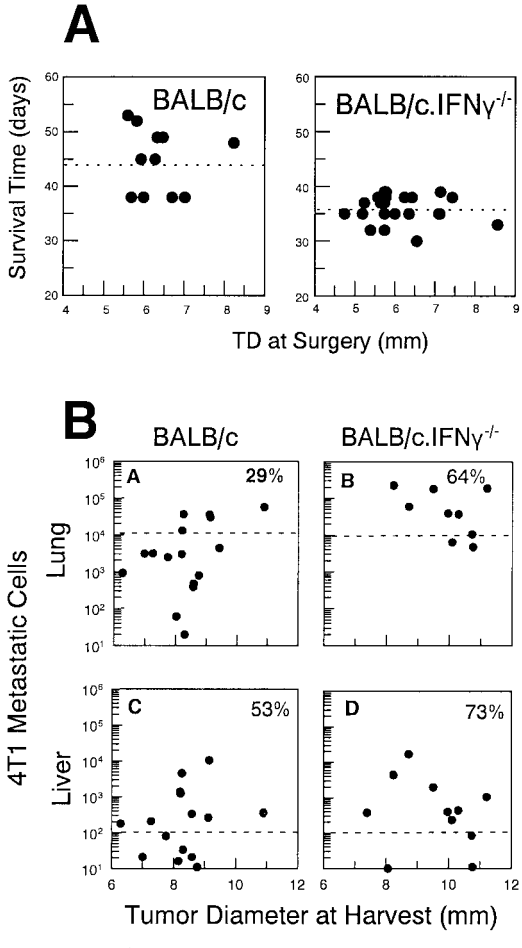


Fig. 1. BALB/c.IFNγ$^{-/-}$ mice have a shortened survival time and increased numbers of metastatic cells after removal of primary tumor, relative to wild-type BALB/c mice. BALB/c and BALB/c.IFNγ$^{-/-}$ mice were inoculated s.c. in the abdominal mammary gland with $7 \times 10^3$ 4T1 cells on day 1. *A*, primary tumors were measured and surgically removed on days 21–24, and mice were followed for survival. *Data points*, the survival time of an individual mouse. · · · ·, the mean survival times. *B*, 35 days after 4T1 inoculation, mice were sacrificed, and the number of metastatic 4T1 cells in the lungs and liver quantified using the clonogenic assay. *Data points*, the number of 4T1 metastatic cells in an individual mouse. · · · ·, the lethal levels of metastatic disease in these organs.

surgical removal of primary tumor does not alter the survival time or the number of disseminated metastases (13).

To determine whether primary tumor size impacts the level of metastatic disease, the data were plotted as the number of clonogenic metastatic cells *versus* TD at the time of harvest (day 35). Because 10,000 and 100 tumor cells in the lungs and liver, respectively, indicated lethal levels of metastatic disease (12), a line indicating these values was included in the plots. As shown in Fig. 1*B* and Table 1, 64% of BALB/c.IFN$\gamma^{-/-}$ *versus* 29% of BALB/c mice contained >10,000 clonogenic lung metastases and 73% of BALB/c.IFN$\gamma^{-/-}$ *versus* 53% of BALB/c mice contained >100 clonogenic liver metastases. To determine whether increased metastasis is specific to the BALB/c.IFN$\gamma^{-/-}$ mice, BALB/c.IL-4$^{-/-}$ mice were also tested. BALB/c.IL-4$^{-/-}$ mice did not develop lung and liver metastases faster than BALB/c mice (data not shown). Thus, deletion of IFN-γ increases the number of spontaneous metastatic tumor cells, and this increase is most likely responsible for the heightened lethality of 4T1 in BALB/c.IFN$\gamma^{-/-}$.

**Primary Tumor Growth Is Marginally Increased in BALB/c.IFN$\gamma^{-/-}$ Mice as Compared with Normal BALB/c Mice.** Because previous immunotherapy studies with 4T1 showed disparate effects on primary and metastatic tumor cells (17), we also analyzed the effects of IFN-γ on primary tumor development in the mice used for Fig. 1. Primary tumors were palpable within 6–13 days of inoculation and although the TDs between BALB/c and BALB/c.IFN$\gamma^{-/-}$ mice were significantly different at day 35 ($P = 0.045$), the overall kinetics of primary tumor growth did not differ (Table 2 and data not shown). Therefore, IFN-γ is a critical mediator of metastatic tumor progression and has only marginal effects on primary tumor growth.

**Generation of IFN-γ-nonresponsive 4T1 Tumor Cells.** Because IFN-γ has multiple immune and nonimmune targets *in vivo*, we tested for whether IFN-γ was inhibiting metastasis progression by acting directly on the tumor cells. Several studies have shown that IFN-γ can alter tumor cells directly *in vitro* with correlative reduction in primary tumorigenicity *in vivo* (19, 20). To examine this possibility, we generated 4T1 transfectants that were insensitive to IFN-γ (4T1/IRt transfectants). If IFN-γ affects metastatic tumor growth by directly acting on the tumor cells, then the growth kinetics of 4T1/IRt in BALB/c mice will be similar to the growth kinetics of 4T1 in BALB/c.IFN$\gamma^{-/-}$ mice.

To generate an IFN-γ-insensitive cell line, 4T1 was transfected with the pEF2.mugR plasmid, which contains a cDNA for a truncated IFN-γR$_\alpha$ chain. High levels of truncated IFN-γR$_\alpha$ chain compete with endogenous full-length IFN-γR$_\alpha$ and yield cells that are nonresponsive to IFN-γ (5). Transfectants were selected in G418 and analyzed for overexpression of IFN-γR (CD119) using flow cytometry. As shown in Fig. 2*A*, the transfectants (4T1/IRt line) express approximately two logs more CD119 than the parental 4T1 cells. The sensitivity of 4T1/IRt cells to IFN-γ was first examined by measuring the

Table 1 *4T1 mammary tumor cells metastasize more rapidly to the lungs and liver of BALB/c.IFN$\gamma^{-/-}$ mice than of wild-type BALB/c mice*

BALB/c and BALB/c.IFN$\gamma^{-/-}$ mice were inoculated s.c. in the abdominal mammary gland with $7 \times 10^3$ 4T1 cells and were killed on day 35. The number of lung and liver metastatic cells was determined using the clonogenic assay.

| | BALB/c | BALB/c.IFN$\gamma^{-/-}$ |
|---|---|---|
| Mice with >10,000 lung metastases | 29% (5/17) | 64% (7/11) |
| Mice with >100 liver metastases | 53% (9/17) | 73% (8/11) |

Table 2 *Primary 4T1 mammary tumors grow slightly faster in BALB/c.IFN$\gamma^{-/-}$ mice as compared with wild-type BALB/c mice; however, growth is not affected by depletion of CD4, CD8, NK, or phagaocytic cells*

Mice were inoculated s.c. in the abdominal mammary gland with $7 \times 10^3$ 4T1 cells. Primary tumors were measured every 3–4 days. Numbers in parentheses indicate the number of mice per group. Two separate experiments are shown.

| Strain | Tumor onset (days) | Tumor diameter at day 35 (mm) |
|---|---|---|
| BALB/c (25) | 6–13 | 8.5 ± 1.4 |
| BALB/c.IFN$\gamma^{-/-}$ (11) | 7–10 | 9.5 ± 1.3[a] |
| BALB/c (27) | 7–12 | 9.3 ± 1.5 |
| BALB/c.Pfp$^{-/-}$ (8) | 10–12 | 8.5 ± 0.4[b] |
| anti-asialo GM1 (8) | 7–9 | 9.5 ± 1.1 |
| CD4-depleted (9) | 7–10 | 9.2 ± 0.9 |
| CD8-depleted (10) | 10–14 | 8.5 ± 1.3 |
| Carrageenan (9) | 7–11 | 9.0 ± 0.7 |

[a] Statistically significantly different from that of control BALB/c mice ($P < 0.05$).
[b] Statistically significantly different from that of control BALB/c mice ($P < 0.025$).

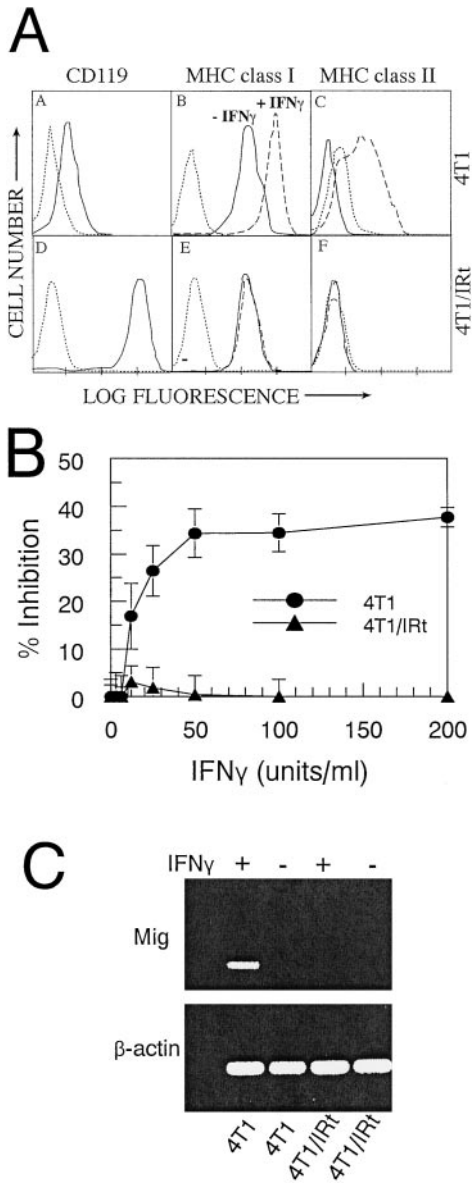


Fig. 2. 4T1 tumor cells expressing an IFN-γ receptor with a truncated α chain (4T1/IRt cells) are not responsive to IFN-γ. *A*, 4T1 and 4T1/IRt transfectants were stained by indirect immunofluorescence for IFN-γ receptor (CD119 mAb), MHC class I (34-5-8 mAb), or MHC class II (MKD6 mAb). · · · ·, staining by fluorescent conjugate alone; — — —, staining after a 3-day *in vitro* treatment with 100 units/ml IFN-γ; —, staining in the absence of IFN-γ treatment. The *X* axis shows four log cycles of fluorescence intensity. *B*, 4T1 and 4T1/IRt cells ($1–10 \times 10^3$/well) were cultured *in vitro* in the presence of various levels of IFN-γ for 3 days, and the % inhibition determined using an MTT assay. *Error bars*, SDs of the mean. *C*, RNA from 4T1 and 4T1/IRt tumor cells cultured in the presence or absence of 100 units/ml IFN-γ for 2 h was reverse-transcribed and amplified using Mig-specific or β-actin-specific primers.

induction of MHC class I and class II molecules. Parental 4T1 cells were induced to express MHC class II and increased levels of MHC class I molecules after incubation with 100 units/ml IFN-γ for 3 days *in vitro*. In contrast, IFN-γ did not induce the 4T1/IRt line to express MHC class II or increase MHC class I levels.

To further evaluate the responsiveness of 4T1 and the insensitivity of 4T1/IRt to IFN-γ, proliferation and chemokine expression in the presence and absence of IFN-γ were determined. As shown in Fig. 2B, 100 units/ml IFN-γ inhibited the proliferation of wild-type 4T1 *in vitro* by 34.5% and has no effect on 4T1/IRt. Because chemokine expression by tumor cells can be induced by IFN-γ and has been suggested to regulate tumor growth *in vivo* through antiangiogenic mechanisms, induction of Mig in 4T1- and 4T1/IRt-IFN-γ-treated cells was also measured. IP-10 induction was not measured because 4T1 was previously shown not to express IP-10 in response to IFN-γ *in vitro* (17). 4T1 was induced by IFN-γ to express Mig, whereas Mig was not induced in IFN-γ-treated 4T1/IRt cells (Fig. 2C). Thus, 4T1/IRt cells are not responsive to IFN-γ and could be used to distinguish whether IFN-γ acts directly on the tumor cells or via the host's system *in vivo*.

**IFN-γ Does Not Act Directly on 4T1 Tumor Cells.** To determine whether IFN-γ directly acts on 4T1 tumor cells, 4T1 and 4T1/IRt cells were injected at $7 \times 10^3$ into BALB/c and BALB/c.IFNγ$^{-/-}$ mice. As shown in Fig. 3*A*, there are no significant differences in the growth kinetics of primary tumors in any of the tumor-cell/mouse-strain combinations tested. Therefore, primary solid tumor growth is not affected by IFN-γ directly acting on 4T1 tumor cells.

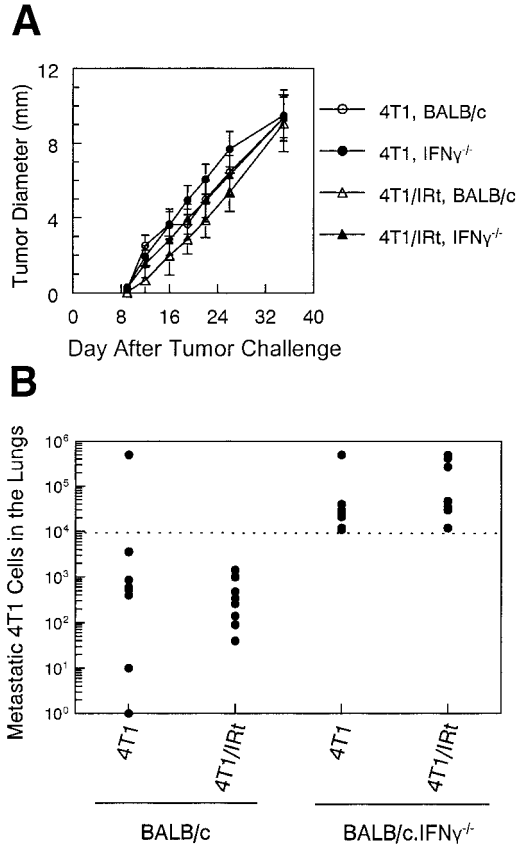


Fig. 3. 4T1 and 4T1/IRt cells have similar primary and metastatic growth kinetics. *A*, BALB/c and BALB/c.IFNγ$^{-/-}$ mice were inoculated s.c. in the abdominal mammary gland with $7 \times 10^3$ 4T1 or 4T1/IRt cells on day 1. Primary tumors were measured every 3–4 days thereafter. *Data points*, the average TD of 5–7 mice. *Error bars*, the SD of the mean. *B*, the mice used in *A* were sacrificed on day 35 after tumor cell inoculation, and the number of 4T1 or 4T1/IRt metastatic cells in their lungs was determined using the clonogenic assay. *Data points*, the number of metastatic 4T1 cells in the lungs of an individual mouse. ····, the lethal level of metastatic cells in the lungs.

To determine whether IFN-γ acts directly on metastasizing 4T1 tumor cells to reduce metastatic disease, the BALB/c and BALB/c.IFNγ$^{-/-}$ mice of Fig. 3A were sacrificed on day 35 after 4T1 inoculation, and the number of metastatic cells in their lungs determined using the clonogenic assay. 4T1 and 4T1/IRt tumor cells produced comparable numbers of metastatic cells in the lungs of BALB/c mice and much higher levels of metastatic cells in the lungs of BALB/c.IFNγ$^{-/-}$ mice (Fig. 3B). Hence, the antitumor effects of IFN-γ are most likely mediated by host-derived factors, rather than by IFN-γ directly altering tumor cell proliferation.

**Perforin-mediated Effector Mechanisms Minimally Contribute to the IFN-γ-dependent Antitumor Immune Response.** Several studies have shown that IFN-γ activates T and NK cell responses *in vitro* and *in vivo* (2). T and NK cells mediate their effects through perforin-mediated lysis (21). To determine whether T and/or NK cells play a role in IFN-γ-mediated responses to 4T1, BALB/c.Pfp$^{-/-}$ mice and BALB/c mice depleted for NK cells, CD4$^+$ or CD8$^+$ T cells were injected s.c. in the abdominal mammary gland with $7 \times 10^3$ parental 4T1 cells, and primary tumor growth monitored. Although a small decrease in tumor size was seen in the BALB/c.Pfp$^{-/-}$ *versus* wild-type BALB/c mice at day 35 ($P = 0.025$), the growth kinetics of 4T1 primary tumors in wild-type BALB/c, BALB/c.Pfp$^{-/-}$, and depleted BALB/c mice were very similar (Table 2 and data not shown). To ascertain whether T and/or NK cells are involved in the IFN-γ-mediated antimetastatic response, the numbers of clonogenic metastatic cells in the lungs and/or livers of these animals were analyzed (Fig. 4). Statistically significant differences were not seen in the anti-asialo-GM1-treated, CD4-depleted, and CD8-depleted BALB/c mice. Spontaneous lung and liver metastases in BALB/c.Pfp$^{-/-}$ mice were slightly increased relative to metastases in BALB/c mice (Table 3, $P = 0.049$ and $P = 0.007$, respectively). Survival time of BALB/c.Pfp$^{-/-}$ mice, however, does not differ from that of BALB/c mice (data not shown), suggesting that the minimal difference in lung and liver metastases is not physiologically important. Therefore, perforin-dependent mechanisms may modestly reduce the numbers of metastatic cells; however, the increased survival of BALB/c *versus* BALB/c.IFNγ$^{-/-}$ mice cannot be attributed to perforin-dependent effector activities. Likewise, because depletion of CD4$^+$, CD8$^+$, or NK cells did not affect tumor growth or survival time, these cell populations cannot account for the increased survival time of wild-type mice *versus* IFN-γ-deficient mice. Therefore, it is likely that non-perforin-dependent mechanisms and cells other than CD4$^+$, CD8$^+$, and NK cells are responsible for increased survival mediated by IFN-γ.

**IFN-γ-dependent Phagocytic Cells Are Primarily Responsible for Controlling Spontaneous Metastatic Tumor Growth and Survival.** IFN-γ also activates phagocytic cells, such as macrophages and dendritic cells (2). Macrophages possess potent tumoricidal activity (18), and dendritic cells are critical cells for antigen presentation (22). To determine whether these cells are responsible for increased spontaneous metastatic disease in BALB/c.IFNγ$^{-/-}$ mice, BALB/c and BALB/c.IFNγ$^{-/-}$ mice were treated with carrageenan, a drug that depletes phagocytic cells (23). Mice were sacrificed at day 35, and the number of metastatic tumor cells in the lungs and liver was quantified using the clonogenic assay. As shown in Fig. 4*A* and Table 3, the number of metastatic cells in the lungs is significantly increased in carrageenan-treated BALB/c mice as compared with untreated BALB/c mice ($P = 0.017$). Although the number of metastatic cells in the livers of carrageenan-treated mice seems to be increased relative to BALB/c mice, the trend was not statistically significant ($P = 0.156$). Fig. 4*A* also shows that the levels of metastatic cells in

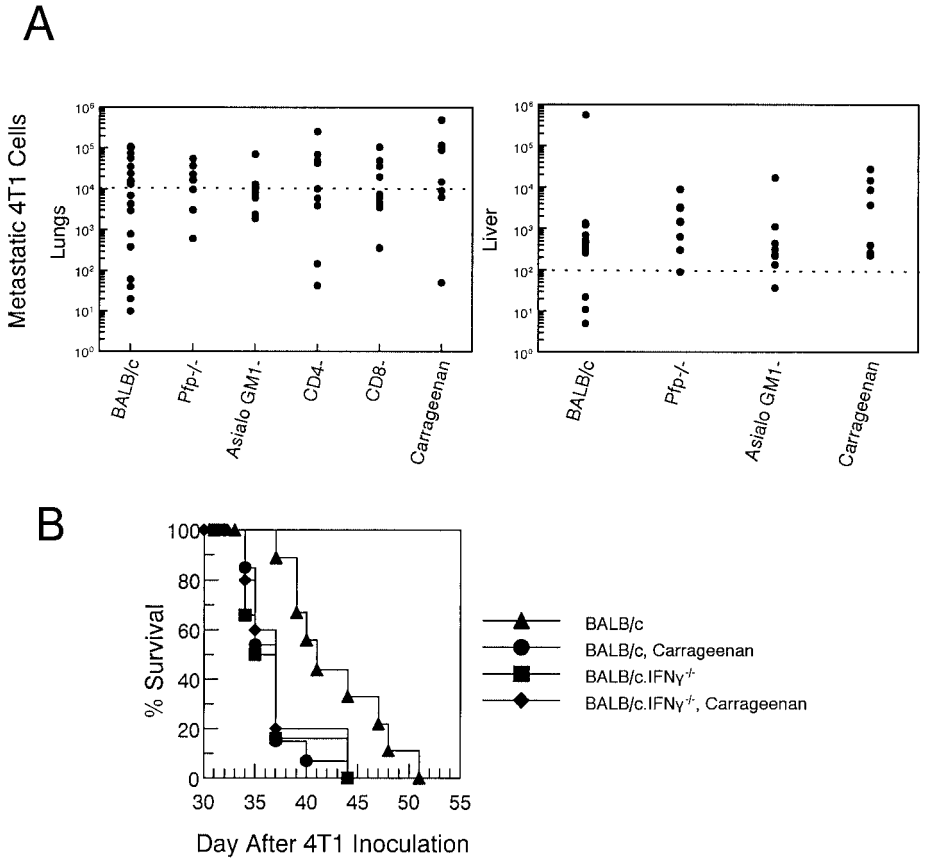


Fig. 4. Carrageenan-treated BALB/c mice have increased metastatic disease and decreased survival time. *A*, BALB/c, BALB/c.Pfp$^{-/-}$, anti-asialo-GM1-treated, CD4-depleted, CD8-depleted, and carrageenan-treated BALB/c mice were inoculated s.c. in the abdominal mammary gland with $7 \times 10^3$ 4T1 cells on day 1. Mice were sacrificed on day 35, and the number of metastatic 4T1 tumor cells in the lungs and liver were quantified using the clonogenic assay. Data points, the number of metastatic 4T1 cells in an individual mouse. · · · ·, the lethal levels of metastatic cells in these organs. *B*, BALB/c (▲), BALB/c.IFNγ$^{-/-}$ (■), carrageenan-treated BALB/c (●), and carrageenan-treated BALB/c.IFNγ$^{-/-}$ (◆) mice were inoculated s.c. in the abdominal mammary gland with $7 \times 10^3$ 4T1 tumor cells on day 1. Primary tumors were surgically removed on days 21–24, and the mice were followed for survival time.

carrageenan-treated BALB/c mice is similar to that found in BALB/c.IFN$^{-/-}$ mice (see Fig. 1*B*).

To determine whether the increase in metastatic cells in carrageenan-treated mice results in decreased survival, BALB/c and carrageenan-treated BALB/c mice were injected with $7 \times 10^3$ 4T1 cells in the abdominal mammary gland, their primary tumors removed on days 21–24, and the mice followed for survival. A group of carrageenan-treated and a group of non-carrageenan-treated BALB/c.IFNγ$^{-/-}$ mice were also included to compare the effects of carrageenan treatment *versus* IFN-γ-deficiency. As shown in Fig. 4*B*, carrageenan-treated BALB/c mice died within 37 (±3) days, which is identical to the survival time of 4T1-challenged BALB/c.IFNγ$^{-/-}$ mice and significantly shorter than the survival time of wild-type BALB/c mice ($P = 0.005$). In contrast, the survival time of carrageenan-treated BALB/c.IFNγ$^{-/-}$ mice was the same as the survival time of carrageenan-treated BALB/c and BALB/c.IFNγ$^{-/-}$ mice. Therefore, the depletion of host phagocytic cells by carrageenan treatment resulted in accelerated tumor growth and decreased survival, which mimics the tumor-growth kinetics seen in BALB/c.IFN$^{-/-}$γ mice. Because the carrageenan-treatment and the IFN-γ-depletion effect were not additive, it is likely that these agents mediate their effects via a common mechanism.

Table 3 *Depletion of phagocytic cells by carrageenan treatment increases the number of metastatic cells in the lungs*

BALB/c, BALB/c.Pfp$^{-/-}$, anti-asialo-GM1-treated, CD4-depleted, CD8-depleted, or carrageenan-treated BALB/c mice were inoculated s.c. in the abdominal mammary gland with $7 \times 10^3$ 4T1 cells. Mice were killed on day 35, and the numbers of metastatic cells in the lungs and liver were determined using the clonogenic assay.

| Mice | Mice with >10,000 metastatic cells in the lungs | Mice with >100 metastatic cells in the liver |
|---|---|---|
| BALB/c | 33% (9/27) | 59% (16/27) |
| BALB/c.Pfp$^{-/-}$ | 63% (5/8) | 88% (7/8)[a] |
| anti-asialo-GM1 | 63% (5/8) | 88% (7/8) |
| CD4-depleted | 56% (5/9) | ND[b] |
| CD8-depleted | 40% (4/10) | ND |
| Carrageenan | 80% (8/10)[a] | 88% (7/8) |

[a] Statistically significantly different from those of wild-type, untreated BALB/c mice ($P < 0.018$).
[b] ND, not determined.

## DISCUSSION

Multiple strategies have been used to evaluate the role of IFN-γ in tumor immunity, *e.g.*, (*a*) tumor cells have been cultured with IFN-γ *in vitro* (3, 5, 7, 17, 23); (*b*) IFN-γ has been administered systemically to mice and patients (24–26); (*c*) tumor cell lines have been transfected with the genes encoding IFN-γ or IFN-γR (5, 7, 19, 20, 23); (*d*) antibodies to IFN-γ have been administered systemically to mice (23); and (*e*) knockout mice deficient for IFN-γ, IFN-γR, and IFN-γ-dependent transcription factors (STAT-1) have been challenged with transplantable syngeneic tumors (6–8, 27). Most of these studies have been conducted using primary solid-tumor models, have only examined a few aspects of immunity (*e.g.*, T cells or NK cells or angiogenesis), and/or have not discriminated as to whether IFN-γ is acting on host cells or directly on tumor cells. As a result, it is difficult to compare results from the various studies and to identify the relevant effector mechanisms that mediate the IFN-γ effect. The shortage of experimental studies in metastatic tumor models means that even less is understood about the role of IFN-γ in metastatic disease. In the present studies, we have used a realistic metastatic mammary carcinoma model in which the primary tumor is surgically removed to model the human disease situation, and have comprehensively examined multiple avenues of IFN-γ activity.

The significant reduction in the survival time and the increase in number of metastatic tumor cells in BALB/c.IFN$\gamma^{-/-}$ mice relative to BALB/c mice demonstrate that IFN-γ plays an important innate role in regulating mammary carcinoma metastasis. It is not surprising that primary 4T1 tumor growth does not differ between BALB/c.IFN$\gamma^{-/-}$ and BALB/c mice, because earlier studies with the 4T1 tumor in an immunotherapy setting demonstrated that primary and metastatic tumor cells frequently respond differently to immune effectors (13, 17).

It is not known whether IFN-γ regulates tumor growth by acting directly on tumor cells or indirectly by modulating host cells and/or factors. Several mechanisms have been proposed by which IFN-γ directly reduces tumor cell growth: (*a*) tumor cells treated *in vitro* with IFN-γ have increased tryptophan metabolism, which leads to tumor cell starvation (3); (*b*) tumor cells treated with IFN-γ are induced to express chemokines that inhibit angiogenesis, thereby depriving the growing tumor of the requisite vasculature (5, 7); (*c*) IFN-γ-treated tumor cells have up-regulated levels of MHC class I and/or class II molecules, making them better targets for $CD4^+$ and $CD8^+$ T cells (23). These proposed mechanisms are not strongly supported by studies in the literature. Although demonstrated *in vitro*, tryptophan starvation has not been found *in vivo*. The results of several studies are consistent with the hypothesis that the production of antiangiogenic factors by tumor cells limits tumor growth (6, 7). However, none of these studies show decreased tumor rejection by blocking antiangiogenesis *in vivo*. Although increased MHC class I and II expression should lead to increased sensitivity to T cells (23), it is unlikely that this mechanism is responsible for the IFN-γ effect, because primary tumor rejection of many tumors is mediated by nonspecific effectors and not by T cells (19, 20). Although these studies suggest that tumor cells are not the immediate target for IFN-γ, they are indirect, and more definitive experiments are necessary. Our findings that 4T1/IRt cells have the same *in vivo* growth kinetics as wild-type 4T1 cells demonstrate that direct interactions of IFN-γ with tumor cells are not responsible for decreased metastatic disease. Therefore, direct action of IFN-γ on tumor cells is probably not involved in the IFN-γ effect on the 4T1 mammary carcinoma.

It is more likely that IFN-γ mediates its tumor effect by acting on host cells that secondarily produce factors that diminish tumor growth. CD119 is expressed on many cells (28), and IFN-γ is known to up-regulate the transcription of hundreds of genes (1), thereby producing multiple host-derived effector cells. Different studies support a role for IFN-γ-activated NK cells (8), $CD4^+$ and/or $CD8^+$ T cells (6, 7), B cells (9), macrophages (26), and/or non-hematopoietic-derived cells (6, 7). With the exception of IFN-γ-activated NK cells (8), a role for more than one cell type was implicated in each of these studies.

In contrast, the observation reported here that the deletion of phagocytic cells gives a tumor phenotype completely overlapping with IFN-γ-deficient mice strongly suggests that phagocytic cells are a central cell population for IFN-γ-mediated innate immunity. Because mice doubly depleted for phagocytic cells and IFN-γ (carrageenan-treated BALB/c.IFN$\gamma^{-/-}$ mice) have the same tumor phenotype as singly depleted mice, IFN-γ and phagocytic cells do not appear to act additively, which suggests that they control tumor growth via the same pathway. Therefore, phagocytic cells may be the critical cell population through which IFN-γ mediates its effects.

Previous studies have identified NK and/or NKT cells (8, 29) and perforin-mediated cytotoxicity (16) as critical components of innate immunity against metastatic tumor. In contrast, the present study did not find any effect of NK cells, and perforin-mediated mechanisms were only marginally involved. Differences in the present studies and previous studies may be the result of several factors: (*a*) previous studies used i.v.-induced experimental metastases (8). The present study uses spontaneous metastases. There may be significant physiological differences between metastases that are established as the result of spontaneous disease *versus* experimental metastases, and these differences may dictate the type of effector mechanisms to which the metastatic cells are susceptible; (*b*) in the present report, primary tumor was surgically removed, and spontaneous metastatic disease was subsequently assessed. In contrast, earlier studies were performed with primary tumor *in situ*, although one study used a postsurgery model followed by i.v.-induced metastases (8). Surgical removal of primary tumor may create a novel *in vivo* environment that is not present when the primary tumor remains in place. For example, large 4T1 primary tumor burdens are systemically immunosuppressive.[4] This immunosuppression may interfere with or block some effector mechanisms, but not others; (*c*) the 4T1 line used in the present report expresses high levels of MHC class I molecules (see Fig. 2*A*). This high level expression may render 4T1 resistant to NK cell lysis, and, hence, the deletion of NK cells does not greatly impact 4T1 growth. Indeed, 4T1 is not killed by NK cells in *in vitro* NK cell assays (14); and (*d*) earlier reports did not assess the potential involvement of macrophages, and additional experiments with these other tumor systems are needed to clarify the role of host phagocytic cells.

Carrageenan deletes/inactivates phagocytic cells such as macrophages, immature DCs, and neutrophils. A major role of DCs is to phagocytose antigen for presentation to T cells. The finding that T cells are largely unimportant in the IFN-γ effect implies that DCs are also not involved. Additional experiments assessing antigen presentation activity of DCs derived from BALB/c.IFN$\gamma^{-/-}$ *versus* BALB/c mice showed no diminished activity,[5] further indicating that DCs are not a key player.

Neutrophils and macrophages are also active phagocytic cells. Although neutrophils are key cells for Fas/FasL-mediated tumor rejection (30, 31), they principally ingest bacteria. Activated macrophages directly kill tumor cells by releasing incompletely reduced oxygen intermediates, such as hydrogen peroxide and nitric oxide, which are directly toxic to target cells (1). At least two observations support the hypothesis that IFN-γ mediates its antitumor effects via phagocytic cells releasing hydrogen peroxide and nitric oxide: (*a*) mice with iNOS-targeted mutations show reduced inflammatory responses to carrageenan and are resistant to LPS-induced mortality (1). Similarly, carrageenan-treated BALB/c mice have reduced LPS-induced mortality.[6] Therefore, carrageenan treatment and iNOS deficiency both result in macrophage dysfunction, which supports the idea that iNOS production and phagocytic cells are linked; (*b*) IL-13 suppresses innate immunity (32) and antagonizes IFN-γ-mediated induction of iNOS (1). These observations tie together IFN-γ, IL-13, iNOS production, and carrageenan-treatment, and suggest the following model for the role of IFN-γ in innate immunity to metastatic tumor. In the presence of IFN-γ, macrophages are activated to make iNOS and $H_2O_2$ that directly kill tumor cells. In carrageenan-treated mice, macrophages are eliminated therefore, iNOS is not produced, and metastatic tumor cells proliferate. In addition to activating macrophages, IFN-γ also activates NKT cells. Either activated NKT cells activate antigen-presenting cell to produce IL-12, which feeds back to induce more IFN-γ, or they produce IL-13, which blocks iNOS production. Therefore, up-regulation of IL-13 and/or treatment with carrageenan prevents iNOS production and limits macrophage-induced control of metastatic disease.

[4] E. Danna, M. Gilbert, B. Pulaski, and S. Ostrand-Rosenberg, unpublished observations.
[5] B. Pulaski and S. Ostrand-Rosenberg, unpublished results.
[6] B. Pulaski, E. Danna, and S. Ostrand-Rosenberg, unpublished results.

Although innate immunity triggered by IFN-γ succeeds in limiting tumor growth, it is not sufficient to mediate complete tumor destruction. Hence, immunocompetent mice display delayed metastatic disease and longer survival times relative to IFN-γ-deficient mice, but they still die from metastases. As with most immune responses, the optimal situation would be the development of an adaptive immune response against tumor that would take over when the innate response was no longer capable of curtailing tumor progression.

The immunosurveillance hypothesis states that the immune system destroys or inactivates newly transformed cells, thereby preventing the outgrowth of malignant tumors. This concept has been controversial; however, it has recently regained support through experiments with T-cell- deficient and knockout mice (27, 33). If immunosurveillance occurs, components of the innate immune response are likely to be involved. Although IFN-γ may not be exclusively responsible for tumor immunosurveillance, it is a strong candidate as a component of innate immunity that contributes to protection against the proliferation of transformed cells.

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