

T cells require TRAIL for optimal graft-versus-tumor activity

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Published online 11 November 2002; doi:10.1038/nm797

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily that exhibits specific tumoricidal activity against a variety of tumors^{1,2}. It is expressed on different cells of the immune system and plays a role in natural killer cell-mediated tumor surveillance³⁻⁵. In allogeneic hematopoietic-cell transplantation, the reactivity of the donor T cell against malignant cells is essential for the graft-versus-tumor (GVT) effect⁶. Cytolytic activity of T cells is primarily mediated through the Fas-Fas ligand and perforin-granzyme pathways. However, T cells deficient for both Fas ligand and perforin can still exert GVT activity *in vivo* in mouse models^{7,8}. To uncover a potential role for TRAIL in donor T cell-mediated GVT activity, we compared donor T cells from TRAIL-deficient and wild-type mice in clinically relevant mouse bone-marrow transplantation models. We found that alloreactive T cells can express TRAIL, but the absence of TRAIL had no effect on their proliferative and cytokine response to alloantigens. TRAIL-deficient T cells showed significantly lower GVT activity than did TRAIL-expressing T cells, but no important differences in graft-versus-host disease, a major complication of allogeneic hematopoietic cell transplantation, were observed. These data suggest that strategies to enhance TRAIL-mediated GVT activity could decrease relapse rates of malignancies after hematopoietic cell transplantation without exacerbation of graft-versus-host disease.

Several studies in mice and humans have demonstrated that activated, but not resting, CD4⁺ and CD8⁺ T cells express TRAIL (refs. 5,9,10). We first used 2PK-3 cells transfected with mouse TRAIL to show that human TRAIL-R2 Fc-immunoglobulin (FcIg) fusion protein can be used to detect T-cell surface expression of mouse TRAIL by flow cytometry (Fig. 1a). Freshly isolated mouse splenic T cells did not express detectable levels of TRAIL on their surface even after stimulation with IFN- α , (Fig. 1b). In contrast, activated splenic T cells expressed TRAIL after IFN- α stimulation (Fig. 1c). Activated T cells from TRAIL^{-/-} mice did not express TRAIL even after IFN- α stimulation.

To assess whether alloreactive donor T cells, which are primarily responsible for graft-versus-host disease (GVHD) and graft-versus-tumor (GVT) activity in allogeneic hematopoietic cell transplantation (AHCT), express TRAIL *in vivo*, we isolated splenic T cells of donor origin from lethally irradiated recipients

treated with allogeneic T cell-depleted (TCD) bone marrow (TCD-BM) plus T cells. We detected TRAIL expression on these alloreactive T cells after IFN- α stimulation *in vitro* (Fig. 1d). In some experiments alloreactive T cells expressed low levels of TRAIL even before IFN- α stimulation. In contrast, alloreactive splenic T cells from recipients of allogeneic TCD-BM plus TRAIL-deficient T cells had no detectable TRAIL expression even after IFN- α stimulation.

We found that TRAIL-deficient T cells have an intact proliferative and cytokine response to mitogens and alloantigens *in vitro* and *in vivo*: TRAIL^{-/-} T cells showed intact *in vitro* proliferation in response to mitogen (Fig. 1e) and alloantigen (Fig. 1f), and normal *in vivo* alloreactive expansion (Fig. 1g), and the kinetics of early *in vivo* expansion as well as the expression of activation markers and were very similar to wild-type T cells (Fig. 1h). The production of IFN- γ , TNF, IL-4 and IL-10 by alloreactive CD4⁺ and CD8⁺ T cells (Fig. 1i and data not shown) was intact.

To assess the role of TRAIL in the development of GVHD, we used two parent \rightarrow F1 models and a major histocompatibility locus (MHC)-matched, minor histocompatibility antigen (mHAg)-mismatched model of AHCT. To exclude potentially confounding variables associated with TRAIL expression on non-T cells, we designed our allograft as a combination of wild-type TCD-BM with TRAIL-deficient T cells and compared these to allografts consisting of wild-type TCD-BM plus wild-type T cells. In the B6 \rightarrow C3FeB6F1 model, we did not find any significant differences in GVHD morbidity and mortality in two independent experiments between recipients of splenic T cells from wild-type B6, B6.pfp^{-/-} or B6.TRAIL^{-/-} donors (Fig. 2a), whereas recipients of B6.gld T cells developed less GVHD, as we have demonstrated previously¹¹. Similar experiments in the B6 \rightarrow B6D2F1 model demonstrated only a slight delay in mortality of recipients that had received TRAIL^{-/-} donor T cells, although all recipients did succumb to GVHD (Fig. 2b). To assess the contribution of the TRAIL pathway to GVHD in an MHC-matched model with mHAg disparity, we used a third GVHD model (B6 \rightarrow LP) and again found no differences in the development of lethal GVHD (Fig. 2c). Finally, we titrated lower doses of T cells in the B6D2F1 GVHD model to uncover a potential role for TRAIL when GVHD mortality is not rapid or uniform (Fig. 2d). We found no difference in GVHD mortality or morbidity (as mea-



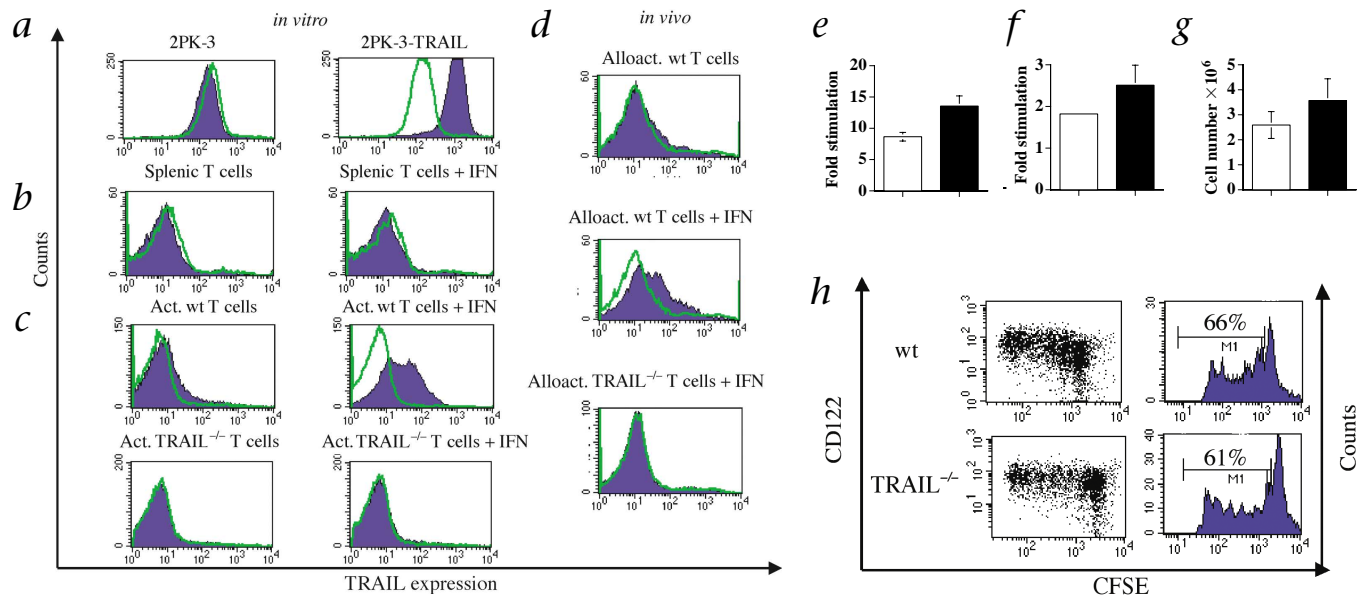
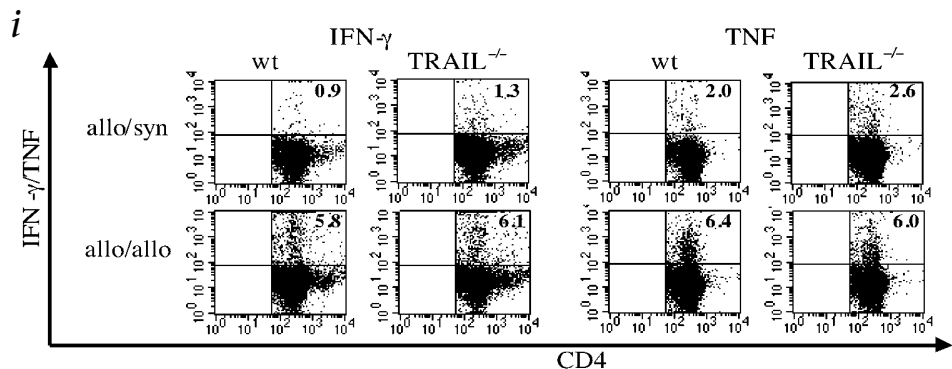


Fig. 1 TRAIL is expressed on the surface of activated and alloreactive murine T cells. Proliferative capacity and cytokine expression is intact in alloreactive TRAIL^{-/-} T cells. **a–d**, Flow-cytometric analysis after staining with TRAIL R2–FcIg fusion protein (purple filled curve) and human Ig isotype control (green unfilled curve) of 2PK-3 cells transfected with murine TRAIL or vector alone (**a**); normal murine splenocytes after 5 h incubation with or without IFN- α (**b**); activated (with antibody against CD3, antibody against CD28 and IL-2 for 7 d) T cells derived from wild-type (wt) or TRAIL^{-/-} splenocytes, after incubation with or without IFN- α (**c**); or allo-activated splenic donor T cells from C3FeB6F1 recipients of B6 TCD-BM plus T cells from wild-type or TRAIL^{-/-} B6 donors (**d**). Cells were collected on day 14 after AHCT and incubated *in vitro* with or without IFN- α before analysis. Representative experiments of at least 3 are shown. **e–g**, Splenocytes from wild-type (□) or TRAIL^{-/-} (■) B6 mice were incubated for 72 h in the presence of ConA (**e**) or for 120 h in the presence of irradiated fully MHC-mismatched BALB/c splenocytes (**f**). Proliferation was measured by [³H]thymidine incorporation. Donor T-cell number (**g**) was determined in spleens of BALB/c recipients on day 14 after fully MHC-mismatched AHCT (B6 BM cells plus B6 T cells of wild-type or TRAIL^{-/-} origin). Results for **e–g** represent average \pm s.d. for 4 mice per group; $P = \text{n.s.}$ **h**, C3FeB6F1 recipients received AHCT with B6 BM



cells plus CFSE-labeled purified B6 T cells (25×10^6) of wild-type or TRAIL^{-/-} background. Splenocytes were collected on day 3 and flow-cytometric analysis was performed. One representative experiment of 3 is shown. **i**, B6 splenocytes of wild-type or TRAIL^{-/-} background were incubated in the presence of irradiated allogeneic C3FeB6F1 TCD splenocytes as stimulators for 5 d, collected and re-incubated for 16 h with syngeneic or allogeneic irradiated TCD splenocytes in the presence of brefeldin A. Flow cytometric analysis for intracellular cytokines was performed on activated/memory CD4⁺ T cells (CD4⁺, CD44^{high}, CD62L^{low}). Percentage of cytokine-positive cells within this population is listed in the right upper quadrant. Data shown were derived from an MLR of combined splenocytes from 4 mice per group and represent 1 of 2 separate experiments with similar results.

sured by weight loss) between recipients of wild-type T cells versus TRAIL-deficient T cells. We therefore conclude from our data in three different GVHD models that TRAIL has no or only a minor role in the GVHD activity of donor T cells. This correlates well with the documented inability of TRAIL to induce apoptosis in most non-transformed cells^{2,12}.

To exclude the possibility that donor T cells require the TRAIL pathway to cause specific GVHD target-organ pathology, we analyzed intestine (small and large), liver and skin of recipients of allogeneic TCD-BM plus T cells at 14 and 28 days after AHCT. We found no significant differences between the GVHD target-organ damage of recipients of TRAIL^{-/-} T cells and recipients of wild-type T cells (Fig. 2e). The absence of a difference in liver

GVHD is noteworthy in the context of considerable controversy regarding the possible sensitivity of hepatocytes to soluble and membrane-bound TRAIL^{13–15}. Our observation is in agreement with findings by us and others, demonstrating the dominant role of the Fas/FasL pathway in liver GVHD (refs. 16,17).

Donor T cells have an important role in establishing full donor T-cell chimerism after AHCT¹⁸. We determined chimerism in C3FeB6F1 recipients of TRAIL^{-/-} donor T cells and found full donor chimerism in all cell populations tested, including T cells, B cells, monocytes/macrophages and red cells, in spleen and thymus at days 14 and 28 after AHCT (data not shown).

We tested in ⁵¹Cr-release assays the cytolytic activity of membrane-bound mouse TRAIL (stably expressed on 2PK-3-TRAIL

transfectants (Fig. 1a) against a panel of mouse tumor cells, including the two cell lines used in our GVT models (32Dp210 and P815). We found significant cytotoxicity after 18 hours of incubation against all tumor cells, whereas the untransfected 2PK-3 cells had no cytolytic activity (Fig. 3a).

We tested the role of TRAIL on donor T cells in a well-established mouse model of GVT after AHCT: B6 → C3FeB6F1 with 32Dp210 CML cells (Fig. 3b). Lethally irradiated C3FeB6F1 recipients received TCD-BM from wild-type B6 donors and 32Dp210 leukemic cells. Some recipients also received splenic T cells from B6 wild-type donors or B6.TRAIL^{-/-} donors at a relatively low dose (0.5×10^6) to decrease GVHD mortality in both groups. Recipients of TRAIL^{-/-} T cells had a highly significant decrease in survival ($P < 0.003$) and more recipients died of leukemia compared to recipients of wild-type T cells.

We confirmed these data in a second mouse model of GVHD and GVT after AHCT: B6 → B6D2F1 with P815 mastocytoma cells. Again, recipients of TRAIL^{-/-} T cells had a highly significant decrease in survival ($P < 0.001$) compared to recipients of wild-type T cells (Fig. 3c), and all mortality in this experiment was caused by tumor. We conclude from these data that TRAIL is required for optimal GVT activity by donor T cells after AHCT in these mouse models.

Recent studies have indicated that TRAIL is centrally involved in the innate immune surveillance by NK cells against tumors^{3,4,19}, but it has not been demonstrated that TRAIL has a role in the T cell-mediated immune defense against cancer. We have previously shown in the C3FeB6F1 model by selective depletion that GVT activity depends on CD8⁺ T cells and not CD4⁺ T cells or NK cells¹¹. Also, we found that in both models (C3FeB6F1 and B6D2F1), optimal GVT activity depended on an intact perforin pathway, whereas FasL was not required. To exclude NK cells as mediators of GVT activity and to further define the relative contribution of perforin and TRAIL to GVT, we stringently depleted NK cells *in vitro* before transplantation (final concentration of NK cells in the T-cell graft $\leq 0.1\%$) and used wild-type, perforin-deficient and TRAIL-deficient T cells at a slightly higher T-cell dose (1×10^6 versus 0.5×10^6) and a slightly lower tumor dose (1,000 versus 5,000 P815 cells) than in the previous experiment (Fig. 3c). B6D2F1 recipients of wild-type T cells showed complete protection against leukemia and only 25% mortality from GVHD (Fig. 3d). Recipients of NK-depleted perforin-deficient or TRAIL-deficient T cells both had 100% mortality, and we determined at autopsy that 80% of both groups had evidence of extensive leukemia. Recipients of NK-depleted perforin-deficient T cells died significantly sooner than recipients of NK-depleted

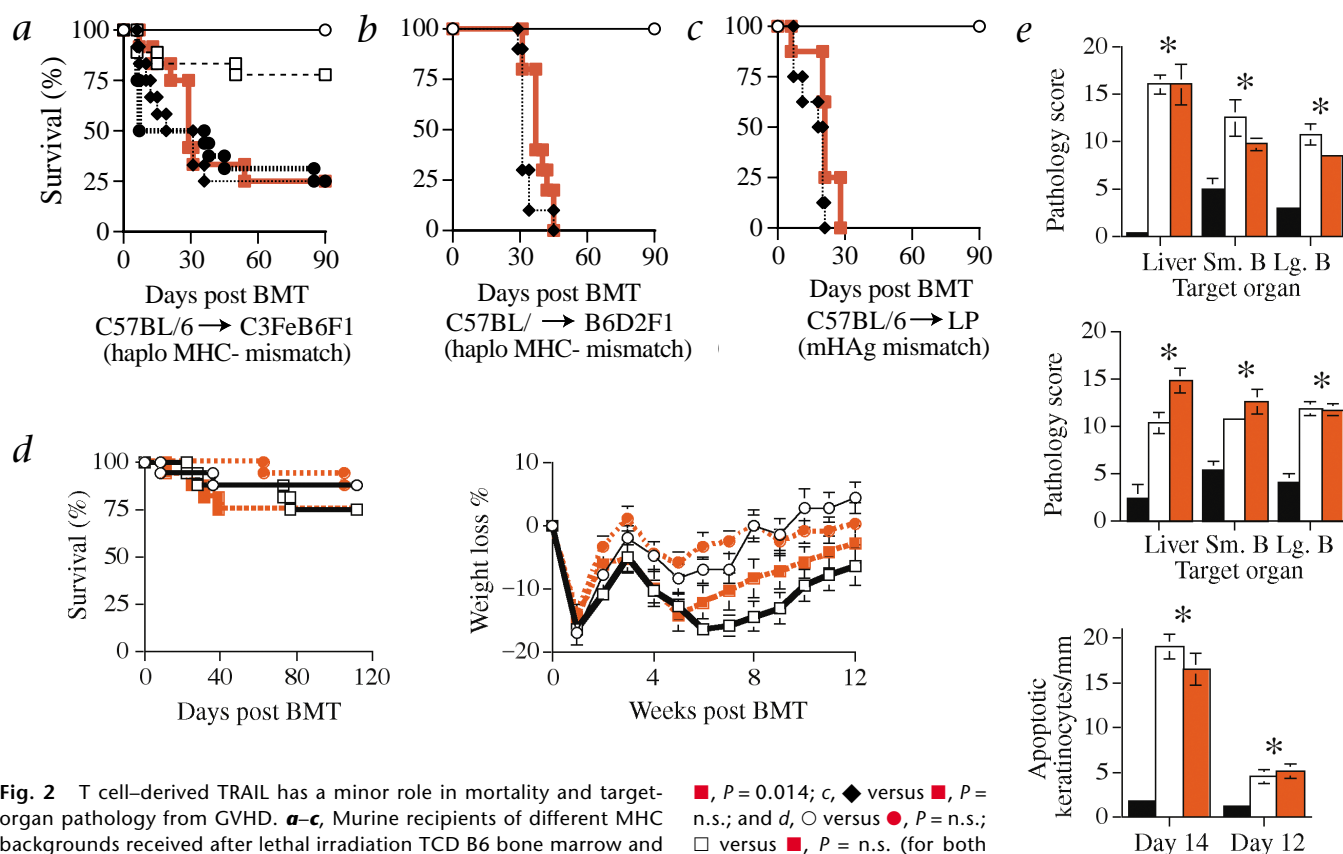


Fig. 2 T cell-derived TRAIL has a minor role in mortality and target-organ pathology from GVHD. **a–c**, Murine recipients of different MHC backgrounds received after lethal irradiation TCD B6 bone marrow and splenic T cells (**a** and **b**, 2×10^6 ; **c**, 5×10^6) from wild-type or TRAIL^{-/-} (or *pfp*^{-/-} or FasL-defective *gld*) B6 donors. Shown are survival curves from 4 (BM only control groups) and 8–18 (T-cell recipients) animals per group from combined experiments. ○, B6 BM; ◆, B6 BM + B6 T; □, B6 BM + *gld* T; ●, B6 BM + *pfp*^{-/-} T; ■, B6 BM + TRAIL^{-/-} T. **d**, Survival and weight loss (\pm s.e.m.) curves are shown for 16 B6D2F1 recipients per group from 2 combined experiments carried out with lower doses of donor T cells. ○, B6 BM + 0.5×10^6 T; □, B6 BM + 1×10^6 T; ●, B6 BM + 0.5×10^6 TRAIL^{-/-} T; ■, B6 BM + 1×10^6 TRAIL^{-/-} T. Statistical analyses: **a**, ◆ versus ■, $P = n.s.$; □ versus ■, $P < 0.01$; ○ versus □, $P = n.s.$; **b**, ◆ versus

■, $P = 0.014$; **c**, ◆ versus ■, $P = n.s.$; and **d**, ○ versus ●, $P = n.s.$; □ versus ■, $P = n.s.$ (for both survival and weight loss).

e, GVHD target organs (skin, liver, small bowel (Sm. B) and large bowel (Lg. B.)) from C3FeB6F1 recipients of AHCT as described above were collected on days 14 and 28. ■, B6 BM; □, B6 BM + B6 T; ●, B6 BM + TRAIL^{-/-} T. H&E-stained slides of liver and intestines were scored for established organ-specific parameters in a blinded fashion. Skin GVHD was assessed by counting apoptotic keratinocytes (as determined by morphology and TUNEL staining) per millimeter of epidermis. Data shown represent average (\pm s.e.m.) for 4–12 mice per group. Statistical analysis: * □ versus ■, $P = n.s.$

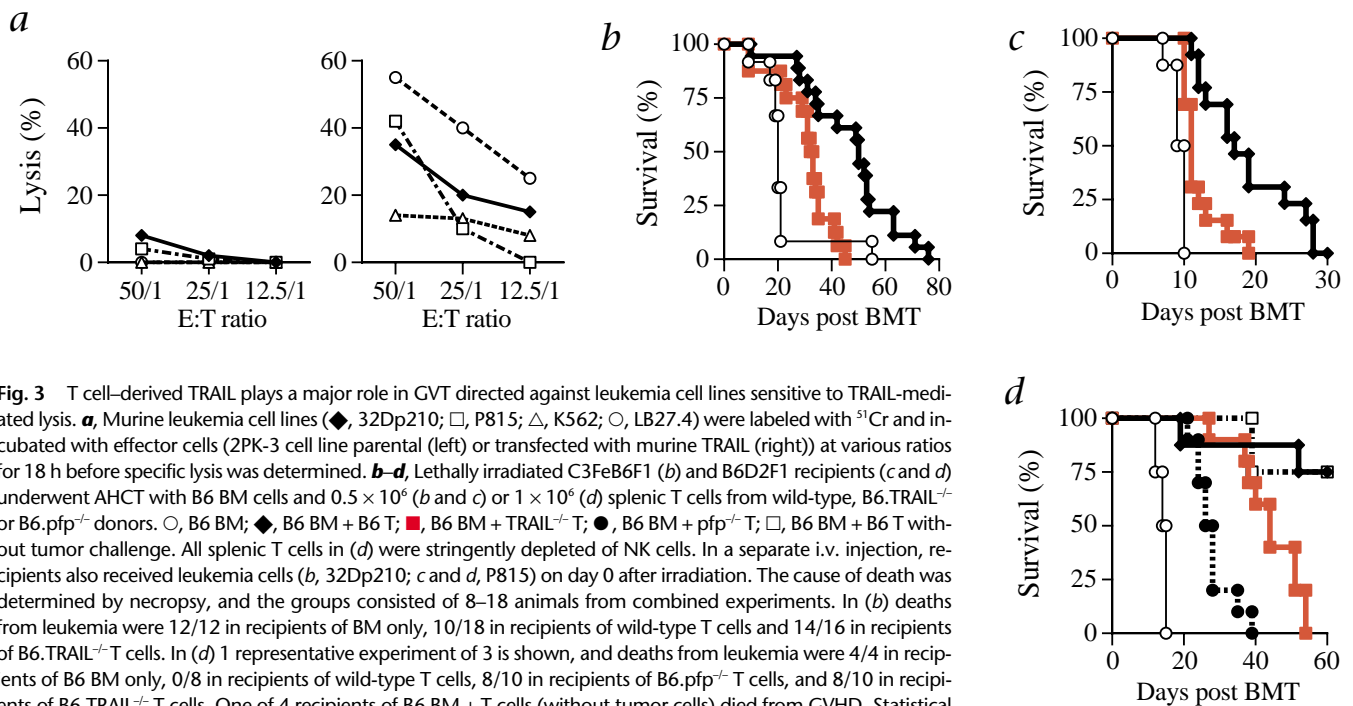


Fig. 3 T cell–derived TRAIL plays a major role in GVT directed against leukemia cell lines sensitive to TRAIL-mediated lysis. **a**, Murine leukemia cell lines (◆, 32Dp210; □, P815; △, K562; ○, LB27.4) were labeled with ⁵¹Cr and incubated with effector cells (2PK-3 cell line parental (left) or transfected with murine TRAIL (right)) at various ratios for 18 h before specific lysis was determined. **b–d**, Lethally irradiated C3FeB6F1 (**b**) and B6D2F1 recipients (**c** and **d**) underwent AHCT with B6 BM cells and 0.5×10^6 (**b** and **c**) or 1×10^6 (**d**) splenic T cells from wild-type, B6.TRAIL^{-/-} or B6.pfp^{-/-} donors. ○, B6 BM; ◆, B6 BM + B6 T; ■, B6 BM + TRAIL^{-/-} T; ●, B6 BM + pfp^{-/-} T; □, B6 BM + B6 T without tumor challenge. All splenic T cells in (**d**) were stringently depleted of NK cells. In a separate i.v. injection, recipients also received leukemia cells (**b**, 32Dp210; **c** and **d**, P815) on day 0 after irradiation. The cause of death was determined by necropsy, and the groups consisted of 8–18 animals from combined experiments. In (**b**) deaths from leukemia were 12/12 in recipients of BM only, 10/18 in recipients of wild-type T cells and 14/16 in recipients of B6.TRAIL^{-/-} T cells. In (**d**) 1 representative experiment of 3 is shown, and deaths from leukemia were 4/4 in recipients of B6 BM only, 0/8 in recipients of wild-type T cells, 8/10 in recipients of B6.pfp^{-/-} T cells, and 8/10 in recipients of B6.TRAIL^{-/-} T cells. One of 4 recipients of B6 BM + T cells (without tumor cells) died from GVHD. Statistical analysis: (**b**) ◆ versus ■, $P = 0.003$; (**c**) ◆ versus ■, $P < 0.001$; (**d**) ◆ versus ■, $P = 0.01$; ● versus ■, $P < 0.002$.

TRAIL-deficient T cells. We conclude the following in the B6D2F1 GVHD/GVT model from these results: (i) NK-depleted T cells mediate potent GVT activity; (ii) both perforin and TRAIL are required for optimal GVT activity by T cells; and (iii) the perforin pathway has a greater relative contribution to GVT activity than the TRAIL pathway.

In conclusion, our data are the first to establish a significant role for TRAIL in T-cell-mediated tumor immunity *in vivo* in general, and in GVT activity after AHCT in particular. The requirement for TRAIL to achieve optimal GVT activity suggests that strategies aimed at increasing TRAIL expression and/or activity of donor T cells could enhance the GVT effect of AHCT.

Methods

TRAIL-deficient mice. TRAIL^{-/-} mice²⁰ were backcrossed for 5–10 generations in a C57BL/6 background. To confirm that TRAIL^{-/-} N5 mice (which were used for most of our experiments) were congenic with C57BL/6 mice for transplantation purposes, we performed 3 AHCTs with very high doses (10^7) of wild-type T cells into TRAIL^{-/-} N5 recipients and vice versa, which resulted in 0% mortality and only transient morbidity equal to or less than that of the control group of syngeneic T-cell recipients. The key experiments relating to GVT activity (Fig. 3) were repeated with N8 and N10 mice and resulted in very similar survival curves.

Cell lines, mice, AHCT and CFSE labeling. 32Dp210 (H-2^b), a myeloid leukemia cell line of C3H/He mouse origin, transfected with the p210 *bcr/abl* oncogene²¹, was provided by J. Griffin; P815 (H-2^d), a mastocytoma of DBA/2 mouse origin, LB27.4, a murine B cell hybridoma, and K562, a human erythroleukemia, were obtained from the ATCC (Manassas, Virginia).

Female C57BL/6 (B6, H-2^b), B6.Smn.C3H-Fas^{bl/d}, C57BL/6-Pfp^{tm15sz} (B6.pfp^{-/-}, H-2^b), C3FeB6F1 (H-2^{b/h}), LP (H-2^b) and B6D2F1 (H-2^{b/d}) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Mice were used for experiments at between 7 and 12 wk of age. All experiments with mice were performed in accordance with the guidelines of the American Association for the Advancement of Laboratory Animal Care and were approved by the Institutional Animal Care and Use Committee of the Memorial Sloan-Kettering Cancer Center.

AHCT was performed as described¹¹. Briefly, BM cells were removed from femurs and tibias of killed donor mice, and then depleted of T cells with antibody against Thy-1.2 and complement lysis. T cells were obtained by nylon-wool passage (NWP) of splenocytes, and in some cases depleted of NK cells by negative magnetic cell separation (Miltenyi, Auburn, California) as indicated (final NK-cell concentration in T-cell graft $\leq 0.1\%$). 5×10^6 BM cells and indicated doses of T cells per animal were infused into the tail vein of recipients.

For CFSE labeling, NWP T cells were further purified by depletion of macrophages, B cells, NK cells and granulocytes using magnetic-cell separation (Miltenyi, Auburn, California) and labeled with 2.5 μ M CFSE at 37 °C for 15 min before injection.

Leukemia induction and assessment of leukemic death versus death from GVHD. Mice received 5×10^3 (Fig. 3c) or 1×10^3 (Fig. 3a) P815 cells or 2×10^4 32Dp210 cells intravenously in a separate injection on day 0 of AHCT after radiation. Survival was monitored daily and the cause of each death after AHCT (leukemia versus GVHD) was determined by necropsy and histopathology as previously described¹¹. Briefly, all animals with (i) hind leg paralysis (P815), (ii) macroscopic liver or spleen metastases at autopsy (P815), or (iii) hepatosplenomegaly with spleen weight >300 mg (32Dp210 leukemia) (all criteria previously established as highly specific for leukemic infiltrations) were recorded as undergoing leukemic death. Spleen and liver tissue from all dead animals without these macroscopic signs was submitted for histopathological examination to a veterinary pathologist (H. Nguyen). In a few cases, mice without any macroscopic signs of leukemia were found on microscopic examination to have leukemic infiltration and deaths were subsequently recorded as leukemic. Death from GVHD was defined as the absence of macroscopic and microscopic evidence of leukemia and correlated with presence of GVHD symptoms as assessed by a clinical GVHD scoring system²².

Flow-cytometric analysis (including intracellular cytokine staining). Flow-cytometric analysis of splenocytes was performed with fluorochrome-labeled antibodies (all antibodies were purchased from Pharmingen, San Diego, California) or TRAIL-R2-FcIg fusion protein (initially provided by H.W., later purchased from Alexis, San Diego, California) as described¹¹ and analyzed on a FACSCalibur (Becton Dickinson, San Jose, California) with CellQuest software. TRAIL-R2-FcIg was visualized with secondary biotinylated goat antibody against human and tertiary streptavidin-phycoerythrin. In some cases, cells were incubated for 5 h at 37 °C with IFN- α (200 U/ml;

R & D Systems, Minneapolis, Minnesota) in some cases preceded by incubation for 7 d with antibody against CD3 (1 µg/ml; 145.2C11), antibody against CD28 (1 µg/ml; 37.51), and human recombinant IL-2 (100 IU/ml; Chiron, Emeryville, California). For intracellular cytokine staining, cells (stimulated in MLR as described below) were incubated for 16 h with brefeldin A (10 µg/ml) and then stained, fixed and permeabilized with the Cytofix/Cytoperm Kit (Pharmingen) according to the manufacturer's instruction.

⁵¹Cr-release and proliferation assays. These assays were performed as described previously¹¹. MLR for intracellular cytokine stimulation was performed in 24-well plates with a responder:stimulator (C3FeB6F1, H-2^{b/k}) ratio of 1:2 for 5 d. Before cytokine staining on day 6, cells were collected and restimulated for 12–15 h in the presence of brefeldin A (10 µg/ml) with TCD, irradiated (2,000 cGy) splenocytes of allogeneic (C3FeB6F1, H-2^{b/k}) or syngeneic (C57BL/6), H-2^b) origin.

Histopathological analysis. GVHD target-organ pathology for bowel and liver was assessed by J.M.C. and for skin by G.F.M. in a blinded fashion on H&E-stained tissue sections with a validated semiquantitative scoring system as described before^{23,24}.

Statistics. All values are expressed as mean ± s.e.m. The results of the comparison of survival curves were produced using a permutation procedure based on the rank-sum and log-rank statistics. A *P* < 0.05 was considered statistically significant.

Acknowledgments

We thank the staff of the Research Animal Resource Center for animal care; M.-A. Perales for assistance with intracellular cytokine staining; H. Nguyen for expertise with the histopathologic examination of tissue specimen; G. Heller for statistical help; and R.J. O'Reilly and A. Houghton for helpful discussions and support throughout the project. This work was supported by grants HL69929 and HL72412 from the National Institutes of Health (to M.R.M.v.d.B.). C.S. is a Special Fellow of the Leukemia and Lymphoma Society. M.R.M.v.d.B. is the recipient of a Damon Runyan Scholar Award of the Cancer Research Fund and a research award from the V scholar program of the V Foundation.

Competing interests statement

The authors declare competing financial interests: see the website (<http://www.nature.com/naturemedicine>) for details.

RECEIVED 29 JULY; ACCEPTED 8 OCTOBER 2002

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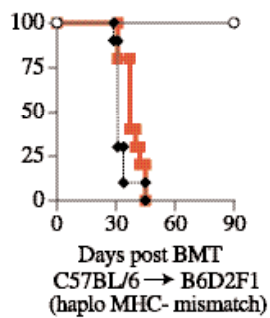
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T cells require TRAIL for optimal graft-versus-tumor activity

C. Schmaltz, O. Alpdogan, B.J. Kappel, S.J. Muriglan, J.A. Rotolo, J. Ongchin, L.M. Willis, A.S. Greenberg, J.M. Eng, J.M. Crawford, G.F. Murphy, H. Yagita, H. Walczak, J.J. Peschon & M.R.M. van den Brink

Nature Med. 8, 1433–1437 (2002); Published online 11 November 2002; corrected 15 November 2002 (details online); doi:10.1038/nm797

In the version of this article originally published online, Figure 2b contained an error: C57BL/ should be C57BL/6. This has been corrected in the HTML and PDF versions, and will appear correctly in the forthcoming print issue. The correct figure is below.



We regret this error.