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Increased Susceptibility to Tumor Initiation and Metastasis in TNF-Related Apoptosis-Inducing Ligand-Deficient Mice[1]

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We have previously implicated TNF-related apoptosis-inducing ligand (TRAIL) in innate immune surveillance against tumor development. In this study, we describe the use of TRAIL gene-targeted mice to demonstrate the key role of TRAIL in suppressing tumor initiation and metastasis. Liver and spleen mononuclear cells from TRAIL gene-targeted mice were devoid of TRAIL expression and TRAIL-mediated cytotoxicity. TRAIL gene-targeted mice were more susceptible to experimental and spontaneous tumor metastasis, and the immunotherapeutic value of α-galactosylceramide was diminished in TRAIL gene-targeted mice. TRAIL gene-targeted mice were also more sensitive to the chemical carcinogen methylcholanthrene. These results substantiated TRAIL as an important natural effector molecule used in the host defense against transformed cells. ***The Journal of Immunology,*** **2002, 168: 1356–1361.**

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)[3] is a type-II membrane protein belonging to the TNF family, which preferentially induces apoptotic cell death in a wide variety of tumor cells but not in normal cells in vitro (1). In humans, TRAIL can bind two death-inducing receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), resulting in receptor cross-linking, recruitment of caspases, and initiation of the caspase cascade (2–4). Two other receptors that may act as a potential decoys, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), and the soluble receptor osteoprotegerin (TRAIL-R5) can also bind TRAIL (4–6). In the mouse, only one receptor has been described that shares some homology to human TRAIL-R2 (mouse DR5) (7).

Recombinant TRAIL has been shown to be nontoxic and to exert potent antitumor functions when administered in vivo to tumor-bearing mice and nonhuman primates (8, 9). Only recently have studies begun to elucidate some physiological roles for TRAIL. The most significant progress has resulted from the development of several key tools to investigate the role of TRAIL in the mouse. These include soluble DR5 (produced in *Pichia*), which has been shown to neutralize mouse TRAIL and to define a role for TRAIL in autoimmune inflammation (10, 11); and a neutralizing rat anti-mouse TRAIL mAb (12) that we have used to define a role of TRAIL in host protection from tumor metastasis (13, 14). Using the anti-TRAIL mAb, we found that some murine liver NK cells constitutively expressed TRAIL, which was at least partly responsible for natural antimetastatic function of liver NK cells against TRAIL-sensitive tumor cells (13). We also demonstrated that IFN-γ-mediated TRAIL induction on NK cells plays some role in IFN-γ-dependent antimetastatic effects of IL-12 and α-galactosylceramide (α-GalCer) (14).

In this study, we describe the initial characterization of TRAIL-deficient mice generated by gene targeting. These mice were used to further substantiate the importance of TRAIL expressed on NK cells in mediating antitumor activity. Some additional roles of TRAIL in protecting mice from spontaneous metastasis of mammary tumors and suppressing chemical carcinogen-induced tumor development were also revealed by the present study.

Materials and Methods

TRAIL-deficient mice

Mice genetically deficient in TRAIL (TRAIL$^{-/-}$) were generated by homologous recombination in 129 derived embryonic stem cells (J. J. Peschon and M. Galccum, unpublished observations). In brief, sequences between nucleotides 274 and 371 encoding amino acids 76–110 of the TRAIL cDNA (1) were replaced with a cassette conferring resistance to G418. The structure of the mutation was confirmed by both genomic Southern blotting and PCR analyses. Chimeras generated from TRAIL-targeted embryonic stem cells were crossed to C57BL/6 to achieve germline transmission of the mutation. The resulting (C57BL/6 $\times$ 129)F_1 hybrids were successively back-crossed to either C57BL/6 or BALB/c as described below.

Mice

Inbred BALB/c and C57BL/6 (B6) wild-type (WT) mice were purchased from the Walter and Eliza Hall Institute (Parkville, Victoria, Australia). The following gene-targeted mice were bred at the Peter MacCallum Cancer Institute: BALB/c perforin (pfp)-deficient (BALB/c pfp$^{-/-}$; Ref. 15), BALB/c IFN-γ-deficient (BALB/c IFN-$\gamma^{-/-}$); BALB/c TRAIL-deficient (BALB/c TRAIL$^{-/-}$), and C57BL/6 TRAIL-deficient (B6 TRAIL$^{-/-}$) mice. B6 TRAIL$^{-/-}$ and BALB/c TRAIL$^{-/-}$ mice had been back-crossed for five generations onto B6 and BALB/c backgrounds, respectively, initiated from random B6 $\times$ 129 hybrids. Mice of 6–12 wk of age were used in all experiments under specific pathogen-free conditions according to animal experimental ethics committee guidelines.

Tumor cells and reagents

The TRAIL-sensitive and Fas ligand (FasL)-insensitive BALB/c-derived renal adenocarcinoma cell line, Renca (H-2^d), has been previously described (13) and was maintained in RPMI 1640 containing 10% FCS and

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[3] Abbreviations used in this paper: TRAIL, TNF-related apoptosis-inducing ligand; α-GalCer, α-galactosylceramide; FasL, Fas ligand; CMA, concanamycin A; i.s., intrasplenically; MCA, 3-methylcholanthrene; MNC, mononuclear cell; pfp, perforin; WT, wild type.

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2 mM L-glutamine. The TRAIL-sensitive and 6-thioguanine-resistant BALB/c-derived mammary carcinoma cell line, 4T1, was provided by Dr. R. Anderson (Peter MacCallum Cancer Institute, Victoria, Australia) and was maintained in α-MEM containing 10% FCS and 2 mM L-glutamine. α-GalCer, a marine sponge glycolipid that activates CD1d-restricted NKT cells (14), was provided by the Pharmaceutical Research Laboratories (Kirin Brewery, Gunma, Japan) and was prepared as described (14). α-GalCer and the control vehicle were resuspended in saline supplemented with 0.5% polysorbate-20. Concanamycin A (CMA), which inhibits pfp-mediated cytotoxicity (13), was purchased from Wako Pure Chemicals (Osaka, Japan). The neutralizing anti-mouse TRAIL mAb (N2B2) was prepared as described previously (12).

Flow cytometric analysis

Mononuclear cells (MNC) were prepared from the spleen and liver as described previously (16). Some mice were treated i.p. with α-GalCer (2 μg) on days 0 and 4, and their splenic and hepatic MNC were isolated on day 5. To avoid the nonspecific binding of mAbs to FcγR, anti-mouse CD16/32 (2.4G2) mAb was added to the mAb mixture. Cells were incubated with PE-conjugated anti-mouse TRAIL (N2B2) mAb (12), FITC-conjugated anti-mouse CD3, and biotinylated anti-NK1.1 mAb before incubation with PerCP-conjugated streptavidin. PE-N2B2 was obtained from e-Bioscience (San Diego, CA), and the remaining reagents were obtained from BD PharMingen (San Diego, CA). After washing the cells with PBS/FCS/azide, the stained cells were analyzed on a FACScan (BD PharMingen) and the data were processed by the CellQuest program (BD PharMingen).

Cytotoxicity assay

Cytotoxic activities of hepatic and splenic MNC were tested against Renca and 4T1 tumor targets by an 8-h ^{51}Cr-release assay as described previously (12, 17). The assay was also performed in the presence of control rat IgG2a (R35-95, 10 μg/ml; BD PharMingen), anti-TRAIL (N2B2) mAb (10 μg/ml), and/or CMA (50 nM). This assay has previously been shown to accurately represent tumor cell death exposed to NK cells, and it tightly correlates with clonogenic potential of target tumor cells in soft agar (18).

Renca tumor metastasis

Male BALB/c WT and TRAIL$^{-/-}$ mice were injected intrasplenically (i.s.) or i.v. with Renca tumor cells as described previously (14). Mice were euthanized 14 days after tumor inoculation, and liver (after i.s.) or lung (after i.v.) metastases were quantified with the aid of a dissecting microscope. Some mice received either anti-mouse TRAIL mAb (250 μg i.p.) on days 0, 1, and 7 after tumor inoculation or polyclonal rabbit anti-asialoGM1 Ab (200 μg i.p.; Wako Pure Chemicals) on days -1, 0, and 7 relative to tumor inoculation. This depletion protocol has been shown to selectively deplete NK cells but not other leukocyte subsets, including NKT cells, in both C57BL/6 and BALB/c mouse strains (19). The anti-metastatic treatment protocol with α-GalCer used 2 μg i.p on days 0, 4, and 8. This regimen was chosen based on the previous efficacy studies in the Renca tumor model (14).

4T1 mammary carcinoma growth and metastasis

To examine primary tumor growth and spontaneous metastasis, female WT or TRAIL$^{-/-}$ BALB/c mice were inoculated in the abdominal mammary gland with 4T1 tumor cells at the doses indicated on day 0. Some groups of mice received either anti-mouse TRAIL mAb (250 μg i.p.) on days 0, 1, 4, 7, 10, 14, and 21; anti-asialoGM1 Ab (200 μg i.p.) on days -1, 0, 7, and 14; and/or α-GalCer (2 μg i.p.) on days 0, 4, 8, 12, and 16. Primary tumors were measured every 4 days following tumor inoculation over the course of 30 days with a caliper square as the product of two perpendicular diameters (cm^2) and represented as the mean $\pm$ SE of 5–10 mice in each group. Tumors >2 mm in diameter and demonstrating progressive growth were recorded as positive. Mice were sacrificed at 30 days, and spontaneous metastasis was also measured by harvesting the lungs and livers as described (20). Clonogenic metastases were calculated on a per organ basis.

Fibrosarcoma induction by MCA

Male WT and TRAIL$^{-/-}$ B6 mice were inoculated s.c. in the hind flank with 5, 25, 100, or 400 μg of 3-methylcholanthrene (MCA; Sigma-Aldrich, St. Louis, MO) in 0.1 ml of corn oil. Development of fibrosarcomas was monitored periodically over the course of 80–180 days. Tumors >2 mm in diameter and demonstrating progressive growth were recorded as positive.

Statistical analysis

Significant differences in incidence at one time point were determined by the Fisher's exact test. Significant differences in metastasis were determined by the unpaired Mann-Whitney U test. Values of $p < 0.05$ were considered significant.

Results and Discussion

TRAIL gene-targeted mice lack TRAIL expression

TRAIL$^{-/-}$ mice were generated from TRAIL$^{+/-}$ intercrosses at the expected frequency and displayed no obvious histological, hematological, or reproductive defects (E. Cretney and J. J. Peschon, unpublished observations). In WT mice, we have previously demonstrated that the only detectable surface TRAIL expression on MNC was observed on a proportion of liver NK cells (14). Liver and spleen MNC from WT and TRAIL$^{-/-}$ mice were analyzed for NK1.1 and CD3 expression. B6 WT and TRAIL$^{-/-}$ mice displayed similar proportions of liver NK, NKT, and T cells (Fig. 1, *A* and *C*). Constitutive TRAIL expression was found on freshly isolated liver CD3$^-$NK1.1$^+$ NK cells (Fig. 1*B*) but not on CD3$^+$NK1.1$^+$ T cells, CD3$^+$NK1.1$^-$ T cells, or CD3$^-$NK1.1$^-$ cells from the liver of WT mice (data not shown). NK cells freshly isolated from the spleen of WT mice did not express TRAIL (data not shown). The lack of TRAIL in B6 TRAIL$^{-/-}$ mice was verified when liver MNC from these mice were similarly analyzed (Fig. 1*D*). Furthermore, α-GalCer, which has been shown to induce NK cell proliferation, IFN-γ production, cytotoxicity, and TRAIL expression on additional NK cells in vivo (14), induced TRAIL on B6 WT splenic NK cells but not on B6 TRAIL$^{-/-}$ liver and spleen NK cells (data not shown). TRAIL expression was also not detected in liver MNC from B6 TRAIL$^{-/-}$ mice that had been permeabilized, and similar data were obtained using DX-5 and CD3 markers in BALB/c mice (data not shown). These data indicated that TRAIL$^{-/-}$ mice did not express the TRAIL protein, at least in NK cells.

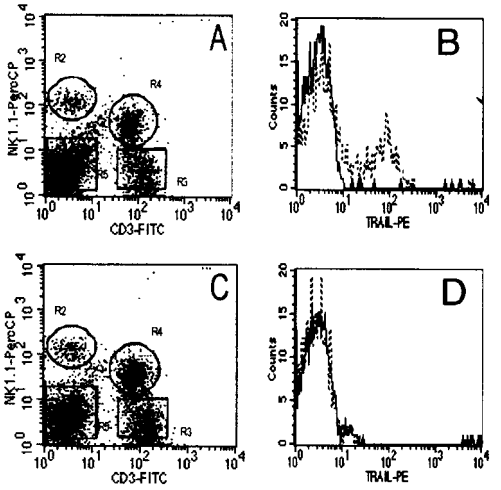

FIGURE 1. Absence of TRAIL expression in TRAIL gene-targeted mice. Liver MNC were isolated from WT or TRAIL$^{-/-}$ B6 mice and were stained with FITC-conjugated anti-CD3 mAb, PE-conjugated anti-TRAIL mAb, and biotin-conjugated anti-NK1.1 mAb followed by streptavidin-PerCP. *A* and *C*, NK1.1/CD3 staining of WT and TRAIL$^{-/-}$ mice, respectively, with R2 representing the gated NK cell population. *B*, TRAIL expression on WT liver NK cells (R2 gated). *D*, Absence of TRAIL on TRAIL$^{-/-}$ liver NK cells (R2 gated). Solid lines represent staining with isotype-matched control mAb; dotted lines represent staining with anti-mouse TRAIL mAb. These analyses have been performed on more than three occasions and these profiles are representative.

TRAIL contributes to NK cell cytotoxicity of TRAIL-sensitive tumor cells

To confirm that TRAIL$^{-/-}$ mice lacked functional TRAIL, the contribution of TRAIL to the cytotoxicity of hepatic and splenic NK cells was assessed against TRAIL-sensitive tumor targets. We have previously shown that NK cells completely account for the lysis of these target tumors by liver or spleen MNC from untreated or α-GalCer-treated mice (Ref. 13 and data not shown). The cytotoxicity of liver MNC from untreated BALB/c WT mice was inhibited partially by anti-TRAIL mAb alone and was inhibited completely by the combination with a pfp inhibitor, CMA, against TRAIL-sensitive 4T1 (Fig. 2*A*) or Renca (Fig. 2*B*) tumor cells. The cytotoxicity of spleen MNC from untreated BALB/c WT mice was completely abrogated by CMA (Fig. 2, *A* and *B*) and BALB/c pfp$^{-/-}$ spleen MNC did not lyse 4T1 or Renca tumor targets, indicating that pfp was the only mediator of cytotoxicity used by spleen NK cells against both targets. Liver and spleen MNC from IFN-$\gamma^{-/-}$ and TRAIL$^{-/-}$ mice displayed very similar patterns of cytotoxicity against 4T1 and Renca tumor targets, further substantiating the role of IFN-γ in constitutive TRAIL expression on NK cells (Ref. 13; Fig. 2, *A* and *B*). The cytotoxicity of liver MNC from TRAIL$^{-/-}$ mice was reduced compared with those from WT mice and was completely inhibited by CMA. Administration of α-GalCer augmented the cytotoxicity mediated by WT liver (Fig. 2*C*) and spleen (data not shown) MNC, which was also inhibited partially by anti-TRAIL mAb alone and inhibited completely by combination with CMA. The liver MNC from TRAIL$^{-/-}$ mice did not display TRAIL-mediated cytotoxicity, and their cytotoxicity was completely abrogated by CMA (Fig. 2*C*). Therefore, the anti-TRAIL mAb neutralization experiments were completely consistent with those obtained using the TRAIL$^{-/-}$ mice and confirmed the importance of TRAIL as a mechanism used by NK cells to kill some tumor targets.

TRAIL contributes to NK cell suppression of experimental Renca metastases to the liver

To test the role of TRAIL in NK cell surveillance of tumor metastasis, increasing doses of Renca cells were inoculated i.s. into BALB/c WT and TRAIL$^{-/-}$ mice. At the lower tumor doses administered, significantly increased numbers of liver metastases were observed in TRAIL$^{-/-}$ mice (Fig. 3*A*). These data were entirely consistent with the increased number of liver metastases observed in WT mice treated with anti-TRAIL mAb (Fig. 3*A*). The even greater effect of depleting NK cells on increasing Renca liver metastases was consistent with our previous observations that NK cells control Renca metastasis by both pfp- and TRAIL-dependent mechanisms (14). An inoculum of 3×10^5 Renca tumor cells that metastasized equivalently in untreated WT or TRAIL$^{-/-}$ mice was used for the subsequent α-GalCer therapy experiments. α-GalCer has been demonstrated to possess potent antimetastatic activity against Renca liver metastasis (14). After i.s. inoculation of 3×10^5 Renca cells, the therapeutic administration of α-GalCer significantly reduced the numbers of Renca liver metastases ($p < 0.05$; Fig. 3*B*). This antimetastatic activity was completely abolished by anti-asGM1 Ab, indicating that α-GalCer exerted its activity via NK cell effector function. A significant proportion of the antimetastatic activity was due to TRAIL, as evidenced by the increased number of liver metastases in TRAIL$^{-/-}$ mice or anti-TRAIL mAb-treated WT mice that received α-GalCer (Fig. 3*B*). Because TRAIL expression was not induced on lung NK cells by α-GalCer (14), we reasoned that TRAIL function might not be observed in the Renca lung metastasis model. Although α-GalCer treatment significantly reduced lung metastasis ($p < 0.05$), the number of lung metastases was not significantly different among WT mice, TRAIL$^{-/-}$ mice, or anti-TRAIL mAb-treated WT mice (Fig. 3*C*).

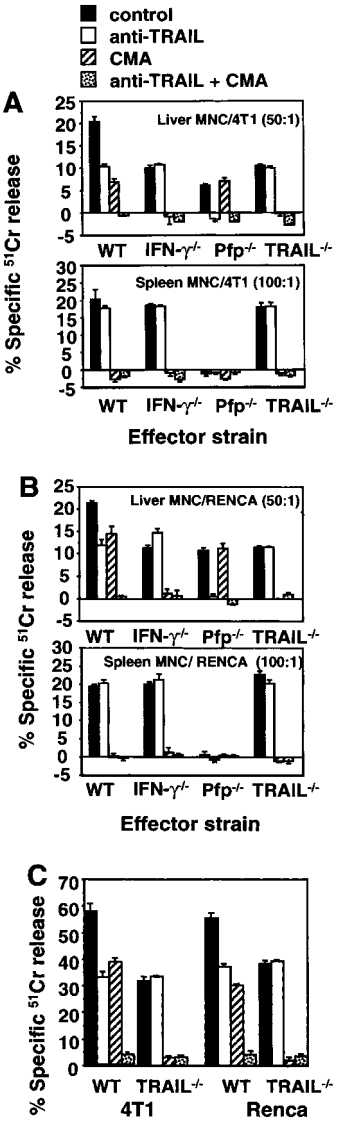

FIGURE 2. TRAIL gene-targeted mice do not exert TRAIL-mediated cytotoxicity. Liver and spleen MNC were isolated from untreated BALB/c WT, BALB/c pfp$^{-/-}$, BALB/c IFN-$\gamma^{-/-}$, or BALB/c TRAIL$^{-/-}$ mice (*A* and *B*), and liver MNC were isolated from α-GalCer (2 μg i.p. on days -5 and -1)-treated BALB/c WT or BALB/c TRAIL$^{-/-}$ mice (*C*). Their cytotoxic activities were tested against TRAIL-sensitive 4T1 (*A* and *C*) and Renca (*B* and *C*) tumor cells in the presence or absence of 50 nM CMA, 10 μg/ml anti-TRAIL mAb, or 10 μg/ml control rat IgG2a (control) by an 8-h ^{51}Cr release assay at three different E:T ratios (highest, 100:1 for spleen MNC; highest, 50:1 for liver MNC, shown) as indicated. Data are representative of those across the E:T ratio range (100 to 5:1, spleen; 50 to 5:1, liver) and are recorded as the mean ± SE of triplicate samples. Similar results were obtained in two independent experiments.

TRAIL contributes to NK cell suppression of 4T1 tumor growth and spontaneous metastasis

Previous studies by Miller and colleagues (21, 22) have established that the 4T1 mammary carcinoma is highly tumorigenic and spontaneously metastatic in syngeneic BALB/c mice. This model is perhaps the best mouse model of metastatic disease available and has proven very useful in defining the efficacy of immunotherapy

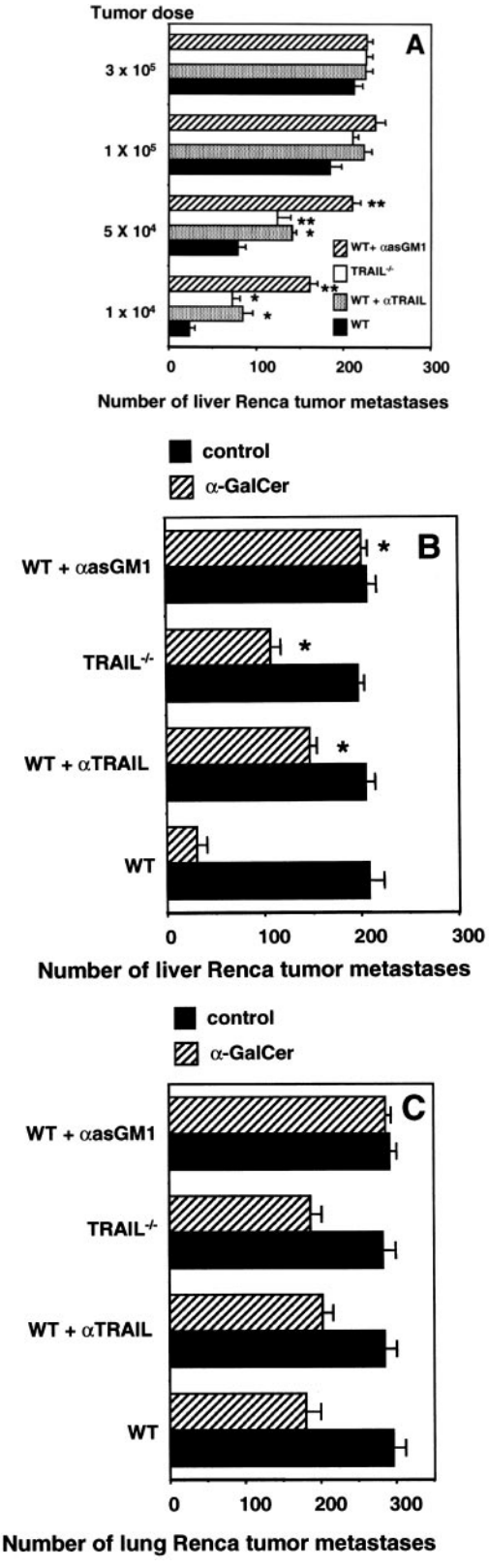

FIGURE 3. TRAIL contributes to NK cell suppression of experimental Renca metastases to the liver. Groups of WT or TRAIL$^{-/-}$ BALB/c mice were inoculated i.s. with Renca tumor cells ranging from 1×10^4–3×10^5 (*A*), i.s. with 3×10^5 Renca tumor cells (*B*), or i.v. with 3×10^5 Renca tumor cells (*C*) on day 0. As indicated, some groups of mice were treated with 200 μg of anti-asGM1 Ab i.p. on days -1, 0, and 7 (*A*–*C*), 0.25 mg of anti-TRAIL mAb i.p. on days 0, 1, and 7 (*A*–*C*), and 2 μg of α-GalCer i.p. on days 0, 4, and 8 (*B* and *C*). The livers (*A* and *B*) or lungs (*C*) were removed from mice on day 14, and the metastatic nodules were quantified as described in *Materials and Methods*. Data are indicated as the mean $\pm$ SE of five mice in each group, with the significance compared with untreated (*A*) or α-GalCer-treated (*B* and *C*) WT mice as defined by the Mann-Whitney *U* test. $*$, $p < 0.01$; $**$, $p < 0.05$. α-GalCer was also statistically effective alone compared with no treatment in the livers and lungs of WT mice ($p < 0.01$). We have previously shown no significant effects of rabbit and mouse control Igs in these models (14).

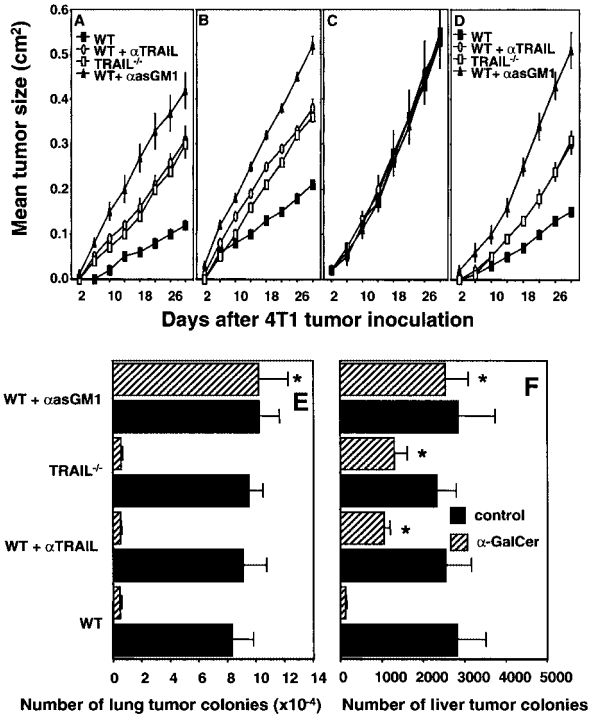

FIGURE 4. TRAIL contributes to the suppression of primary 4T1 tumor growth and spontaneous metastasis. *A–C*, Groups of female BALB/c WT or TRAIL$^{-/-}$ mice were inoculated on day 0 into the abdominal mammary fat pad with 5×10^3 (*A*), 1×10^4 (*B*), or 2.5×10^4 (*C*) 4T1 mammary carcinoma cells. Some groups of mice were treated with 200 μg of anti-asGM1 Ab i.p. on days -1, 0, 7, and 14, or 0.25 mg of anti-TRAIL mAb i.p. on days 0, 1, 4, 7, 10, 14, and 21. Tumor size was measured over the course of 30 days. Data are shown as the mean $\pm$ SE of five tumor-bearing mice in each group. *D–F*, Groups of female BALB/c WT or TRAIL$^{-/-}$ mice were inoculated on day 0 into the abdominal mammary fat pad with 2.5×10^4 4T1 mammary carcinoma cells and were i.p. administered with 2 μg of α-GalCer on days 0, 4, 8, 12, and 16. Some groups of mice were treated with 200 μg of anti-asGM1 Ab i.p. on days -1, 0, 7, and 14 or 0.25 mg of anti-TRAIL mAb i.p. on days 0, 1, 4, 7, 10, 14, and 21. Primary tumor growth in the mammary gland was measured over the course of 30 days (*D*). Lung metastases (*E*) and liver metastases (*F*) were measured as described in *Materials and Methods*. Data are indicated as the mean $\pm$ SE of five mice in each group, with the significance compared with α-GalCer-treated WT mice as defined by the Mann-Whitney *U* test. $*$, $p < 0.01$. α-GalCer was also statistically effective alone compared with no treatment in the livers and lungs of WT mice ($p < 0.01$).

in the context of organ-specific tumor metastasis (20, 23–26). The 4T1 tumor spontaneously metastasizes to lung, liver, lymph nodes, bone, brain, and peripheral blood, and appears to be as TRAIL-sensitive as the Renca renal carcinoma in vitro (data not shown). We first assessed the primary growth of 4T1 tumor cells injected into the mammary fat pad of WT and TRAIL$^{-/-}$ mice (Fig. 4, *A–C*). At the lower doses of 4T1 tumor cells inoculated (5×10^3 and 10^4; Fig. 4, *A* and *B*), tumor growth was retarded in WT mice compared with TRAIL$^{-/-}$ mice or anti-TRAIL mAb-treated WT mice. Consistent with the above experiments in the Renca tumor

models (Fig. 3), the depletion of NK cells enhanced tumor growth even further. There was no significant difference in tumor growth among the groups examined at the highest dose (2.5×10^4) of 4T1 tumor cells inoculated (Fig. 4*C*). These data demonstrated for the first time that TRAIL could naturally suppress 4T1 tumor growth in vivo and particularly function in the mammary gland, a site of potential TRAIL action that has not previously been examined. A subsequent α-GalCer therapy experiment was then performed in mice inoculated in the mammary fat pad with 2.5×10^4 4T1 tumor cells. α-GalCer significantly retarded the primary growth of 4T1 tumor in WT mice (compare filled squares in Fig. 4, *C* and *D*). However, α-GalCer was without effect in WT mice depleted of NK cells and only partially effective in TRAIL$^{-/-}$ mice or anti-TRAIL mAb-treated WT mice (Fig. 4*D*). In these same α-GalCer-treated WT mice, both lung and liver metastases were significantly reduced ($p < 0.05$; Fig. 4, *E* and *F*). The antimetastatic effect was completely abolished by anti-asGM1 Ab, indicating the critical contribution of NK cells. Clearly, although TRAIL played no role in the antimetastatic effect of α-GalCer in the lung (Fig. 4*E*), liver metastasis in the same mice was significantly suppressed by TRAIL, as demonstrated in TRAIL$^{-/-}$ mice and anti-TRAIL mAb-treated WT mice (Fig. 4*F*). These data further substantiated an important role for NK cell TRAIL as an antimetastatic effector molecule in the liver, not only in the Renca experimental metastasis model but also in the 4T1 spontaneous metastasis model.

TRAIL suppresses MCA-induced fibrosarcoma development

We next examined the role of TRAIL during the primary tumor development induced by a chemical carcinogen MCA. We and others have previously shown that MCA induction of fibrosarcomas is dose dependent and is primarily controlled by NK cells (18), NKT cells (27), and the effector molecules, pfp and IFN-γ (28). B6 WT and TRAIL$^{-/-}$ mice were s.c. inoculated with MCA ranging from 5 to 400 μg. Inoculation of 400 μg of MCA induced fibrosarcomas in almost all WT or TRAIL$^{-/-}$ mice, but there was an earlier onset of fibrosarcomas in the TRAIL$^{-/-}$ mice (Fig. 5). As the dose of MCA was reduced, a difference in the susceptibility of WT and TRAIL$^{-/-}$ mice to tumor onset and development was demonstrated (Fig. 5). Notably, 100 μg of MCA induced fibrosarcomas in 7 of 10 TRAIL$^{-/-}$ mice, but only in 6 of 30 WT mice, and their onset was earlier in TRAIL$^{-/-}$ mice (Fig. 5). These data clearly indicated that TRAIL also plays an important role in natural host protection from tumor initiation by MCA.

In the present study, we demonstrated a substantial contribution of TRAIL to NK cell-mediated protection from tumor metastasis and development by using the recently generated TRAIL-deficient mice. In particular, we illustrated the natural role of TRAIL in suppressing primary 4T1 tumor growth in the mammary gland. In addition, spontaneous metastasis of 4T1 from the mammary gland to the liver or lung was inhibited by the CD1d ligand, α-GalCer; however, TRAIL only affected the antimetastatic activity of α-GalCer in the liver. More importantly, TRAIL-deficient mice also showed an increased frequency of fibrosarcomas following s.c. inoculation of MCA, indicating the tumor suppressor function of TRAIL against primary tumor development in vivo. Although we previously illustrated a substantial contribution of TRAIL to NK cell-mediated protection from Renca tumor metastasis in the liver using a neutralizing anti-mouse TRAIL mAb (13, 14), the use of TRAIL$^{-/-}$ mice definitively supported our previous findings. Interestingly, there was little phenotypic difference observed between TRAIL$^{-/-}$ mice and anti-TRAIL mAb-treated WT mice in all experiments where these groups were compared. These data indicate that both the TRAIL$^{-/-}$ mice and the neutralizing anti-TRAIL mAb will be useful tools with which to further dissect the

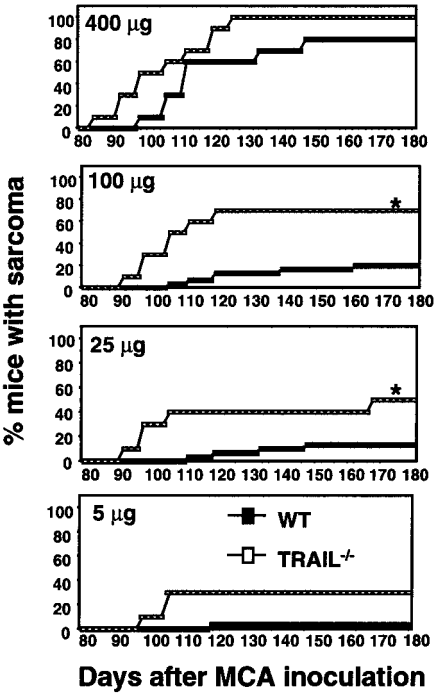

FIGURE 5. TRAIL suppresses MCA induction of fibrosarcomas. Groups of 10–30 B6 WT (■) or TRAIL$^{-/-}$ (□) mice were inoculated s.c. in the hind flank with 400, 100, 25, or 5 μg of MCA in 0.1 ml of corn oil. Development of sarcomas was monitored over the course of 80–160 days, and the percentage of mice with sarcoma was recorded. *, Statistically increased sarcoma incidence above that observed in WT (Fisher's exact test, $p < 0.05$).

physiological and pathological roles of TRAIL. The use of the neutralizing mAb will be particularly useful in peculiar mouse strains (e.g., NOD/Ltz and MRL/*gld*) of special interest in autoimmunity where back-crossing multiple generations may take several years. In contrast, the TRAIL$^{-/-}$ mice will be particularly useful for the long-term monitoring of spontaneous tumor development.

Little is known concerning immune control in the mammary gland, despite its being a common site for human neoplasia. Similarly, most studies have evaluated the antimetastatic activity of α-GalCer rather than its ability to control primary tumor growth. Our study has indicated that α-GalCer can stimulate NK cells and TRAIL to control primary tumor growth in the mammary gland. The 4T1 model has proven to be a very useful model for assessing spontaneous mammary tumor metastasis, and our data suggest that the liver is a particularly active site for the antimetastatic activity of α-GalCer mediated by TRAIL. Future experiments will now focus on the relative role of TRAIL, pfp, FasL, and IFN-γ in immune control of metastasis to other sites such as peripheral blood, lymph nodes, bone, and brain.

TRAIL is the first TNF superfamily member that has been demonstrated to participate in the host protection from tumor initiation in the MCA-induced sarcoma model. FasL was previously shown to be irrelevant (19). From other tumor models (14) we deduce that TRAIL is acting as a substantial part of the IFN-γ-dependent pathway of host protection from MCA-induced sarcoma. The susceptibility of TRAIL$^{-/-}$ mice to MCA-induced sarcoma was almost similar with that observed in IFN-$\gamma^{-/-}$ mice (28). It remains to be determined whether TRAIL also plays a substantial role in natural protection from primary tumor development induced by other oncogenic events. Our preliminary studies in p53 mutant mice using

the neutralizing anti-TRAIL mAb suggest that TRAIL may suppress the spontaneous development of sarcomas and lymphomas (our unpublished observation). Of particular interest will be the role of TRAIL in spontaneous tumors occurring in Her2/neu transgenic mice, where IFN-γ may control tumor development (29). Further studies are now under way to address these issues by using the TRAIL$^{-/-}$ mice and the neutralizing anti-TRAIL mAb.

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