

## Invariant Natural Killer T Cells Regulate Breast Cancer Response to Radiation and CTLA-4 Blockade

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**Abstract Purpose:** Immunoregulatory and suppressive mechanisms represent major obstacles to the success of immunotherapy in cancer patients. We have shown that the combination of radiotherapy to the primary tumor and CTL-associated protein 4 (CTLA-4) blockade induces antitumor immunity, inhibiting metastases and extending the survival of mice bearing the poorly immunogenic and highly metastatic 4T1 mammary carcinoma. Similarly to patients with metastatic cancer, however, mice were seldom cured. Here we tested the hypothesis that invariant natural killer T (iNKT) cells, a subset with unique regulatory functions, can regulate the response to radiotherapy and CTLA-4 blockade.

**Experimental Design:** The growth of 4T1 primary tumors and lung metastases was compared in wild-type and iNKT cell – deficient (iNKT<sup>-/-</sup>) mice. Treatment was started on day 13 when the primary tumors were palpable. Mice received radiotherapy to the primary tumor in two doses of 12 Gy in combination or not with 9H10 monoclonal antibody against CTLA-4. Response to treatment was assessed by measuring primary tumor growth delay/regression, survival, and number of lung metastases.

**Results:** The response to radiotherapy plus 9H10 was markedly enhanced in the absence of iNKT cells, with 50% of iNKT<sup>-/-</sup> versus 0% of wild-type mice showing complete tumor regression, long-term survival, and resistance to a challenge with 4T1 cells. Administration of the iNKT cell activator  $\alpha$ -galactosylceramide did not enhance the response of wild-type mice to radiotherapy plus 9H10. Tumor-infiltrating iNKT cells were markedly reduced in wild-type mice treated with radiotherapy plus 9H10.

**Conclusions:** iNKT cells play a major role in regulating the response to treatment with local radiotherapy and CTLA-4 blockade.

Preclinical models and clinical trials have provided the proof of principle that immunotherapy can treat cancer (reviewed in ref. 1). However, despite the development of multiple vaccination strategies to induce antitumor T cells, objective responses are seen only in a small fraction of patients, and it remains unclear what factors determine the success of any given treatment. Increased understanding of the complex networks of immune cells and cytokines that control immune system function has led to the identification of several regulatory and

suppressive mechanisms as major obstacles to the success of immunotherapy (2). These are of two types: those that are tumor-induced and those preexisting in the host as the result of genetic predisposition, age, concurrent diseases, or previous therapies (3). Myeloid-derived suppressor cells (MDSC; ref. 4) belong to the first category because their accumulation in tumor-bearing mice and cancer patients is driven by tumor growth (5). Regulatory T cells play a key role in the maintenance of self-tolerance as well as tolerance to tumors (6). Natural regulatory T cells develop in the thymus and belong to the second category (7). In contrast, adaptive regulatory T cells are generated in the periphery from mature T cells, and their differentiation is induced by the tumor microenvironment (8, 9).

Another T cell subset with regulatory function, natural killer T (NKT) cells, has been implicated in both up- and down-regulation of immune responses (10). NKT cells have unique properties in that they can rapidly produce both Th1 and Th2 cytokines upon activation, and function as a powerful switch to turn on or off the innate and adaptive immune response in various diseases and conditions (10–12). NKT cells recognize glycolipid antigens presented by CD1d molecules, and in mice most express a canonical  $\alpha$ -chain (V $\alpha$ 14J $\alpha$ 18) and are known as invariant NKT (iNKT) or type I NKT cells. In humans, the homologous population of iNKT cells expresses V $\alpha$ 24 (10). iNKT cells react with  $\alpha$ -galactosylceramide, a

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### Translational Relevance

Metastatic breast cancer still represents a therapeutic challenge. Immunotherapy could offer an important alternative or adjunct to current treatments. Using as a model a known mouse tumor that behaves like aggressive metastatic breast cancer, we have previously shown that it is possible to successfully combine a standard treatment modality, local radiotherapy, with a novel antibody-based therapeutic agent that is currently being tested in clinical trials for cancer and targets the CTL-associated protein 4 (CTLA-4) receptor on T cells. The combination of local radiation to the primary tumor with CTLA-4–blocking antibodies elicited a CD8-mediated immune response inhibiting metastases and extending the survival of the animals. In preparation for the translation of this therapeutic approach to the clinic, we have investigated the regulatory mechanisms that determine the response to treatment. Data presented here indicate that invariant natural killer T (NKT) cells play a major role in down-regulating the response to radiation and CTLA-4 blockade in mice. This information will guide future studies in patients treated with a similar therapeutic strategy, and the potential clinical application of NKT cell–based immunotherapy against cancer.

synthetic CD1d-binding glycolipid that has a strong agonistic activity, whereas the noninvariant or type II NKT cells, although restricted by CD1d, do not bind  $\alpha$ -galactosylceramide (13).

In cancer, NKT cells have been associated with spontaneous immunosurveillance in some experimental systems (14, 15), but have been shown to suppress immunosurveillance in others (16, 17). Recent work by Terabe et al. (18), suggests a functional difference between the two subsets of NKT cells in the regulation of antitumor immunity, with type II NKT cells being mostly responsible for immunosuppression. An immunoregulatory pathway that links the production of interleukin (IL)-13 by NKT cells to the induction of transforming growth factor  $\beta$  (TGF $\beta$ ) production by MDSC has been shown to be responsible for the inhibition of antitumor CTL that results in tumor recurrence in some tumor models (16, 19, 20) but not in others (21, 22). Overall, the mechanisms of NKT cell–mediated immunoregulation in cancer involve multiple pathways and remain largely undefined.

Activation of iNKT cells by systemic administration of  $\alpha$ -galactosylceramide to mice and humans has been shown to induce the rapid production of IFN- $\gamma$  and IL-4 and other Th1 and Th2 cytokines (10). Importantly, in several preclinical cancer models this resulted in effective antitumor immunity mediated by the downstream activation of antigen-presenting cells, and NK and CD8-positive T cells (23). In initial clinical trials, however,  $\alpha$ -galactosylceramide has not shown significant anticancer activity, possibly due to partially impaired NKT cell function in cancer patients (24).

Accumulated evidence in experimental models supports the development of a novel therapeutic approach based on the combination of ionizing radiation therapy with immunotherapy for the treatment of cancer (reviewed in refs. 25, 26). In most cases clinical trials testing the efficacy of novel treatments are done in patients with advanced-stage disease, a population

for which the need for new therapies is most urgent, but that also presents with an immunologic environment largely altered by the tumor. To mimic this situation, we have employed as a model the poorly immunogenic 4T1 mouse mammary carcinoma. After s.c. inoculation 4T1 cells grow to form a highly invasive primary tumor that early on sheds spontaneous metastases to the lungs and other organs (27). Mice usually die of metastatic disease to the lungs. We previously tested the combination of radiotherapy with CTL-associated protein 4 (CTLA-4) blockade approximately two weeks after implantation in mice, when primary tumors are palpable and metastatic cells have already spread systemically (28). Whereas single modality treatment was ineffective, the combination of local radiotherapy to the primary tumor and CTLA-4 blockade elicited CD8 T-cell–dependent antitumor immunity. The immune response effectively inhibited the growth of spontaneous lung metastases, prolonging the survival time of animals. Cure was rare, however, and most mice eventually succumbed to their disease (28).

In this study we investigated whether the disruption of immunoregulatory circuits can improve the response to treatment with radiotherapy and CTLA-4 blockade. Our data indicate that whereas 4T1 tumors grew equally well in wild-type mice and mice lacking iNKT cells (iNKT $^{-/-}$ ), the latter developed a CD8 response that partially inhibited metastases but not primary tumor growth. This observation is in agreement with a minor role of iNKT cells in regulating spontaneous immunosurveillance in the 4T1 model (18). Remarkably, as compared with wild-type mice, iNKT $^{-/-}$  mice showed a marked improvement in survival and cure rate following treatment with radiotherapy and CTLA-4 blockade, suggesting that iNKT cells can play a major role in regulating the response to treatment. Administration of  $\alpha$ -galactosylceramide did not improve the response of wild-type mice to radiotherapy and CTLA-4 blockade. Despite the differential response to treatment, wild-type and iNKT $^{-/-}$  mice showed a similar systemic and intratumoral increase in MDSC around the time treatment was started, suggesting that the degree of MDSC accumulation does not predict the response to treatment.

### Materials and Methods

**Mice.** Six- to eight-week-old BALB/c mice were obtained from Taconic Animal Laboratory. iNKT $^{-/-}$  (V $\alpha$ 14 J $\alpha$ 18-deficient) mice (29) in the BALB/c background obtained from M. Taniguchi (RIKEN Research Center for Allergy and Immunology) were bred at New York University and used between 6 and 12 wk of age. All experiments were approved by the Institutional Animal Care and Use Committee of New York University.

**Cells and reagents.** 4T1 is a BALB/c mouse–derived mammary carcinoma cell line (provided by Fred Miller, the Michigan Cancer Center; ref. 27) and A20 is a BALB/C mouse–derived B-cell leukemia/lymphoma (30). 4T1 cells were grown in DMEM (Invitrogen Corporation) supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin,  $2.5 \times 10^{-5}$  mol/L 2-mercaptoethanol, and 10% fetal bovine serum (Gemini Bio-Products; complete medium). These cells were found to be free of contamination by mycoplasma by the Mycoplasma detection kit (Roche Diagnostics). Anti-CTLA-4 hamster monoclonal antibody (mAb) 9H10 was purified as previously described (28). Control hamster IgG was purchased from Jackson Immunoresearch Laboratories. Purified anti-CD4 (GK1.5), anti-CD8 (2.43) rat mAb and control rat IgG were purchased from BioExpress, Inc.

**Tumor challenge and treatment.** The mice were injected s.c. in the right flank with  $5 \times 10^4$  4T1 cells in 0.1 mL of DMEM without additives on day 0. Perpendicular tumor diameters were measured with a Vernier caliper, and tumor volumes were calculated as length  $\times$  width<sup>2</sup>  $\times$  0.52. On day 13, when tumors reached the average diameter of 5 mm (approximately 65 mm<sup>3</sup> in volume) animals were randomly assigned to various treatment groups, as indicated. Radiotherapy was administered as previously described (28). Briefly, all mice (including mice receiving mock radiation) were lightly anesthetized by i.p. injection of avertin (240 mg/kg) then positioned on a dedicated plexiglass tray, and the whole body was protected by lead shielding, except for the area of the tumor to be irradiated. Radiotherapy was delivered to a field including the tumor with 5-mm margins using a <sup>60</sup>Co radiation source by two fractions of 12 Gy each on days 13 and 14. Control hamster IgG and 9H10 were given i.p. at 200  $\mu$ g at 1, 4, and 7 d after radiotherapy.  $\alpha$ -Galactosylceramide (Alexis Biochemicals) was dissolved in DMSO and diluted in PBS supplemented with 0.5% polysorbate-20 (w/v) prior to injection and given i.p. at 100 ng/mouse twice a week, starting on day 1 postradiotherapy. Tumor growth was evaluated every 2 to 3 d until death or sacrifice when tumor dimensions exceeded 5% body weight or mice showed dyspnea, abnormal posture, >20% body weight loss, difficulty with ambulation, or any other clinical sign of metastatic disease causing significant pain or distress, according to institutional guidelines. In some experiments, mice that rejected the tumors and survived tumor-free until day 120 were injected in the controlateral flank with a tumorigenic inoculum of either 4T1 or A20 cells and followed for up to 60 d for tumor development.

**Clonogenic lung metastases assay.** For determination of the number of clonogenic lung metastases, lungs were harvested on day 33 or 35 post-s.c. injection in the right flank with  $5 \times 10^4$  4T1 cells and processed as previously described (27). Briefly, lungs from each individual animal were minced into 1-mm pieces, and digested with 5 mL enzyme cocktail containing 1 mg/mL collagenase IV and 6 units/mL elastase, both from Sigma Chemical Company, in PBS for 1 h at 4°C with rotation. Cell suspensions were filtered through 70- $\mu$ m nylon cell strainers and washed two times with HBSS, then resuspended in complete medium. Serial 3- to 5-fold dilutions were plated in 10-cm tissue culture dishes in the presence of 60  $\mu$ mol/L 6-thioguanine (2-amino-6-mercaptopurine; Sigma Chemical Company) to allow only the growth of 4T1 cells which are resistant to this drug (27). When colonies of growing 4T1 cells became visible (8-14 d) the plates were washed with PBS, fixed with methanol, and stained with Crystal violet. The colonies were counted independently by two to three investigators, blinded to the group to which each mouse belonged, and the total number/lungs was calculated for each animal.

**In vivo T-cell subset depletion.** Depletion of CD4 and CD8 T-cell subsets was done by injecting GK1.5 or 2.43 mAb i.p. at 100  $\mu$ g/mouse on 3 consecutive d, starting 1 d before the s.c. injection in the right flank with  $5 \times 10^4$  4T1 cells. The depletion was maintained by repeated weekly injections of mAb. Depletion was confirmed by testing spleen cells from control animals for the presence of CD4 and CD8 T-cells using non-cross-reactive FITC-RMA4-4 and PE-anti-CD8 $\beta$  mAb (BD Pharmingen).

**Mice vaccination.** Wild-type and iNKT<sup>-/-</sup> mice were vaccinated with 10<sup>6</sup> irradiated (100 Gy) 4T1 cells s.c. in the right flank 3 times at weekly intervals. Control mice received DMEM. Seven days after the last vaccination the mice were challenged with a tumorigenic inoculum ( $5 \times 10^4$ ) of 4T1 cells s.c. in the opposite flank and followed for tumor development.

**Analysis of MDSC and iNKT cells infiltrating 4T1 tumors.** The tumors were dissected carefully removing all surrounding normal tissue, minced into 1-mm pieces, and digested with collagenase D (400 U/mL) for 25 min at 37°C in a shaker. Ten milliliters of the enzyme solution were used for every gram of tumor tissue. Obtained cell suspensions were filtered through 40- $\mu$ m nylon cell strainers and washed two times with HBSS. Aliquots of 10<sup>6</sup> tumor-derived cells were incubated with antimouse CD16/32 (Fc block) for 10 min followed by staining at 4°C with mAb against mouse Gr-1-Cy-Chrome and CD11b-PE (BD

PharMingen) to identify MDSC, or with PE-conjugated mCD1d/PBS57 tetramer (provided by the NIH Tetramer Facility, ref. 31) and FITC-conjugated CD3 to identify iNKT cells. Unloaded PE-conjugated mCD1d tetramer was used as control. Samples were analyzed using a FACScan flow cytometer and FlowJo version 6.4.4 (Tree Star).

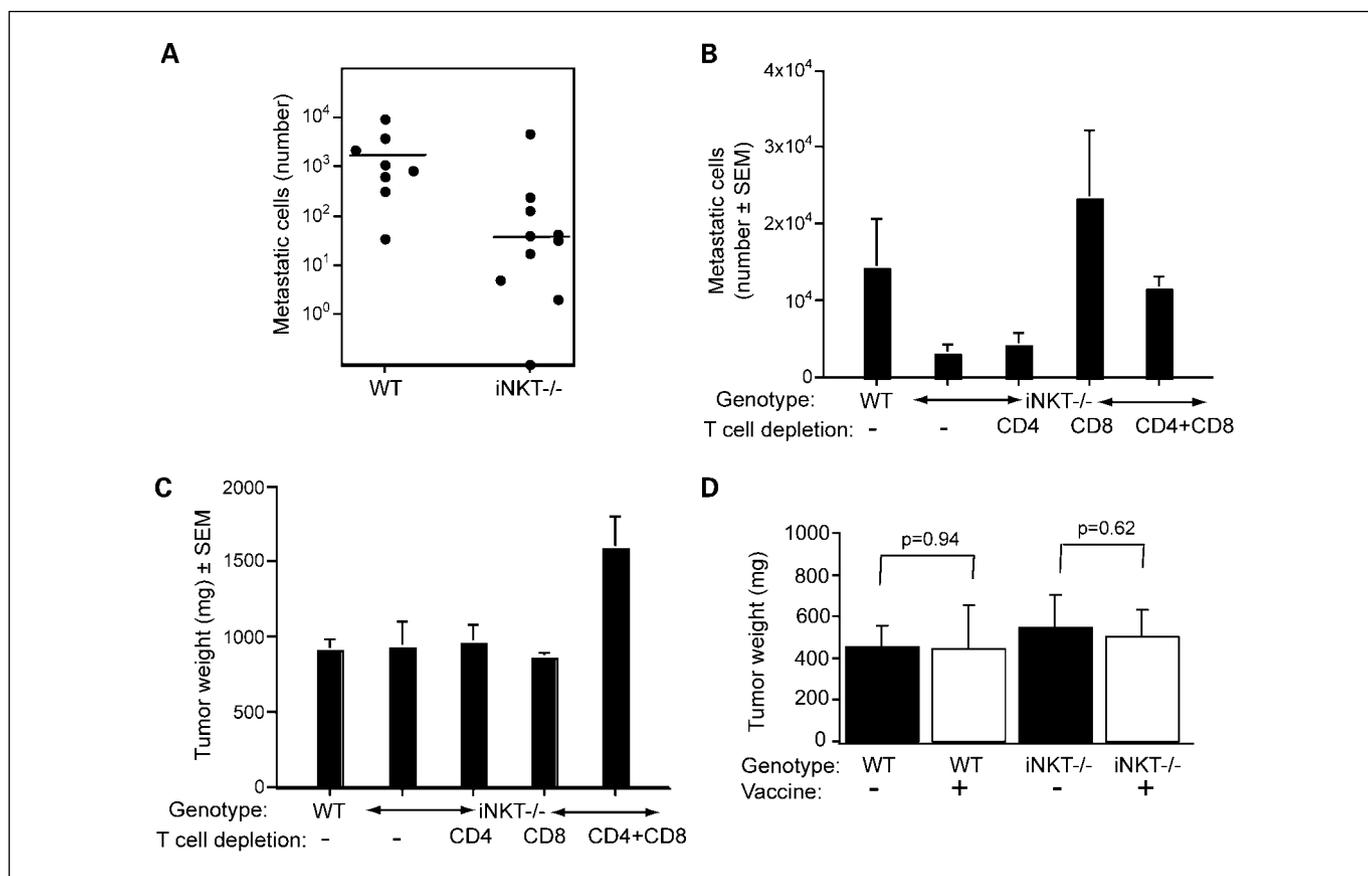
**Measurement of TGF  $\beta$ 1 production.**  $1 \times 10^6$  spleen cells from individual mice were cultured o.n. in RPMI 1640 medium supplemented with 1% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin in a 24-well plate. Supernatants were harvested and stored at -80°C. The concentration of TGF  $\beta$ 1 was determined in duplicate samples using the Quantikine TGF  $\beta$ 1 Immunoassay Kit (R&D Systems) following the manufacturer's instructions. Background readings for culture medium were subtracted from all samples. As previously reported (19), acidification of the samples was required to detect the latent form of TGF  $\beta$ 1 produced in vitro by MDSC.

**Immunostaining of tumor sections.** 4T1 tumors from treated and untreated wild-type and iNKT<sup>-/-</sup> mice were harvested on day 29 posttumor inoculation, fixed for 1 h at 4°C in 4% paraformaldehyde followed by overnight incubation in 30% sucrose, and frozen in optimum cutting temperature medium. Sections (8  $\mu$ m) were incubated with 0.1% Tween-20 and 0.01% Triton-X100 for 20 min, followed by 4% rat serum in 4% bovine serum albumin/PBS for an additional 30 min. Sections were stained with PE-Texas-Red-conjugated rat antimouse CD4 or PE-conjugated rat antimouse CD8 $\alpha$  (Caltag), and counterstained with 5  $\mu$ g/mL 4', 6-diamidino-2-phenylindole (Sigma). Images were obtained using a Nikon Eclipse 800 deconvolution microscope. The CD4 and CD8 T cells were counted in three randomly selected (20 $\times$ ) fields in each tumor.

**Statistical analysis.** ANOVA based on ranks was used to assess differences among animal groups defined by genotype and/or treatment received with respect to tumor weight, tumor volume, or the number of lung metastases at a fixed time point. Specifically, each end point (tumor weight, tumor volume, number of metastases) was first converted to ranks within each experiment and the ranks were then used as the dependent variable in the analysis of variance. Ranks were used in place of the observed values to better satisfy underlying distributional assumptions. When the treatment groups constituted a 2  $\times$  2 factorial design (presence/absence of CTLA-4 blockade/radiotherapy), the analysis examined the main effects for each treatment modality (radiotherapy, CTLA-4 blockade) and the interaction between the modalities. The log-rank test was used to compare animal groups in terms of overall survival, defined as time to death or sacrifice. The median and mean survival times within each treatment arm were estimated using the Kaplan-Meier product-limit method, and a 95% confidence interval for each median survival time was derived on the basis of a sign test. All reported *P* values are two-sided and were declared statistically significant at the 5% level. The statistical computations were carried out using SAS for Windows, version 9.0 (SAS Institute).

## Results

**iNKT<sup>-/-</sup> mice develop a spontaneous CD8-dependent antitumor immune response inhibiting metastases.** To investigate the role of the host immune status in tumor growth and response to treatment we injected mice lacking iNKT cells with 4T1 cancer cells. Whereas primary tumors grew at the same rate in iNKT<sup>-/-</sup> and wild-type mice (not shown) and primary tumor weight was not significantly different on day 34 (mean  $\pm$  SD, 1,364  $\pm$  895 and 873  $\pm$  234 mg for iNKT<sup>-/-</sup> and wild-type mice, respectively; *P* = 0.41), the number of metastatic cells in the lungs was significantly lower in iNKT<sup>-/-</sup> mice (mean  $\pm$  SD, 525.4  $\pm$  1461.9 and 1,364.0  $\pm$  895.5 for iNKT<sup>-/-</sup> and wild-type, respectively; *P* = 0.0009; Fig. 1A). The depletion of CD8 T cells or CD4 and CD8 T cells abrogated the difference in lung



**Fig. 1.** CD8 T cell – mediated inhibition of metastases in untreated tumor-bearing mice lacking iNKT cells. *A*, mice received  $5 \times 10^4$  4T1 cells s.c. on day 0. The number of metastatic cells in the lungs on day 34 was significantly lower for the iNKT<sup>-/-</sup> than for the wild-type mice ( $P = 0.0009$ ). Each symbol represents one animal. *B*, *C*, mAb-mediated CD4 and CD8 T cell depletion was started on day-1, and maintained up to the day of sacrifice. Mice were sacrificed on day 33 to determine the number of metastatic cells in the lungs (*B*) and tumor weight (*C*). Bars, mean  $\pm$  SE of 8 to 10 animals/group. *B*, the number of metastases in iNKT<sup>-/-</sup> mice depleted of CD8 T cells (*CD8*) or CD4 and CD8 T cells (*CD4+CD8*) was significantly higher than in nondepleted iNKT<sup>-/-</sup> mice ( $P = 0.004$  and  $0.001$ , respectively). In contrast, CD4 depletion did not significantly increase the number of metastases ( $P = 0.94$ ). Whereas there was a significant difference between wild-type and nondepleted iNKT<sup>-/-</sup> mice in the number of metastases ( $P = 0.043$ ), no significant difference was seen between wild-type and iNKT<sup>-/-</sup> mice after CD8 T cell depletion by itself or in combination with CD4 T cell depletion. *C*, there was no significant tumor weight difference ( $P > 0.6$ ) between wild-type and iNKT<sup>-/-</sup> mice depleted or not depleted of CD8 or CD4 T cells. However, tumor weight was significantly increased in mice depleted of both T cell subsets ( $P < 0.01$  when compared with all other groups). *D*, 4T1 cells are not intrinsically immunogenic in iNKT<sup>-/-</sup> mice. Wild-type and iNKT<sup>-/-</sup> mice ( $n = 5$ /group) were vaccinated weekly for 3 wk with  $10^6$  irradiated (100 Gy) 4T1 cells s.c. in the left flank (+) or mock vaccinated with DMEM (-). Seven days after the last vaccination mice were challenged with  $5 \times 10^4$  viable 4T1 cells and followed for tumor development. All mice developed tumors and there was significant difference in tumor weight in vaccinated wild-type ( $P = 0.94$ ) or iNKT<sup>-/-</sup> ( $P = 0.62$ ) mice on day 26.

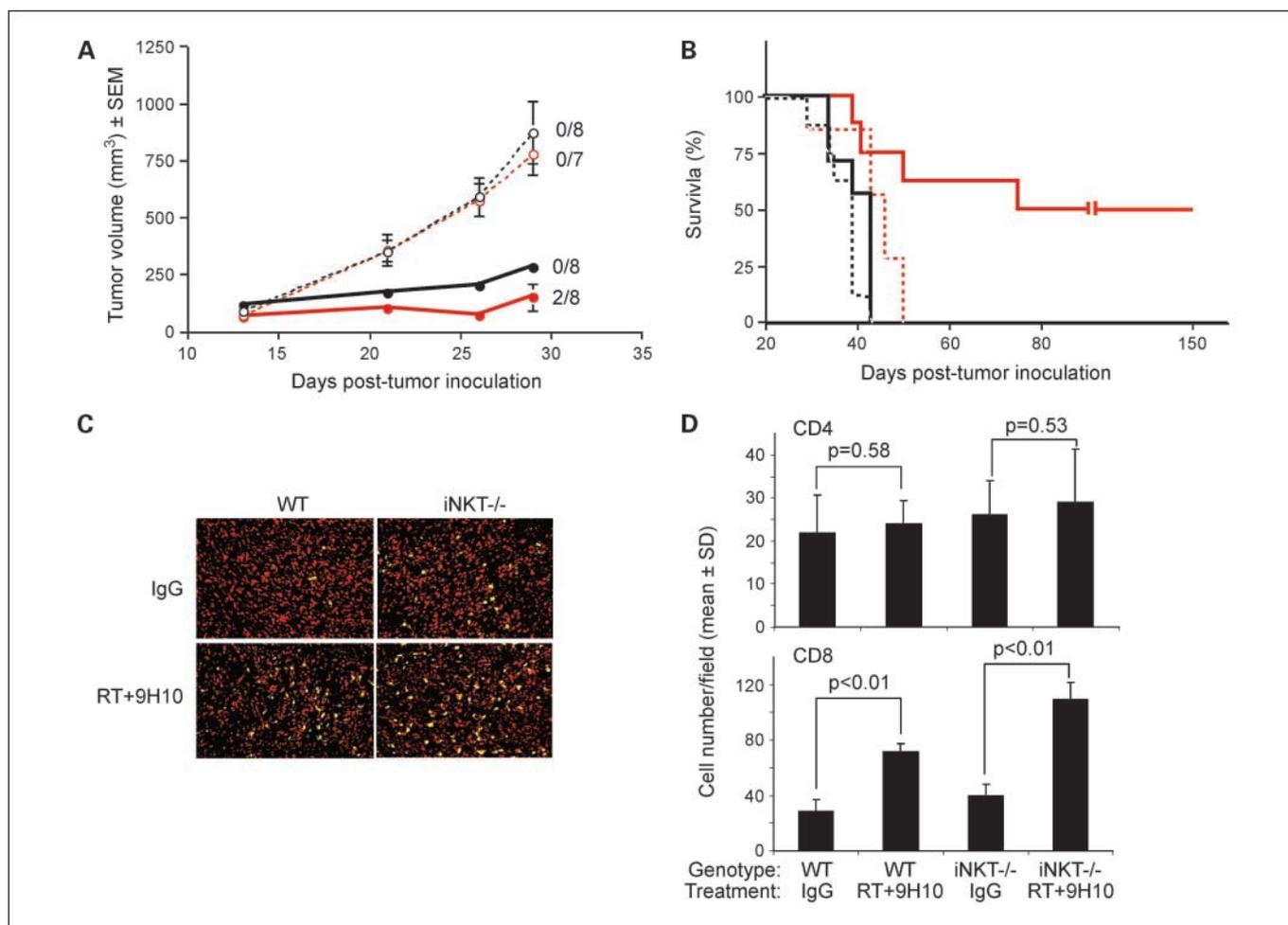
metastases between wild-type and iNKT<sup>-/-</sup> mice, whereas the depletion of CD4 T cells did not have any effect, indicating that CD8 T cells are required for the inhibition of lung metastases observed in iNKT<sup>-/-</sup> mice (Fig. 1B). Interestingly, primary tumor growth was not affected by depletion of CD8 or CD4 T cells but a statistically significant increase in tumor weight ( $P < 0.01$ ) was seen in double-depleted mice (Fig. 1C).

The spontaneous development of antitumor CD8 T cells could be due to increased intrinsic immunogenicity of 4T1 cells in iNKT<sup>-/-</sup> mice. To test this hypothesis, wild-type and iNKT<sup>-/-</sup> mice were immunized repeatedly with 4T1 cells inactivated by irradiation, followed 7 days after the last immunization by challenge with  $5 \times 10^4$  live 4T1 cells. If antitumor CD8 cells had developed following vaccination, they would prevent or inhibit growth of the early small tumors. In contrast, all wild-type and iNKT<sup>-/-</sup> mice developed tumors, and there was no significant difference in tumor weight between 4T1-vaccinated and nonvaccinated mice (Fig. 1D). Therefore, 4T1 cells do not show increased immunogenicity in iNKT<sup>-/-</sup> mice.

*In the absence of iNKT cells, mice bearing established 4T1 mammary carcinoma show a markedly enhanced therapeutic response to treatment with local radiotherapy and CTLA-4 blockade.* We have shown that wild-type mice bearing the poorly immunogenic 4T1 mammary carcinoma develop antitumor CD8 T cells inhibiting spontaneous lung metastases and extending their survival when treated with the combination of local radiotherapy to the primary tumor and CTLA-4–blocking mAb 9H10, whereas each single modality did not have an effect on survival (28). However, complete cure of mice with well-established disease remained rare: 0% to 15% of the mice were cured in different experiments (28).<sup>4</sup> This suggested that the duration and/or potency of the antitumor immune response elicited by treatment was limited in most animals. To test if iNKT cells play a role in response to treatment, iNKT<sup>-/-</sup> mice with established 4T1 tumors were randomly assigned to be treated with radiotherapy,

<sup>4</sup> Our unpublished results.





**Fig. 3.** Comparison between wild-type and iNKT<sup>-/-</sup> mice with established 4T1 carcinoma in the response to treatment with local radiotherapy and CTLA-4 blockade. Comparison of response to treatment with radiotherapy and 9H10 (solid lines) or IgG (broken lines) in wild-type (black) and iNKT<sup>-/-</sup> (red) 4T1 tumor-bearing mice. Treatment was started on day 13 post-s.c. inoculation in the flank. Radiotherapy was delivered in two fractions of 12 Gy to the s.c. tumors on days 13 and 14. Antibodies were given i.p. 1, 4, and 7 d postradiotherapy. **A**, tumor volume is shown as the mean ± SE for animals with tumors in each treatment group up to day 29 when all animals were alive. The number of mice with complete tumor regression by day 29 over the total number of mice per group is indicated. Two additional iNKT<sup>-/-</sup> mice had complete tumor regression after day 29. Primary tumor growth was not significantly different in wild-type and iNKT<sup>-/-</sup> mice receiving the control IgG ( $P > 0.05$ ) at all time points. In contrast, iNKT<sup>-/-</sup> mice receiving radiotherapy plus 9H10 had a significantly lower tumor volume than wild-type mice receiving radiotherapy plus 9H10 ( $P < 0.05$ ) from day 21. Tumor volume differences between radiotherapy plus 9H10 and control (IgG) mice were statistically significant ( $P < 0.05$ ) from day 21 within each genotype. **B**, data are shown as the percentage of surviving mice over time. Surviving tumor-free mice were followed up to day 150. iNKT<sup>-/-</sup> mice treated with radiotherapy plus 9H10 showed a statistically significant improvement in overall survival ( $P < 0.05$ ) as compared with all other groups. Half of the iNKT<sup>-/-</sup> mice but none of the wild-type mice were cured from established 4T1 tumors by treatment with radiotherapy and CTLA-4 blockade. **C, D**, in a separate group of mice treated as above, tumors were excised at day 29 and analyzed by fluorescence microscopy for the presence of CD4-positive and CD8-positive T cells. **C**, representative fields showing CD8-positive T cells (green) infiltrating 4T1 tumors in wild-type and iNKT<sup>-/-</sup> mice treated as indicated. Nuclei were stained with 4',6-diamidino-2-phenylindole (red). **D**, mean numbers of CD4-positive and CD8-positive tumor-infiltrating lymphocytes in three mice per group. In both wild-type and iNKT<sup>-/-</sup> mice, CD8-positive but not CD4-positive tumor-infiltrating lymphocytes were increased by radiotherapy plus 9H10 treatment, but the increase was more pronounced in iNKT<sup>-/-</sup> mice ( $P = 0.006$ ).

achieve complete tumor regression and cure, as shown by the absence of tumor on day 150 in 50% of the animals (Fig. 3A and B). None of the wild-type mice survived long-term, suggesting that only in iNKT<sup>-/-</sup> mice was the duration and potency of the antitumor immune response sufficient to completely eradicate the tumor.

Analysis of tumor-infiltrating lymphocytes on day 29 showed that CD4-positive cells were present in similar quantities in 4T1 tumors growing in wild-type and iNKT<sup>-/-</sup> mice, and were not significantly increased by treatment with radiotherapy and CTLA-4 blockade (Fig. 3C). In contrast, following treatment there was a significant increase in the number of CD8-positive cells infiltrating 4T1 tumors in both wild-type and iNKT<sup>-/-</sup> mice ( $P < 0.01$  in treated versus untreated mice of both genotype; Fig. 3D). Importantly, the number of CD8-positive tumor-

infiltrating lymphocytes was higher in iNKT<sup>-/-</sup> mice than in wild-type mice, and this difference was highly significant following treatment ( $P = 0.006$  in treated iNKT<sup>-/-</sup> versus treated wild-type mice). These data confirm our previous observations that CD8-positive but not CD4-positive T cells are responsible for 4T1 tumor inhibition induced by treatment with radiotherapy and CTLA-4 blockade (28, 32). In addition, they suggest that the antitumor response induced by treatment is stronger in the absence of iNKT cells.

*The iNKT cell-specific activator  $\alpha$ -galactosylceramide does not improve the response of tumor-bearing wild-type mice to treatment with radiotherapy and CTLA-4 blockade.*  $\alpha$ -Galactosylceramide, a potent activator of iNKT cells, has been shown to induce powerful antitumor immune responses in several tumor models (23). Moreover, in a burn injury model, administration

of  $\alpha$ -galactosylceramide was able to prevent the immunosuppression that is mediated by iNKT cells (33). To determine whether administration of  $\alpha$ -galactosylceramide to 4T1 tumor-bearing mice could activate iNKT cells to carry out stimulatory rather than inhibitory functions, and improve the response of wild-type mice to radiotherapy and CTLA-4 blockade, mice were given  $\alpha$ -galactosylceramide starting one day postradiotherapy. As a single modality  $\alpha$ -galactosylceramide did not have any effect on tumor growth, and it did not improve the tumor growth delay or survival caused by treatment with radiotherapy and radiotherapy plus 9H10 (Fig. 4 and data not shown). However, a modest but statistically significant tumor growth delay was seen in mice treated with  $\alpha$ -galactosylceramide and 9H10 ( $P = 0.0396$  compared with control vehicle-treated mice; Fig. 4).

Overall, these data indicate that administration of  $\alpha$ -galactosylceramide to wild-type mice with established tumors

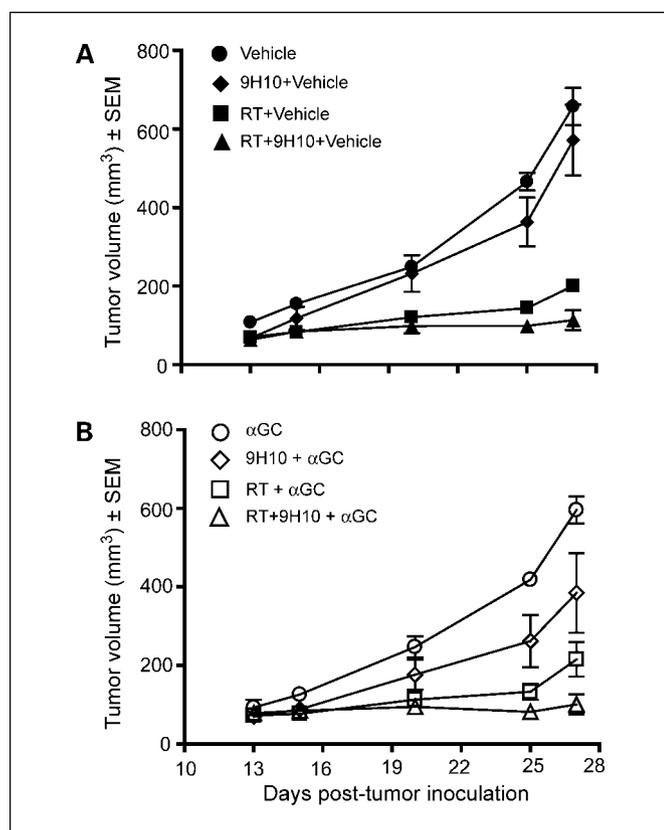
does not improve their response to treatment with radiotherapy and CTLA-4 blockade.

**Reduction in tumor-infiltrating iNKT cells after treatment with radiotherapy and CTLA-4 blockade.** The presence within tumors of T cells with regulatory function has been shown to play an important role in the suppression of antitumor immunity (8). To determine whether iNKT cells were present within 4T1 tumors growing in wild-type mice, cell suspensions prepared from 4T1 tumors and lungs were stained with CD3 and CD1d tetramers loaded with the  $\alpha$ -galactosylceramide analog PBS-57 (31). In untreated mice iNKT cells represented ~4% of T cells infiltrating 4T1 tumors and 3% of T cells isolated from the lungs (Fig. 5A and B). Importantly, there was a pronounced decline in iNKT cells in both primary tumor and lungs following treatment with radiotherapy plus 9H10 (Fig. 5B and C). Overall, these data indicate that iNKT cells are recruited to "primary" 4T1 tumors and their metastases, and that a treatment stimulating antitumor immunity leads to a relative decline in iNKT cell numbers, an observation that supports a regulatory role for iNKT cells.

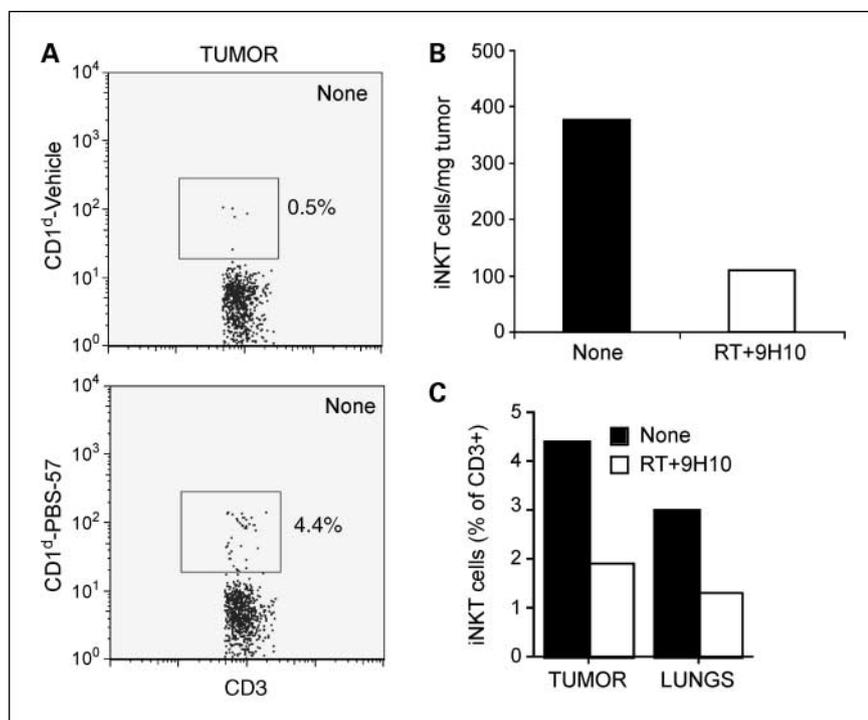
**Wild-type and iNKT<sup>-/-</sup> mice do not differ in the tumor-driven expansion and recruitment of MDSC to tumors.** Suppression of antitumor immune responses by NKT cells has been linked to their ability to induce TGF $\beta$  production by MDSC (19). To determine whether the markedly increased response to treatment in iNKT<sup>-/-</sup> mice was due to decreased accumulation of MDSC, the numbers of MDSC in the spleen and primary tumors of wild-type and iNKT<sup>-/-</sup> mice were analyzed on day 14 of tumor growth, corresponding approximately to the time therapy was initiated. MDSC accumulation in spleens as well as primary tumors was similar between wild-type and iNKT<sup>-/-</sup> mice (Fig. 6A and B). Next, spleen cells of tumor-bearing wild-type and iNKT<sup>-/-</sup> mice were tested for secretion of TGF $\beta$  in short-term cultures. As previously reported for the 15-12RM tumor model (19), the secretion of TGF $\beta$  by spleen cells from 4T1 tumor-bearing wild-type mice was significantly increased compared with healthy mice ( $P < 0.05$ ). This was true also for cells derived from iNKT<sup>-/-</sup> mice (Fig. 6C). Although the baseline production of TGF $\beta$  did not differ significantly between wild-type and iNKT<sup>-/-</sup> healthy mice ( $P = 0.35$ ), tumor-bearing iNKT<sup>-/-</sup> mice produced more TGF $\beta$  than tumor-bearing wild-type mice, indicating that the production of TGF $\beta$  was not dependent on the presence of iNKT cells. Overall, these results are consistent with a recent report showing that NKT cells are not required to activate MDSC (34). They also indicate that the improved response to treatment with radiotherapy and CTLA-4 blockade seen in tumor-bearing iNKT<sup>-/-</sup> mice cannot be explained by differences in MDSC numbers or functional activation to produce TGF $\beta$ .

## Discussion

In this study, we show that mice lacking the iNKT cell subset and bearing well-established 4T1 tumors respond to treatment with the combination of local radiotherapy and CTLA-4 blockade with markedly increased overall survival and cure rate as compared with wild-type mice (Figs. 2 and 3 and ref. 28). The improved response to treatment was not a result of increased immunogenicity of 4T1 cells in iNKT<sup>-/-</sup> mice because the growth of 4T1 tumors was similar in wild-type and iNKT<sup>-/-</sup> mice and vaccination with irradiated tumor cells did not induce



**Fig. 4.** Administration of  $\alpha$ -galactosylceramide does not enhance the response of 4T1 tumor-bearing wild-type mice to local radiotherapy and CTLA-4 blockade. Treatment was started on day 13 post-s.c. inoculation in the flank. Radiotherapy was delivered in two fractions of 12 Gy to the s.c. tumors on day 13 and 14. 9H10 was given i.p. 1, 4, and 7 d postradiotherapy.  $\alpha$ -Galactosylceramide (100 ng) was given i.p. twice per week starting on day 1 postradiotherapy. Wild-type mice ( $n = 5$ /group) were treated with (A) vehicle (closed circles), 9H10 plus vehicle (closed diamonds), radiotherapy plus vehicle (closed squares), radiotherapy plus 9H10 plus vehicle (closed triangles), (B)  $\alpha$ -galactosylceramide (open circles), 9H10 plus  $\alpha$ -galactosylceramide (open diamonds), radiotherapy plus  $\alpha$ -galactosylceramide (open squares), or radiotherapy plus 9H10 plus  $\alpha$ -galactosylceramide (open triangles). Tumor volume is shown as the mean  $\pm$  SE for animals with tumors in each treatment group up to day 27 when all animals were alive. Radiotherapy caused a significant ( $P < 0.001$  compared with control) tumor growth delay that was slightly enhanced by 9H10 but not by  $\alpha$ -galactosylceramide. Although tumor volume differences between mice treated with  $\alpha$ -galactosylceramide plus 9H10 and control were statistically significant ( $P = 0.0396$ ), the triple combination of  $\alpha$ -galactosylceramide, radiotherapy, and 9H10 did not show better tumor control than the combination of radiotherapy plus 9H10, and no complete tumor regression was observed.



**Fig. 5.** iNKT cells infiltrating 4T1 tumors are reduced by treatment with local radiotherapy and CTLA-4 blockade. Wild-type mice were injected with 4T1 cells on day 0 and left untreated (None) or treated with local radiotherapy and 9H10 (radiotherapy plus 9H10) as described in the legend of Fig. 3. Tumors and lungs were harvested on day 29, and obtained single cell suspensions stained with FITC-anti-CD3 mAb and CD1d-PBS-57 PE or control CD1d-Vehicle PE tetramers to identify iNKT cells, followed by flow cytometry analysis. The lymphocyte gate was set based on the scattered plots in the spleen. *A*, histograms showing tumor-derived cells from untreated mice stained with CD3 and CD1d-PBS-57 or control tetramers, as indicated, and gated on CD3-positive cells. Numbers indicate the percentage of cells in the square gate. *B*, the percentage of cells in the lymphocyte gate positive for CD3 and CD1d-PBS-57 was multiplied for the percentage of cells in the lymphocyte gate and for the total number of viable cells isolated from the tumors, and divided for the tumor weight to obtain the number of cells per milligram of tumor in treated (radiotherapy plus 9H10, white bar) and untreated (None, black bar) mice. *C*, percentage of CD3-positive T cells that bind the CD1d-PBS-57 tetramers in tumor and lungs of treated (radiotherapy plus 9H10, white bars) and untreated (None, black bars) mice, as indicated. All data are from 4 to 5 mice of each group. Error bars are absent because pooling of tumors and lungs within each group was necessary to obtain sufficient cells for analysis.

a protective antitumor response (Fig. 1D). However, iNKT<sup>-/-</sup> mice developed a spontaneous CD8 response inhibiting lung metastases (Fig. 1).

The development of an antitumor immune response that is at least partially effective against metastases in face of a poorly immunogenic growing primary tumor is an example of concomitant tumor immunity (35). This well-known phenomenon is consistent with the notion that invasive cancers cause tissue damage and the generation of inflammatory signals attracting innate immune cells and eventually leading to the activation of the adaptive immune system (36). Regulatory T cells have been shown to suppress concomitant immunity to the poorly immunogenic B16 melanoma (37). Our data suggest that iNKT cells play a similar role in the case of the poorly immunogenic 4T1 carcinoma and are consistent with a previous observation that iNKT<sup>-/-</sup> mice have improved immunosurveillance against 4T1 metastases compared with wild-type mice (18). Importantly, whereas Terabi et al. (18) detected the immune response against metastases after surgical removal of the primary tumor, our work provides the evidence that it is generated in the absence of any surgical manipulation that could potentially contribute to the development of the antitumor response (38).

Treatment of iNKT<sup>-/-</sup> mice with local radiotherapy used as a single modality caused a reduction in lung metastases that, however, was not statistically significant (Fig. 2C). Consistent with this, despite the complete "cure" obtained in ~20% of the mice, the effect of radiotherapy alone on survival was not significant ( $P = 0.16$ ; Fig. 1B). This suggests that radiotherapy by itself was either not sufficient to induce a T cell response against this poorly immunogenic tumor, or that if induced it was suppressed (39). However, in combination with CTLA-4 blockade there was a highly significant antitumor effect leading to complete clearance of primary tumor and metastatic disease in over half of the mice (Figs. 2 and 3). Overall, these data are

consistent with the hypothesis that radiotherapy causes changes in the tumor microenvironment that can promote the afferent and efferent phases of the antitumor immune response (26, 32, 40).

CTLA-4 blockade used as a single modality had a modest but detectable effect in inhibiting tumor growth and extending the survival of iNKT<sup>-/-</sup> but not wild-type mice (Fig. 2 and ref. 28). This result was confirmed in two additional experiments (data not shown). CTLA-4 blockade used as a single treatment is known to be effective only against more immunogenic tumors in wild-type mice (41). This suggests that besides the intrinsic immunogenicity of a tumor, preexisting host-specific factors can determine the response to CTLA-4 blockade. It is intriguing to consider whether the number or activity of iNKT cells could be a determinant of therapeutic responses mediated by anti-CTLA-4 antibodies in cancer patients (42).

Immunosuppression by NKT cells has been previously shown to be mediated by MDSC and TGF $\beta$  in some tumor models (16, 19, 20). In the 4T1 model, the increase in splenic MDSC in tumor-bearing mice was shown to correlate with the suppression of the immune response against metastases (43). Given the development of a spontaneous CD8 response inhibiting metastases in iNKT<sup>-/-</sup> mice, we expected to find less MDSC in iNKT<sup>-/-</sup> than in wild-type mice. In contrast, neither MDSC nor TGF $\beta$  production was reduced in the spleen of iNKT<sup>-/-</sup> mice (Fig. 6). This suggests that MDSC likely exert their suppression locally, within the growing primary tumors, as previously shown by Kusmartsev et al. (44). In mice treated with radiotherapy, sensitization of CD11b-positive MDSC by tumor-derived antigens that are released by radiation (40) could promote their destruction by antitumor CTL activated by anti-CTLA-4 antibodies leading to complete regression of established primary tumors.

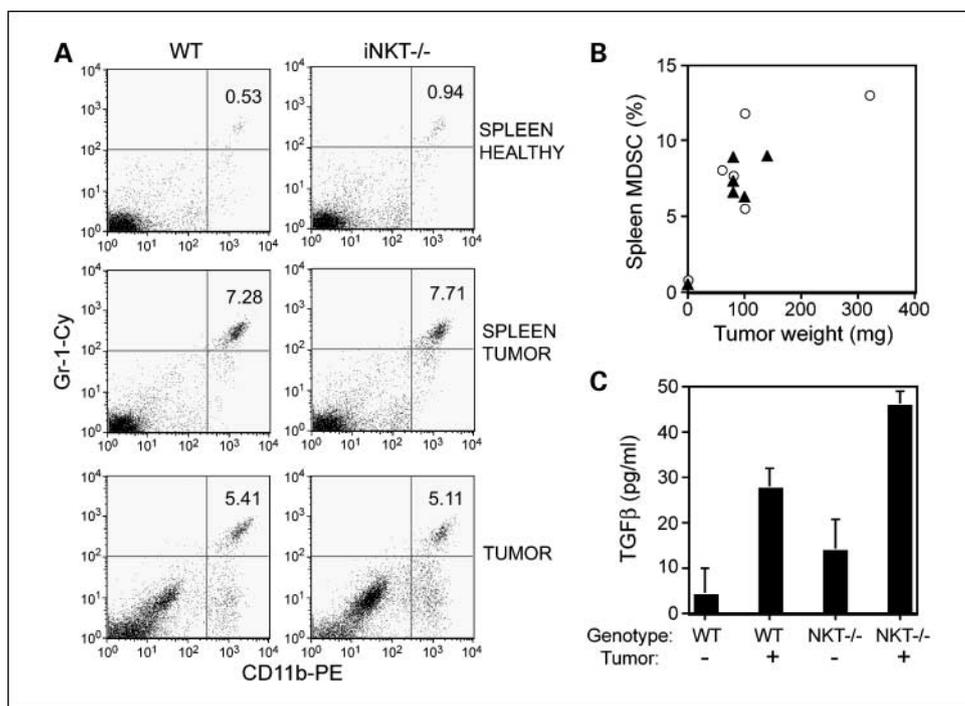
Administration of the potent iNKT cell activator  $\alpha$ -galactosylceramide did not improve the response of wild-type mice to

treatment with local radiotherapy and CTLA-4 blockade (Fig. 4). In a recent report, 4T1 tumor-bearing mice given  $\alpha$ -galactosylceramide in a similar dose/schedule (100 ng every 4 days) in combination with anti-DR5 mAb to induce tumor cell apoptosis and anti-4-1BB mAb to provide costimulatory signals to T cells, showed significant rates of tumor rejection (45). This suggests that iNKT cell immunostimulatory function can be "rescued" by activation with  $\alpha$ -galactosylceramide, but the outcome of this activation is influenced by the immunologic environment in which this takes place. Indeed,  $\alpha$ -galactosylceramide has been shown to promote the regulatory functions of iNKT cells when given to mice with autoimmune diseases (11). Therefore, it will be important to determine which immunotherapy strategies may be successfully combined with  $\alpha$ -galactosylceramide to elicit antitumor immune responses in cancer patients.

It has been proposed that there is a dichotomy between the two main subsets of NKT cells, noninvariant (or type II) NKT cells being sufficient for negative regulation of the antitumor immune response and iNKT cells being mostly responsible for protection (18). Our study shows that the selective absence of iNKT cells is sufficient to dramatically enhance the response to treatment with radiotherapy and CTLA-4 blockade in mice with an established metastatic mammary carcinoma. Importantly, at least in this tumor model, an antitumor immune response was detectable even in the absence of treatment, consistent with a role for iNKT cells in the suppression of antitumor immunity.

Therefore, it is likely that in cancer, similarly to other autoimmune/inflammatory diseases (10), the regulatory role of invariant and noninvariant NKT cells is not absolute, but is influenced by both the immunologic environment of the tissue/tumor and unknown factors of the host. For instance, a glycolipid produced by melanoma cells has been shown to be cross-presented by dendritic cells (DC) and to induce production of IL-10 by NKT cells (46). The presence of iNKT cells in 4T1 tumors (Fig. 5) raises the question of whether they may recognize a tumor-derived glycolipid. Few endogenous CD1d ligands are currently known. Identification of CD1d ligands that are expressed by cancer cells and may be recognized by different NKT cell subsets and/or with different affinity will help clarify the role of NKT cells in regulation of antitumor immunity (47).

Previous studies have shown a marked enhancement of antitumor immunity in CD1d-deficient mice that lack both subsets of NKT cells (18, 21, 22), and these results have been interpreted as evidence of a strong regulatory role of type II NKT cells. However, the recent evidence that some mouse tumors express low levels of CD1d (48) raises the possibility that CD1d may act as a neoantigen in CD1d-deficient mice, similarly to *STAT6* in *STAT6*-deficient mice (49). Although CD1d expression in 4T1 cells has not been detected by immunostaining and flow cytometry (ref. 18 and data not shown), using a more sensitive technique, real time reverse transcription-PCR, we have found that 4T1 cells express CD1d (Supplementary Fig. S1). Because CD1d-reactive T cells may be present in the



**Fig. 6.** Accumulation of MDSC and TGF $\beta$  production in wild-type and iNKT<sup>-/-</sup> mice bearing 4T1 tumors. Wild-type and NKT<sup>-/-</sup> mice were inoculated s.c. with  $5 \times 10^4$  4T1 cells on day 0 ( $n = 5$ /group). Three non-tumor-bearing mice of each genotype served as controls. On day 14 primary tumors and spleens were harvested and obtained cell suspensions were stained with PE-anti-CD11b and Cy-anti-Gr-1 mAbs to detect MDSC. **A**, representative histograms of spleen from one healthy (*top row*) and one tumor-bearing (*middle row*) mouse showing the CD11b-positive Gr-1-positive MDSC (*bottom row*). Numbers indicate the percentage of MDSC. **B**, percentage of spleen MDSC in relation to the tumor weight in wild-type (*closed triangles*) and iNKT<sup>-/-</sup> (*open circles*) mice. Each symbol represents an individual mouse. For control healthy mice, one symbol representing the mean of 3 animals is shown. **C**, splenocytes isolated from tumor-free ( $-$ ;  $n = 3$ ) and tumor-bearing ( $+$ ;  $n = 5$ ) mice were cultured o.n. and the concentration of TGF $\beta$  in the culture supernatants was determined by ELISA. Data are the mean  $\pm$  SE. In both wild-type and iNKT<sup>-/-</sup> mice spleen cells derived from tumor-bearing mice produced significantly more TGF $\beta$  than spleen cells derived from healthy mice ( $P < 0.05$ ). Although the baseline production of TGF $\beta$  did not differ significantly between wild-type and iNKT<sup>-/-</sup> healthy mice ( $P = 0.35$ ), tumor-bearing iNKT<sup>-/-</sup> mice produced more TGF $\beta$  than tumor-bearing wild-type mice ( $P < 0.01$ ).

repertoire of CD1d-deficient mice, their possible role in 4T1 tumor rejection will need to be addressed.

In conclusion, we show that mice developing a detectable antitumor response to a syngeneic poorly immunogenic tumor because their regulatory circuits are “slightly” altered in the absence of the iNKT cells, only show a mild improvement in survival in the absence of treatment. However, their response to immunotherapy is dramatically improved in comparison with wild-type mice. These results model clinical observations that patients with preexisting antitumor immune responses are more likely to respond to immunotherapy (50). Although the intrinsic tumor immunogenicity has often been invoked to

explain these responses, focusing on the “immunologic” make-up of the host may be important for an improved understanding of the determinants of response to immunotherapy.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Clinical Cancer Research

## Invariant Natural Killer T Cells Regulate Breast Cancer Response to Radiation and CTLA-4 Blockade

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