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CUTTING EDGE

Cutting Edge: TRAIL Deficiency Accelerates Hematological Malignancies[1]

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TNF apoptosis-inducing ligand is attracting considerable interest as a potential extrinsic tumor suppressor mechanism, although previous reports have conveyed somewhat contrasting views regarding the likely importance of this pathway. In this study, we provide the first evaluation of spontaneous tumor formation over the life span of TRAIL-deficient mice. Interestingly, >25% of these mice do develop lymphoid malignancies after 500 days of life. TRAIL suppressed the initiation and development of both tumors of lymphoid and stromal origin in the context of the loss of at least one p53 allele. Specific examination of the role of TRAIL in* Her2/neu *oncogene-driven mammary epithelial cancer revealed no critical role for TRAIL despite the inherent TRAIL sensitivity of such mammary carcinomas. Overall, the data indicate an important function of TRAIL in controlling carcinogenesis, but suggest that further examination of this pathway in epithelial malignancies is warranted. The Journal of Immunology, **2005, 175: 5586–5590.**

TNF apoptosis-inducing ligand is a member of the TNF superfamily that induces apoptosis through engagement of the TRAIL-R2 (DR5) receptor in mice (1). TRAIL is expressed constitutively on a subset of liver NK cells (2) and may be induced on other leukocytes, such as NK cells, monocytes, dendritic cells, B cells, neutrophils, and T cells by IFN-$\alpha\beta$, IFN-γ, or signals via TLR (3–6). More recent studies have focused on the role of TRAIL in natural host suppression of tumors (7), including the antimetastatic function of NK cells in mice that is partially TRAIL dependent (2, 3). Further studies using TRAIL-deficient mice supported a role for TRAIL in host immune responses to experimental tumors (8–10). Continued interest in the role of TRAIL expressed by innate leukocytes such as NK cells and neutrophils has been fueled by the discovery that existing and promising new adjuvant therapies may mediate their antitumor activities in part via TRAIL (3, 5, 9). Furthermore, a clear role for TRAIL in the T cell-mediated immune defense against tumor was formally shown in various graft vs leukemia models in mice (11).

Although convincing, past studies were limited by only examining the role of TRAIL in host protection from transplanted experimental tumors. Thus far only three studies have addressed the role of the TRAIL-DR5 pathway in tumor immunosurveillance (9, 12, 13). Two studies using either neutralizing anti-TRAIL Ab (12) or TRAIL-deficient mice (9) have shown that TRAIL suppresses methylcholanthrene (MCA)[4]-induced sarcoma, a mouse tumor initiation model where multiple innate and adaptive immune cells have been implicated in control of tumor initiation. A substantial contribution of TRAIL to immune surveillance against spontaneous tumor development caused by p53 mutation was demonstrated using long-term administration of anti-TRAIL Ab (12). By contrast, a recent study using TRAIL-R (DR5)-deficient mice bred with $p53^{-/-}$ or adenomatous polyposis coli mutant mice did not indicate any significant role for the DR5 pathway in tumor control (13).

To compare these contrasting results further, we now present evidence from three distinct mouse strains that shed more light on the role of TRAIL in host protection from malignancy. Most surprisingly, loss of TRAIL itself is sufficient to lead to the development of spontaneous lymphoma.

Materials and Methods

Mice

Inbred BALB/c and C57BL/6 (B6) wild-type mice were purchased from the Walter and Eliza Hall Institute (Parkville, Australia). The Tnfsf10 (TRAIL) gene-targeted mice (9) were bred at the Peter MacCallum Cancer Centre (East Melbourne, Australia). C.129-Tnfsf10<tmlMjs> (MGI:2179709) (BALB/c $TRAIL^{-/-}$) were 10 generations backcrossed to BALB/c and B6.129-Tnfsf10<tm1Mjs> (B6 $TRAIL^{-/-}$) mice (10 generations backcrossed to B6). Inbred C.Cg-Tg (MMTV-Erbb2)1Pv (BALB/c Her2/neu) (backcrossed to BALB/c for >12 generations and provided by Dr. G. Forni, University of Torino, Torino, Italy) and B6.129S7-Trp53<tm1Brd> (B6 $p53^{+/-}$) (backcross $n > 20$) obtained from Dr. A. Harris (Walter and Eliza Hall Institute) were bred and maintained at the Peter MacCallum Cancer Centre. BALB/c TRAIL-deficient Her2/neu-transgenic mice (BALB/c $TRAIL^{-/-}$ Her2/neu)

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[4] Abbreviation used in this paper: MCA, methylcholanthrene.

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were generated by backcrossing BALB/c Her2/neu with BALB/c TRAIL$^{-/-}$ mice until BALB/c TRAIL$^{-/-}$ Her2/neu mice were generated. Male BALB/c TRAIL$^{-/-}$ Her2/neu mice were then bred with female BALB/c TRAIL$^{-/-}$ mice and offspring were screened for the Her2/neu transgene as described below. B6 TRAIL-deficient and B6 TRAIL-sufficient p53$^{+/-}$ mice (B6 TRAIL$^{-/-}$ p53$^{+/-}$, B6 TRAIL$^{+/+}$p53$^{+/-}$) were generated by B6 TRAIL$^{-/-}$p53$^{+/-}$ × B6 TRAIL$^{-/-}$ p53$^{-/-}$ and B6 TRAIL$^{+/+}$p53$^{+/+}$ × B6 TRAIL$^{+/+}$p53$^{-/-}$ mice, respectively. Genetic screening of tail DNA of mice was performed using the Puregene DNA Purification kit (Flowgen Bioscience) as per the instructions and as previously described (8, 14, 15). All experiments were performed in accordance with guidelines set out by the Peter MacCallum Animal Experimental Ethics Committee.

Tumor monitoring

All mice were routinely screened for viruses, parasites, and other microbes and tested negative over the entire course of the experiment. B6 TRAIL$^{+/+}$, B6 TRAIL$^{-/-}$, B6 TRAIL$^{+/+}$p53$^{+/-}$, and B6 TRAIL$^{-/-}$ p53$^{+/-}$ mice were monitored for health three times weekly as previously described (15). When sacrificed, the mouse age was recorded, a postmortem was performed, and tissues and tumor were stored in formaldehyde for H&E analysis, fresh-frozen or spleen single-cell suspensions were frozen as described elsewhere (16). The preparation and staining of sections for histology were conducted by the Microscopy Imaging and Research Core Facility, Peter MacCallum Cancer Centre. Mean age of death ± SEM was calculated and the probability of significance was determined using a nonparametric Mann-Whitney *U* test. In each group, one to two mice from each strain was excluded after dying of unknown causes (no detectable tumor). No mice became moribund from autoimmunity. The significance of proportions of tumors and, in particular, disseminated lymphomas was determined by a Fisher's exact test. The mammary glands of BALB/c TRAIL$^{+/+}$ Her2/neu mice and BALB/c TRAIL$^{-/-}$ Her2/neu mice were palpated two to three times per week. Mammary glands with growing masses >1 mm in diameter were considered tumors. Tumor multiplicity was calculated as the cumulative number of incidence of tumors/total number of mice and is shown as mean ± SEM. Whole-mount preparations of mammary glands and immunohistochemical analyses were performed as described previously (17). The average age of first tumor onset (one tumor >1 mm in diameter) was also recorded and the mean ± SEM was calculated and probability of significance determined using a Mann-Whitney rank sum *U* test. Mammary tumor cell lines were prepared from small mammary tumors excised from Her2/neu mice as follows. Briefly, excised tumors were cut into small pieces and digested with DNase I and collagenase over a 45-min period with agitation. Cells were washed several times and then incubated for 2–3 days in medium (10% CO_2 at 37°C). Tumor cells were passaged and split into large flasks when confluent and assessed by immunostaining as 100% Her2/neu-positive mammary tumors. These lines all grew as mammary tumors when transplanted into BALB/c mice.

Flow cytometry

Mammary carcinomas derived from mice were assessed for surface phenotype by multiparameter flow cytometric analysis. The following reagents used for flow cytometry were purchased from BD Pharmingen unless indicated otherwise: anti-TCR$\gamma\delta$-FITC (GL3), anti-TCR$\alpha\beta$-allophycocyanin (H57-597), anti-NK1.1-PE (PK136), anti-B220-allophycocyanin (RA3-6B2), anti-CD19-FITC (1D3), anti-CD11b-PE (M1/70), anti-IgM-biotin (R6-60.2), anti-H-2D^d-PE (34-5-8S), anti-H-2K^d-biotin (SF1–1.1); anti-CD1d-PE (1B1), anti-mouse DR5 (MD5-1), anti-pan Rae-1 (clone 186107, rat IgG2a isotype reacts with Rae-1α, β, γ, δ, and ϵ), anti-H60-biotin; and streptavidin-allophycocyanin Abs. Isotype controls included: mouse IgG2a, PK136, rat IgG2a, R35-95, hamster IgG (H57-97), and rat IgG1 (R3-34). Anti-FcR (2.4G2) was used to prevent nonspecific binding by mAb. Analysis was conducted on a LSR II using FACSDIVA software (BD Biociences).

TRAIL-mediated cytotoxicity

2PK3 parental and 2PK3-mTRAIL transfectants were used as effector cells to determine TRAIL sensitivity of Her2/neu mammary carcinomas by an 18-h ^{51}Cr release assay as described elsewhere (9).

Results and Discussion

TRAIL suppresses late-age lymphoma development

Spontaneous tumor development upon aging has now been assessed in a number of mice gene-targeted for various effector molecules and cytokines (15, 16, 18, 19). Given the potential role of TRAIL as an extrinsic tumor suppressor, we aged TRAIL-deficient mice on a B6 background and compared spontaneous tumor formation with B6 TRAIL$^{+/+}$ and B6 TRAIL$^{+/-}$ controls for up to 850 days (Fig. 1*A*). Ten of 31

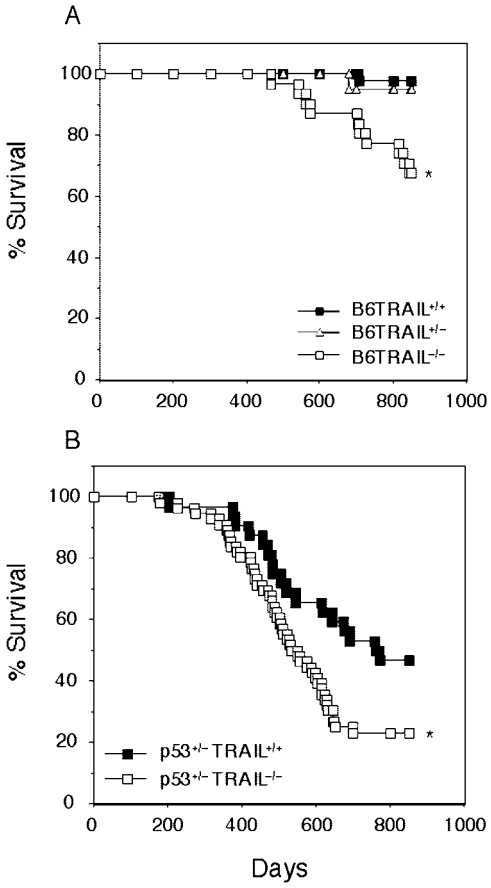

FIGURE 1. *A,* TRAIL protects mice from spontaneous disseminated tumors. Kaplan-Meier survival curve demonstrates groups of B6 TRAIL$^{+/+}$ (n = 41), B6 TRAIL$^{-/-}$ (n = 31), and B6 TRAIL$^{+/-}$ (F_1) (n = 20) mice that were evaluated twice weekly and, when moribund, a full autopsy was performed and tumor type recorded. The significance of enhanced spontaneous tumor formation in B6 TRAIL$^{-/-}$ mice was determined by Fisher's exact test (p = 0.0006). *B,* TRAIL suppresses spontaneous tumors in p53$^{+/-}$ mutant mice. Kaplan-Meier survival curve demonstrates groups of B6 TRAIL$^{+/+}$p53$^{+/-}$ (32 mice) and B6 TRAIL$^{-/-}$p53$^{+/-}$ (56 mice) mice that were evaluated twice weekly and, when moribund, a full autopsy was performed and tumor type recorded. The significance of enhanced spontaneous tumor formation in B6 TRAIL$^{-/-}$p53$^{+/-}$ mice was determined by Fisher's exact test (p = 0.0319).

aged B6 TRAIL$^{-/-}$ mice developed spontaneous lesions, including 8 lymphomas, 1 ovarian cystadenoma, and 1 lacrimal gland tumor. By comparison, only 1 of 41 B6 TRAIL$^{+/+}$ and 1 of 20 B6 TRAIL$^{+/-}$ mice developed disseminated lymphoma over the same period. Lymphomas were verified by histopathology of the primary and subsequent transplant and growth in B6 RAG-1$^{-/-}$ mice. Unfortunately only three lymphomas reproducibly passaged in mice to allow surface phenotyping. One B6 TRAIL$^{-/-}$ lymphoma recovered was a CD19$^+$IgM$^-$ B220$^-$ B cell lymphoma, and each lymphoma derived from B6 TRAIL$^{+/-}$ and B6 TRAIL$^{+/+}$ mice was a TCR$\alpha\beta^+$ T cell lymphoma. None of these lymphomas expressed detectable DR5 and it was not clear whether these tumors were truly clonal (data not shown).

TRAIL suppresses spontaneous tumors following loss of one p53 *allele*

A proportion (35–50%) of mice with a deficiency in one p53 allele typically develop a mixture of sarcomas and disseminated lymphomas (20). In concert with these previous reports, we

found that 17 (53.1%) of 32 of B6 TRAIL$^{+/+}$p53$^{+/-}$ mice developed one or more spontaneous tumors including sarcomas (5 of 17), disseminated lymphomas (9 of 17), with others including a mammary adenocarcinoma, a squamous cell carcinoma, and a hepatoma (Fig. 1*B*). The association between TRAIL deficiency and susceptibility to tumor was strongly reiterated in p53$^{+/-}$ mice with 43 (76.8%) of 56 B6 TRAIL$^{-/-}$ 53$^{+/-}$ mice developing spontaneous tumors including 15 of 43 sarcomas, 23 of 43 disseminated lymphomas, and 6 of 43 other mixed tumors, including adenocarcinoma, hepatoma, and squamous cell carcinoma. The enhanced spontaneous tumor formation in B6 TRAIL$^{-/-}$p53$^{+/-}$ mice was significant by Fisher's exact test ($p = 0.0141$); however, the mean day of survival of these groups (487 ± 19 days vs 529 ± 36 days) was not significantly different ($p = 0.35$). These survival data are very similar to those observed in a previous study comparing survival in B6 perforin$^{-/-}$p53$^{+/-}$ mice (479 ± 22 days) and B6 perforin$^{+/+}$p53$^{+/-}$ mice (569 ± 22 days) (15). Clearly lymphomas arose later and with lower penetrance in B6 TRAIL$^{-/-}$ mice compared with B6 perforin$^{-/-}$ or B6 IFN-$\gamma^{-/-}$ mice (16). We have obtained no evidence to suggest that T cells and NK cells from TRAIL$^{-/-}$ mice have reduced perforin or IFN-γ function (our unpublished data and Refs. 3 and 9).

TRAIL does not affect mammary epithelial carcinoma development

Since there is little evidence for immune surveillance of epithelial malignancies with nonviral etiologies, we sought to investigate this possibility by comparing mammary carcinogenesis in BALB/c Her2/neu mice and BALB/c TRAIL$^{-/-}$ Her2/neu mice (Fig. 2). TRAIL deficiency did not significantly affect the mean day of first onset of BALB/c Her2/neu mice with mammary carcinoma (BALB/c TRAIL$^{-/-}$ Her2/neu mice or BALB/c Her2/neu TRAIL$^{+/+}$ mice, 115.8 ± 2.4 days ($n = 20$) vs 115.7 ± 1.6 days ($n = 29$), respectively, $p = 0.6472$, Mann-Whitney *U* test; Fig. 2*A*). Once the mammary tumors appeared, there was no significant difference in tumor growth rate between each of the groups and day of sacrifice (mortality) was almost identical between BALB/c TRAIL$^{-/-}$ Her2/neu and BALB/c TRAIL$^{+/+}$ Her2/neu mice (data not shown). The mean tumor multiplicity (number of mammary glands involved) was also similar between BALB/c TRAIL$^{-/-}$ Her2/neu and BALB/c TRAIL$^{+/+}$ Her2/neu mice (Fig. 2*B*). Mammary whole mounts of groups of three to five mice, between 3 and 18 wk of age, were prepared to evaluate lesion progression from hyperplasia to neoplasia. There was no discernable difference in the onset or appearance of hyperplastic foci within BALB/c TRAIL$^{-/-}$ Her2/neu and BALB/c TRAIL$^{+/+}$ Her2/neu mice, nor was the progression from the hyperplastic lesions to carcinoma in situ and lobular carcinomas any different in BALB/c TRAIL$^{-/-}$ Her2/neu mice than in BALB/c TRAIL$^{+/+}$ Her2/neu mice (Fig. 2*C* and data not shown). Mammary tumors derived from BALB/c TRAIL$^{-/-}$ Her2/neu and BALB/c TRAIL$^{+/+}$ Her2/neu mice were examined for their expression

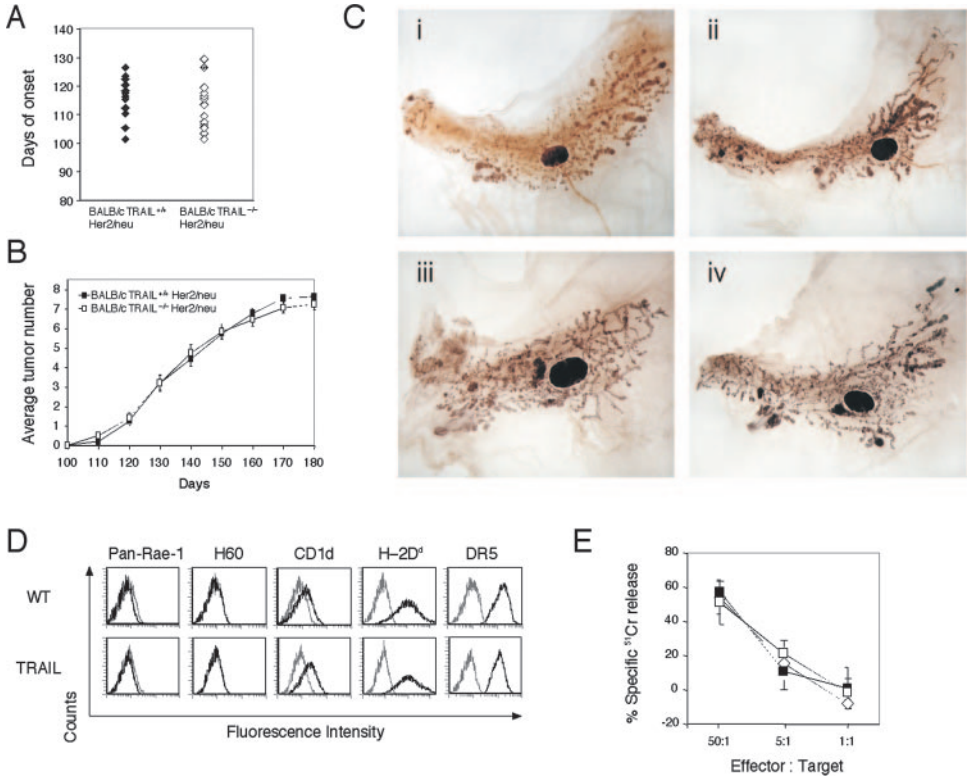

FIGURE 2. TRAIL does not delay the onset of mammary carcinogenesis. *A,* Mammary glands of groups of BALB/c TRAIL$^{+/+}$ Her2/neu ($n = 29$) and BALB/c TRAIL$^{-/-}$ Her2/neu mice ($n = 20$) were palpated two to three times per week and growing tumors >1 mm in diameter were recorded. *B,* Tumor multiplicity was calculated as the cumulative number of tumors/total number of mice and is shown as mean ± SEM. *C,* Whole mounts of mammary glands (three to five in each group) were prepared and stained with ferric hematoxylin from BALB/c TRAIL$^{+/+}$ Her2/neu (*i* and *iii*) and BALB/c TRAIL$^{-/-}$ Her2/neu (*ii* and *iv*) mice at 15 wk of age. Representative photographs of mammary glands (original magnification, ×200). *D,* Mammary carcinomas derived from BALB/c TRAIL$^{+/+}$ Her2/neu and BALB/c TRAIL$^{-/-}$ Her2/neu mice express MHC class I allele D^{d}, CD1d, and DR5, but lack expression of Rae-1 and H60. *E,* TRAIL sensitivity of mammary carcinomas derived from BALB/c TRAIL$^{+/+}$ Her2/neu (□) and BALB/c TRAIL$^{-/-}$ Her2/neu (■) mice in an 18-h ^{51}Cr release assay using TRAIL-2PK3 transfected as effector cells. 4T1 mammary carcinoma target cells are a TRAIL-sensitive control (◇). Data are representative of at least four tumors examined from each strain. WT, Wild type.

of immune recognition molecules and sensitivity to mouse TRAIL ex vivo. Mammary carcinoma cell lines derived from both BALB/c TRAIL$^{-/-}$ Her2/neu and BALB/c TRAIL$^{+/+}$ Her2/neu mice expressed MHC class I (H-2D^{d}), CD1d, and TRAIL receptor DR5, but lacked expression of NKG2D ligands (H60 and Rae-1) (Fig. 2*D*). Interestingly, all such tumors were found to be considerably TRAIL sensitive (Fig. 2*E*). A similar experiment examining BALB/c perforin$^{-/-}$ Her2/neu mice has revealed an earlier mammary tumor onset and greater tumor multiplicity in these mice compared with Her2/neu controls, highlighting that the Her2/neu tumors are subject to immunosurveillance (M. J. Smyth, N. Zerafa, and S. E. A. Street, manuscript in preparation). Collectively, these data suggested that despite the expression of at least some target molecules and intact sensitivity to TRAIL, mammary tumors arising in Her2/neu-transgenic mice were not suppressed by the natural host expression of TRAIL in any discernable way.

Conclusions

In this study, we have presented the first report of spontaneous tumor formation in TRAIL-deficient mice. Interestingly, these mice developed a modest frequency of disseminated lymphomas late in life without any direct induction of carcinogenesis. These data were in concert with the role of TRAIL as a tumor suppressor in mice mutant for one p53 allele, where TRAIL deficiency predisposed mice to a greater number of tumors, including disseminated lymphomas and sarcomas. Our data obtained in TRAIL$^{-/-}$p53$^{+/-}$ mice mimicked what was previously observed in TRAIL$^{+/+}$p53$^{+/-}$ mice that were chronically treated with a neutralizing anti-mouse TRAIL mAb (12). Sarcomas derived from TRAIL$^{-/-}$p53$^{+/-}$ mice were more sensitive to TRAIL ex vivo than those derived from TRAIL$^{+/+}$p53$^{+/-}$ mice (data not shown). These findings are consistent with the fact that soft tissue sarcoma formation following MCA administration has also been shown to be elevated in TRAIL-deficient mice and tumors derived from such mice were found to be more sensitive to TRAIL than those derived from control wild-type mice (9, 12). In the MCA model, it is known that NK cells are important effector cells in controlling tumor initiation (21), whereas the effector cells controlling tumor development in p53$^{+/-}$ mice have not yet been established. Nonetheless, in the absence of data concerning the phenotype and TRAIL sensitivity of lymphomas arising in TRAIL$^{-/-}$ mice, it is possible that multiple defects in these mice could indirectly lead to lymphoma generation. More recently, there was a report that TRAIL-R deficiency did not influence spontaneous tumor formation in p53$^{-/-}$ mice (13); however, complete p53 loss from birth may either effect the normal functioning of the TRAIL-TRAIL-R pathway or the expression of TRAIL by immune cells. The exact reason for the discrepancy between models requires further investigation. In addition, clearly the TRAIL-deficient phenotype is complex considering that, in the absence of CD4^{+} T cell helper function, CD8^{+} T cells may actually have a survival advantage in the TRAIL-deficient mouse (22).

Notably, our study of Her2/neu-induced mammary carcinoma development did not detect a role for TRAIL in tumor suppression, despite the fact that such mammary tumors expressed MHC class I and were decidedly TRAIL-sensitive. It is possible that either TRAIL-expressing effector cells never enter the tumor microenvironment or that endogenous host osteoprotegerin might block host TRAIL activity (23). The striking sensitivity of these and other mammary tumors to TRAIL (24) warrants further attention when designing effective therapies for this disease. Our findings aligned closely with another study that reported that TRAIL-R loss did not significantly affect epithelial tumor development in adenomatous polyposis coli mutant mice (13). Therefore, further assessment in models of epithelial tumor formation will be required to assess its role in controlling more common cancers.

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Disclosures

The authors have no financial conflict of interest.

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