

Brief report

Absence of donor T-cell–derived soluble TNF decreases graft-versus-host disease without impairing graft-versus-tumor activity

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Tumor necrosis factor (TNF) plays an important role in graft-versus-host disease (GVHD) and graft-versus-tumor (GVT) activity after allogeneic bone marrow transplantation (allo-BMT). TNF can be expressed in a membrane-bound form (memTNF) and as a soluble (solTNF) molecule after being cleaved by the TNF- α converting enzyme (TACE). To study the contribution of donor T-cell–derived

memTNF versus solTNF in GVHD and GVT, we used mice containing a noncleavable allele in place of endogenous TNF (memTNF $\Delta\Delta$) as donors in murine BMT models. Recipients of memTNF T cells developed significantly less GVHD than recipients of wild-type (wt) T cells. In contrast, GVT activity mediated by memTNF T cells remained intact, and alloreactive memTNF T cells showed no

defects in proliferation, activation, and cytotoxicity. These data suggest that suppressing the secretion of solTNF by donor T cells significantly decreases GVHD without impairing GVT activity. (Blood. 2007;110:783-786)

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Introduction

Tumor necrosis factor (TNF) is a type I cytokine involved in many pathophysiological processes; it plays a critical role in the regulation of cell trafficking, inflammation, maintenance of lymphoid organ structure, and host defense against various pathogens.¹ TNF can be expressed as a transmembrane molecule (memTNF) on the surface of activated monocytes, macrophages, lymphocytes, and natural killer (NK) cells² and, when cleaved by the TNF- α converting enzyme (TACE), is secreted by the cell in its soluble form (solTNF).^{3,4} Its important role in the development of graft-versus-host disease (GVHD) and graft-versus-tumor (GVT) activity has previously been demonstrated in murine bone marrow transplantation (BMT) models using anti-TNF antibodies,⁵⁻⁸ TNF-deficient donors,⁹ and TNF receptor–deficient recipients.¹⁰ In our previous study, we showed that donor T-cell–derived TNF is required in the development of GVHD, especially in the thymus, small bowel, and large bowel target organs, as well as in GVT activity.⁹ To distinguish between the roles of memTNF and solTNF in contributing to GVHD and GVT, we used memTNF $\Delta\Delta$ mice as the source of donor T cells in our BMT experiments. memTNF $\Delta\Delta$ mice have a combined deletion of wild-type (wt) TNF and a “knocked-in” allele of a mutated TNF resistant to TACE-mediated cleavage.¹¹

line of C57BL/6 origin, were obtained from the ATCC (Manassas, VA). All the fluorochrome-labeled antimurine antibodies were obtained from Pharmingen (San Diego, CA). CFSE labeling of T cells and flow cytometric analysis were performed as previously described¹² using a LSRII cytometer (Becton Dickinson, San Jose, CA) and FlowJo (Treestar, Ashland, OR) for data analysis.

GVHD/GVT experiments

Female C57BL/6 (B6) (H-2^b), BALB/c (H-2^d), C3FeB6F1 (H-2^{b/k}), and B6D2F1 (H-2^{b/d}) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). MemTNF $\Delta\Delta$ mice were obtained from the DNAX Research Institute (Palo Alto, CA). The BMT procedure was performed as previously described.¹² Recipients were monitored for survival, weight loss, and clinical GVHD as previously described.¹² BMT protocols were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee. BMT recipients were killed for blinded histopathologic and flow cytometric analysis of GVHD target organs as previously described.¹²

Cytometric bead array

Serum concentrations of TNF and IFN- γ were determined by cytometric bead array (CBA) and analyzed with BD CBA Software (Becton Dickinson) according to the manufacturer’s protocol.

Cytotoxicity assay

T cells were stimulated in vitro for 5 days with irradiated BALB/c splenocytes and cytotoxicity against ⁵¹Cr-labeled target cells was determined as previously described.¹²

Materials and methods

Cell lines, antibodies, and flow cytometry

P815 (H-2^d), a mastocytoma cell line of DBA/2 origin, A20 (H-2^d), a lymphoma cell line of BALB/c origin, and EL4 (H-2^b), a lymphoma cell

Submitted October 27, 2006; accepted March 19, 2007. Prepublished online as *Blood* First Edition paper, March 29, 2007; DOI 10.1182/blood-2006-10-054510.

The online version of this article contains a data supplement.

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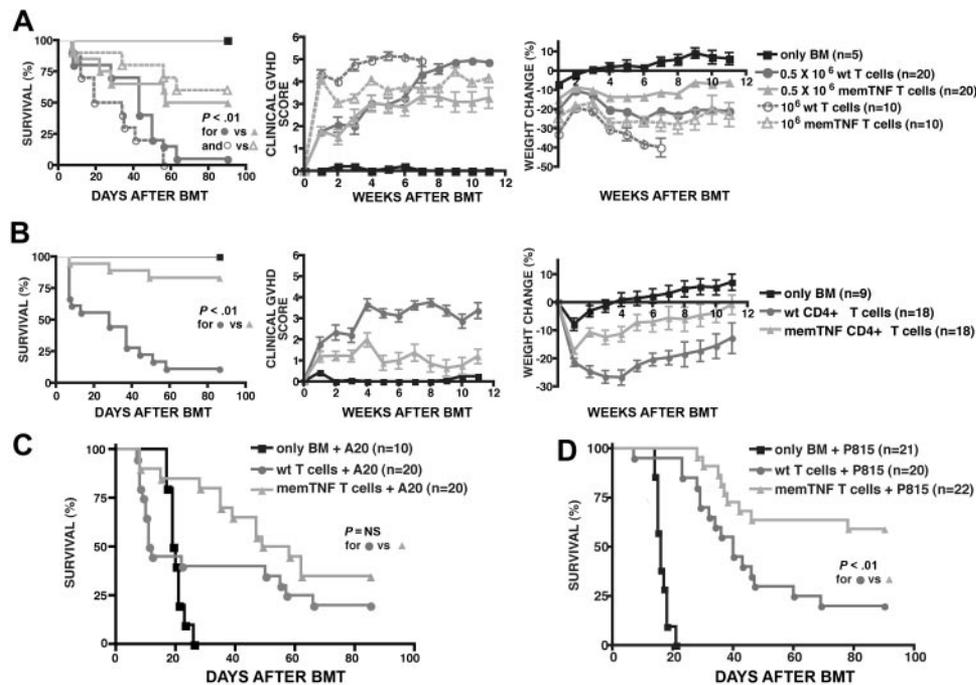


Figure 1. memTNF T cells induce less GVHD mortality and morbidity than wt T cells and have intact GVT activity. (A) Lethally irradiated (850 cGy) BALB/c mice received 5×10^6 TCD wt B6 BM cells alone ($n = 5$) or in combination with 0.5×10^6 ($n = 20$) or 1×10^6 ($n = 10$) wt or memTNF B6 T cells. Data represent 2 combined experiments. (B) Lethally irradiated (1300 cGy) C3FeB6F1 mice received 5×10^6 TCD wt B6 BM cells alone ($n = 9$) or in combination with 1×10^6 purified CD4⁺ T cells from wt or memTNF B6 mice ($n = 18$). Data represent 2 combined experiments. (C) Lethally irradiated (850 cGy) BALB/c mice received 5×10^6 TCD wt B6 BM cells and 1×10^6 A20 lymphoma cells ($n = 10$) with or without the addition of 0.5×10^6 B6 wt or memTNF B6 T cells ($n = 20$). Survival was monitored daily and cause of death (tumor versus GVHD) was determined by necropsy and histopathology. Data represent 2 combined experiments. (D) Lethally irradiated (1300 cGy) B6D2F1 mice received 5×10^6 TCD wt B6 BM cells and 1×10^5 P815 mastocytoma cells ($n = 21$) with or without the addition of 0.5×10^6 B6 wt ($n = 20$) or memTNF B6 T cells ($n = 22$). Survival was monitored daily and cause of death (tumor versus GVHD) was determined by necropsy and histopathology. Data represent 3 combined experiments.

Statistics

All values shown in graphs represent the mean \pm SEM. Survival data were analyzed with the Mantel-Cox log-rank test. For all other analysis, nonparametric unpaired Mann-Whitney *U* test was used.

Results and discussion

To assess the contribution of donor T-cell–derived memTNF in the development of GVHD, we used a major histocompatibility complex (MHC)–mismatched murine BMT model in which BALB/c hosts received a transplant of 5×10^6 T-cell–depleted (TCD) wt B6 bone marrow (BM) cells and 0.5 or 1×10^6 T cells from wt or memTNF B6 mice. Recipients of memTNF T cells showed significantly improved survival and less morbidity compared with recipients of both doses of wt T cells (Figure 1A). The morbidity difference became evident at 5 weeks after BMT with a dose of 0.5×10^6 T cell, suggesting a late effect of memTNF donor T cells. We observed no difference in GVHD between wt or memTNF groups in a murine parent-into-F1 model (B6 \rightarrow C3FeB6F1) (data not shown). However, when we repeated the transplantation using 1×10^6 purified CD4⁺ donor T cells, we found again that recipients of memTNF CD4⁺ T cells showed less GVHD mortality and morbidity than recipients of wt CD4⁺ T cells (Figure 1B). These data are in agreement with previous studies regarding the role of TNF (in particular donor T-cell–derived TNF) in GVHD.^{5,7-9} Our data from 2 GVHD models suggest that solTNF released by donor T cells (especially CD4⁺ T cells) is important for the development of GVHD.

To determine whether the presence of memTNF on T cells alone is sufficient to mediate GVT, we used a well-characterized GVHD/GVT model: B6 \rightarrow BALB/c with A20 lymphoma cells. We observed improved survival in recipients of memTNF T cells compared with recipients of TCD-BM, indicating that memTNF T cells were capable to exert GVT activity. Moreover, overall survival and deaths from lymphoma did not differ between recipients of memTNF versus wt T cells, suggesting

that GVT activity was comparable (Figure 1C, Table 1). Interestingly, in a second GVHD/GVT model (B6 \rightarrow B6D2F1 with P815 mastocytoma cells), the mice receiving memTNF T cells showed significantly improved survival over the mice receiving wt T cells (Figure 1D, Table 1). Analysis of necropsy samples showed only tumor-related deaths with no GVHD-associated mortality (table in Figure 1D, Table 1). Our previous study demonstrated that TNF from donor T cells is required for GVT activity.⁹ We conclude from the current experiments that donor T-cell solTNF is dispensable for GVT activity.

We then performed histopathological analysis of different GVHD organs and found that recipients of memTNF T cells developed significantly less damage to the liver, small bowel, and large bowel (Figure 2A). The role of TNF in intestinal^{5,13,14} and hepatic¹⁵ GVHD has previously been shown, and our data suggest that solTNF secreted by donor T cells is involved in intestinal and hepatic GVHD.

No difference in thymic GVHD (as defined by thymic cellularity and decrease in CD4⁺CD8⁺) was observed (Figure 2B) in contrast from the decrease in thymic GVHD that we observed using TNF^{-/-} mice as the source of donor T cells.⁹ This suggests that the presence of memTNF alone may be sufficient to cause thymic GVHD.

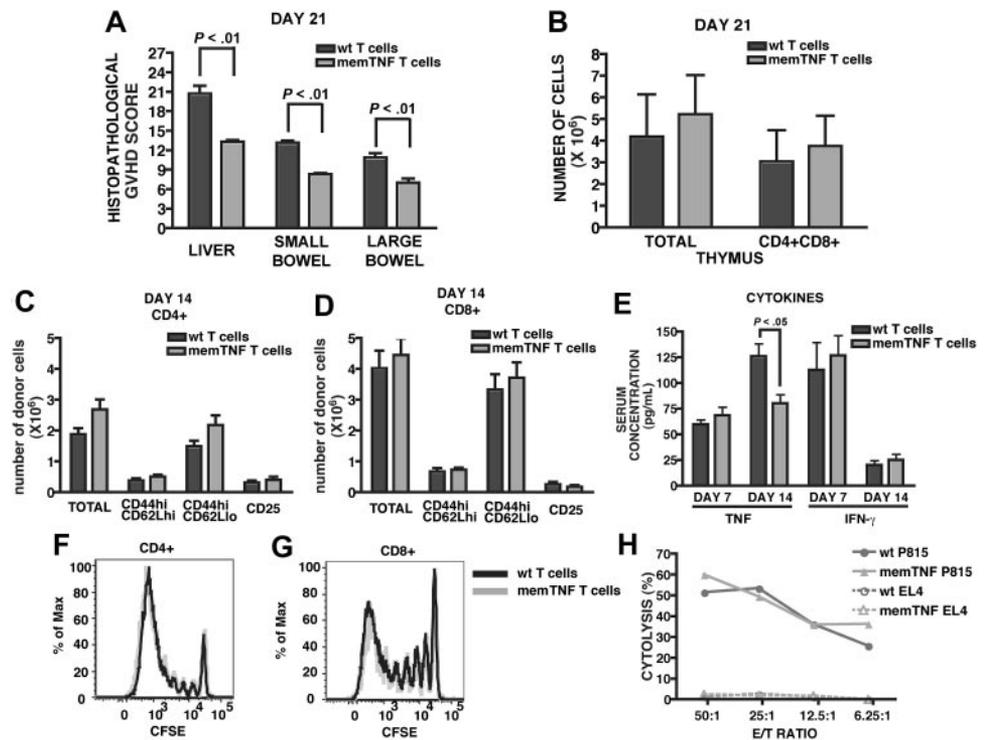
To assess whether memTNF T cells differ in their capacity to undergo alloactivation, we analyzed splenic T cells from

Table 1. Cause of death for mice after lethal irradiation

| Group | Tumor | GVHD | N/A |
|-----------------------|-------|-------|------|
| BALB-C | | | |
| only BM + A20 | 10/10 | 0/10 | 0/10 |
| wt T cells + A20 | 4/20 | 11/20 | 1/20 |
| memTNF T cells + A20 | 3/20 | 5/20 | 5/20 |
| B6D2F1 | | | |
| only BM + P815 | 21/21 | 0/21 | |
| wt T cells + P815 | 16/20 | 0/20 | |
| memTNF T cells + P815 | 9/22 | 0/22 | |

Descriptions as in Figure 1C,D.

Figure 2. Alloreactive memTNF T cells cause less liver and gut damage than wt T cells and have intact proliferation, activation, and cytolytic activity. (A-E) Lethally irradiated (850 cGy) BALB/c mice received 5×10^6 TCD wt B6 BM cells in combination with 1×10^6 wt (black bar) or memTNF (gray bar) B6 T cells. (A) Liver, small bowel, and large bowel were harvested on day 21. Hematoxylin and eosin-stained slides were analyzed and scored for their histopathological damage. Shown is the mean \pm SEM for 7 mice per group. (B) Thymic cellularity (total cell counts and CD4⁺CD8⁺ thymocytes) was determined by flow cytometric analysis on day 21. Shown is the mean (\pm SEM) for 9 mice per group. (C-D) Recipient spleens were harvested at day 14 for flow cytometric analysis of CD44, CD62L, and CD25 expression on CD4⁺ and CD8⁺ T cells. Shown is the mean (\pm SEM) for 10 mice per group. (E) Serum cytokine levels were measured by CBA at days 7 and 14. Shown is the mean (\pm SEM) for 10 mice per group. (F-G) Lethally irradiated (850 cGy) BALB/c mice received 20×10^6 CFSE-labeled wt or memTNF B6 T cells. Recipient spleens were harvested after 72 hours for flow cytometric analysis. Data shown are from 1 representative mouse of 4 mice from 2 experiments. Histogram overlays for CFSE-labeled wt (black line) and memTNF (gray line) are shown for donor CD4⁺ (F) and CD8⁺ (G) T cells. (H) In vitro-activated splenocytes were used as effectors in a ⁵¹Cr cytotoxicity assay. Targets were allogeneic P815 and third-party EL4.



recipients of wt versus memTNF T cells 14 and 21 days after BMT. We observed no difference in the number of splenic donor CD4⁺ and CD8⁺ cells and in the number of their subsets (Figure 2C,D; Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Analysis of serum samples showed lower levels of TNF in the recipients of memTNF T cells than in recipients of wt T cells (Figure 2E) on day 14 (but not on day 7), which confirms the late effect exerted by memTNF donor T cells shown in the morbidity graphs (Figure 1A). Host-derived TNF from irradiation-damaged gut may be exerting a confounding effect on differences in serum TNF levels early after transplantation, whereas the difference observed on day 14 indicates that TNF secretion by donor T cells contributes to the serum TNF levels during GVHD in addition to TNF secretion by monocytes and macrophages.¹⁷ No difference was found in the IFN- γ serum levels between the 2 groups. To further assess alloactivation of memTNF T cells, we infused CFSE-labeled wt or memTNF B6 splenic T cells in BALB/c hosts and harvested spleens 72 hours after adoptive transfer. We found that the proliferation kinetics of CD4⁺ and CD8⁺ T cells were similar between wt and memTNF T cells (Figure 2F-G). Finally, cytotoxic activity of alloreactive memTNF T cells was intact, as measured in a ⁵¹Cr release assay of in vitro-activated T cells against the P815 cell line (Figure 2H). Taken together, these results rule out any intrinsic defects of alloreactive memTNF T cells in activation, cytokine production (except TNF), proliferation, and cytotoxic activity.

In conclusion, our results show that eliminating donor T-cell-derived soluble TNF (especially from CD4⁺ origin) causes a significant delayed decrease in GVHD morbidity and mortality after BMT without impairing the proliferation and activation of donor T cells. At the same time, the presence of membrane-bound TNF alone is sufficient to mediate GVT activity. Therefore, targeting the cleavage of membrane-bound TNF with a TACE inhibitor could be assessed as a new

therapeutic strategy for attenuating gastrointestinal and hepatic GVHD without impacting GVT after allo-BMT.^{18,19}

Acknowledgments

This work was supported by grants HL69929, CA33049, and CA107096 from the U.S. National Institutes of Health, by awards from the Leukemia and Lymphoma Society, the Ryan Gibson Foundation, the Emerald Foundation, and The Experimental Therapeutics Center of Memorial Sloan-Kettering Cancer Center funded by Mr. William H. Goodwin and Mrs. Alice Goodwin and the Commonwealth Foundation for Cancer Research (M.R.M.v.d.B.), P20-CA103694 from the National Institutes of Health (O.A.), and Deutsche Krebshilfe (T.D.K.). C.B. is the recipient of a grant from the Università degli Studi di Milano and from the Andrea and Libi Lorini Foundation.

The authors would like to thank the staff of the Research Animal Resource Center for animal care and Dr LaPerle for the histopathologic analysis.

Authorship

Contribution: C.B. designed and performed research, analyzed data, and wrote the paper; S.X.L. and O.M.S. performed research and wrote the paper; A.R.K.F., T.D.K., D.S., C.G.K., and A.C. performed research and analyzed data; C.L. analyzed data; O.A. designed and performed research; and M.R.M.v.d.B. designed research and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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2007 110: 783-786
doi:10.1182/blood-2006-10-054510 originally published
online March 29, 2007

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