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ORIGINAL ARTICLE

Thrombomodulin alleviates murine GVHD in association with an increase in the proportion of regulatory T cells in the spleen

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Recombinant human soluble thrombomodulin (rTM), a potent anticoagulant, has been used for the treatment of disseminated intravascular coagulation in Japan since 2008. Interestingly, rTM possesses anti-inflammatory and cytoprotective functions. This study examined whether rTM alleviates GVHD in a murine hematopoietic SCT (HSCT) model. Use of rTM significantly improved the survival of mice on day 28 of transplantation (survival rate 70% in rTM - treated mice vs 35% in control, $P < 0.05$) in association with a significant decrease in plasma levels of IL-6, IFN-γ and high-mobility group B1 DNA-binding protein on day 7 of HSCT. Intriguingly, the proportion of regulatory T cells in the spleen was significantly increased in rTM-treated mice on day 7 of transplantation compared with control diluent-treated mice. In addition, elevated plasma levels of TM and fibrin/fibrinogen degradation product were noted in HSCT-recipient mice, suggesting coagulopathy caused by endothelial cell damage in this GVHD model. The use of rTM potently decreased these levels. Importantly, rTM did not hamper the anti-GVL and engraftment of hematopoietic cells. Taken together, the use of rTM may prevent GVHD and serve as a potential therapeutic strategy to improve clinical outcomes in individuals who receive HSCT.

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INTRODUCTION

Recombinant human soluble thrombomodulin (rTM) comprises the active, extracellular domain of thrombomodulin, and inactivates coagulation by binding to thrombin.[1] In addition, the thrombin–rTM complex activates protein C to produce activated protein C (APC), which inactivates factors VIIIa and Va in the presence of protein S, thereby inhibiting further thrombin formation.[2] The use of rTM for the treatment of disseminated intravascular coagulation (DIC) was approved in Japan in 2008.[3] Interestingly, the lectin-like domain of rTM possesses anti-inflammatory activity through both protein C-dependent and -independent mechanisms.[4,5] For example, the lectin-like domain of thrombomodulin (TM) binds to and inactivates high-mobility group box 1 protein (HMGB-1), a nuclear architectural chromatin-binding protein that stimulates the production of inflammatory cytokines such as IL-6 and TNFα via TOLL-like receptor 4 and the receptor for advanced glycation end products after being released from necrotic cells or secreted from inflammatory cells such as activated macrophages.[4] In addition, rTM possesses cytoprotective effects against inflammatory cytokines or calcineurin inhibitors that cause endothelial cell damage via upregulation of myeloid cell leukemia sequence 1 (Mcl-1) proteins, which are mediated by extracellular signal-regulated kinase (ERK) signal transduction pathways. This cytoprotective effect is mediated by the E45 repeats of the epidermal growth factor (EGF)-like domain and is independent of APC.[6] Use of rTM successfully rescues individuals with DIC complicated by potentially fatal HSCT-associated complications such as sinusoidal obstruction syndrome (SOS), transplantation-associated microangiopathy (TAM) and engraftment syndrome (ES).[7–9] We have recently performed the retrospective cohort study that showed that the use of rTM significantly decreases the mortality rate at day 100 of HSCT and improves the OS of transplant patients who developed DIC within 28 days of HSCT compared with those who did not receive rTM.[10] We thus expected that the use of rTM would decrease the incidence of acute GVHD (aGVHD) owing to its anti-inflammatory activity. Contrary to our expectation, a decrease in the incidence of either aGVD or chronic GVHD (cGVHD) was not noted. One possible explanation of this result is that the initiation and duration of rTM treatment varied between each case. Recent studies suggested that vascular endothelial vulnerability and endothelial dysfunction are involved in the pathogenesis of GVHD.[11–13] Elevated pretransplant serum levels of angiopoietin-2, a hormone mediating vascular vulnerability, and nitrate are noted in steroid-refractory GVHD patients.[11,12] A marker of endothelial damage including soluble TM is elevated in patients with steroid-refractory GVHD.[13] These observations encouraged us to hypothesize that the prophylactic use of rTM with cytoprotective as well as anti-inflammatory activity would decrease the incidence of GVHD in transplant patients. In the present study, we examined the prophylactic use of rTM on cytokine production, coagulopathy and survival in a murine GVHD model.

MATERIALS AND METHODS

Induction of GVHD and treatment with rTM

Six-week-old female C57BL/6 (B6, H-2b) and (C57BL/6 X DBA/2) F1 (BDF1, H-2b/d) mice were purchased from Japan SLC (Shizuoka, Japan) and maintained in our animal facilities. For induction of lethal GVHD, recipient mice were exposed to 300 cGy of TBI from an X-ray source at a dose rate of 50 cGy/min, after which the BM cells (5×10^6) plus spleen cells (2×10^7) from B6 donors were injected via the tail veins. rTM was provided by Asahi Kasei Pharma (Tokyo, Japan). Either rTM (1 mg/kg in 100 μL RPMI-1640) or control diluent (100 μL RPMI-1640) was given to mice by i.p. injection five times a week for 4 weeks. The dose of rTM was decided according to

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previous studies.[14] The mortality of mice was evaluated until day 28 after HSCT. The experiments were repeated twice independently.

Engraftment assay

Immunodeficient NOD.Cg-Rag1tm1Mom Il2rgtm1Wjl/SzJ mice[15] (Stock number: 007799) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and bred in a pathogen-free environment. Six-week-old mice were injected with $CD34^+$ hematopoietic stem/progenitor cells (5×10^4) isolated from peripheral blood (PB) of a healthy donor after mobilization with G-CSF via the tail vein and assigned into two groups. Mice were treated with either rTM (1 mg/kg) or control diluent (RPMI-1640) five times a week for 6 weeks. Engraftment was examined by quantifying the population of human $CD45^+$ cells in PB every week up to 6 weeks. At the end of the experiments, mice were euthanized and the BM and spleen were removed and subjected to FACS to quantify the proportion of human $CD45^+/CD19^+$ and $CD45^+/CD33^+$ cells. Human CD45 PE-Cy5-conjugated mAb (Dako, Glostrup, Denmark), human CD19 PE-conjugated mAb (Biolegend, San Diego, CA, USA) and human CD33 PE-conjugated mAb (Becton Dickinson Biosciences, San Jose, CA, USA) were used. Animal experiments were approved by the ethical committee of Kochi University.

Measurement of cytokines, TM, HMGB-1 and fibrinogen/fibrin degradation product

Murine plasma levels of IL-6, IL-10, IFN-γ, TM, HMGB-1 and fibrinogen/fibrin degradation product (FDP) were measured by murine-specific ELISA kits. IL-6, IL-10 and IFN-γ ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). FDP and TM ELISA kits were purchased from USCN Life Science Inc. (Wuhan, China). HMGB1 ELISA kit was purchased from Shino test Corporation (Kanagawa, Japan). Blood samples from mice were obtained retro-orbitally using heparinized tubes and collected in plasma separation tubes, and the centrifuged (3000 *g*, 10 min) supernatants were subjected to ELISA as previously described.[16]

Measurement of regulatory T cells

Spleens were removed from the mice on day 7 after HSCT and subjected to flow cytometry to quantify the proportion of $CD4^+$, $CD25^+$ and $Foxp3^+$ regulatory T (Treg) cells. Anti-CD4 FITC-conjugated, -CD25 PE-conjugated and -Foxp3 PE-Cy5-conjugated antibodies and the respective isotype control antibodies were purchased from eBioscience (San Diego, CA, USA). The mean fluorescence intensity of Foxp3 was defined as the geometric mean of fluorescence within the gated area[17] and represents the average levels of expression of Foxp3 in $CD4^+/CD25^+$ cells.

RNA isolation and RT-PCR

A volume of 100 μL of PB was withdrawn from mice by retro-orbital blood collection. 1×10^4 mononuclear cells were obtained after lysing erythrocytes. RNA was extracted from these cells using CellAmp Direct RNA Prep Kit for RT-PCR (TaKaRa, Tokyo, Japan). Real-time PCR was performed with a One Step SYBR PrimeScript RT-PCR Kit (TaKaRa). Primers used are listed in Table 1.

Table 1. RT-PCR primers

Gene	*Direction*	*Primer*
IL-6	Forward	5′-CCGGAGAGGAGACTTCACAG-3′
	Reverse	5′-TCCACGATTTCCCAGAGAAC-3′
IFN-γ	Forward	5′-TCATCCAAGTGATGGCTGAA-3′
	Reverse	5′-CTTCGACCTCGAAACAGCAT-3′
IL-10	Forward	5′-TGAATTCCCTGGGTGAGAAG-3′
	Reverse	5′-TCACTCTTCACCTGCTCCACT-3′
Mcl-1	Forward	5′-GGGGCAGGATTGTGACTCT-3′
	Reverse	5′-GTCCCGTTTCGTCCTTACAA-3′
GAPDH	Forward	5′-GGTGCTGAGTATGTCGTGGA-3′
	Reverse	5′-GTGGTTCACACCCATCACAA-3′

Abbreviations: Mcl-1 = myeloid cell leukemia sequence 1 proteins; RT = reverse transcription PCR.

Mixed lymphocyte reaction

PBMCs (4×10^6 cells /mL) isolated from healthy mice were plated in flat-bottom 96-well culture plates and cultured with irradiated (300 cGy) allogeneic PBMCs (4×10^6cells/mL) in a final volume of 200 μL. After 72 h of incubation either with or without various concentrations of rTM at 37 °C and 5% CO_2, cultures were pulsed with 1 μCi $[^3H]$-thymidine per well and harvested 16 h later. Triplicate cultures were set up for every cell population tested.

Cell sorting

Lymphocytes were isolated from the PB of healthy mice by Ficoll-Hypaque centrifugation and stained with anti-CD4 PE-conjugated, -CD8 FITC-conjugated and -CD25 FITC-conjugated antibodies (eBioscience). $CD4^-/CD8^+$, $CD4^+/CD25^-$ and $CD4^+/CD25^+$ lymphocytes were isolated by sorting using JSAN (Bay Bioscience Co., Ltd, Kobe, Japan) and subjected to $[^3H]$-thymidine uptake assay.

Functional assay of $CD4^+CD25$ cells

Cultures were set up in triplicates in 96-well round-bottom plates in a total volume of 200 μL, as previously described.[18] In brief, cells were cultured in RPMI - 1640 medium with 10% FCS. Fixed numbers of C57BL/6 $CD4^+CD25^-$ T cells (responder cells) and irradiated allogeneic BDF-1-stimulator cells were mixed with variable numbers of C57BL/6 $CD4^+CD25^+$ T cells that were pretreated with either rTM (100 ng/mL, 48 h) or control diluent to obtain the ratios indicated in the figures. Proliferation was assessed after 5 days by pulsing the cells with 1 μCi-well $[^3H]$-thymidine (Amersham Biosciences, Waltham, MA, USA) for the last 16 h. Cells were harvested onto filter membranes using a Wallac harvester (PerkinElmer, Waltham, MA, USA) and the amount of incorporated $[^3H]$-thymidine was measured with a Wallac Betaplate counter (PerkinElmer).

Generation of donor cytotoxic T cells

Lymphocytes were isolated from the PB of healthy mice by Ficoll–Hypaque centrifugation and were cultured with either control diluent or rTM (100 ng/mL) for 7 days. Irradiated lymphocytes from different healthy volunteers were added on day 1 of the culture to provide responder: stimulator (R:S) ratios that ranged from 5:1 to 20:1 in the presence of recombinant IL-2 (Chiron, Emeryville, CA, USA) at a final concentration of 50 ng/mL.

Cytotoxicity assays

Cytotoxicity was evaluated using the JAM assay.[19] In brief, $[^3H]$-thymidine-labeled target cells (acute monocytic leukemia THP-1 cells, 1.2×10^4 per well) were cocultured with effector cells at the ratios described in 96-well plates. After 4 h at 37 °C, plates were harvested and radioactivity was determined in a β-counter. Cytotoxicity is presented as % cytotoxicity = 100 × (counts in control well − counts in test well)/(total counts per well).

Statistical analysis

The statistical significance of differences between the two groups was assessed by Student's *t*-test. For multiple comparisons, one-way Analysis of variance followed by Dunnett's test was employed.

RESULTS

rTM inhibits the development of GVHD in association with the downregulation of inflammatory cytokines

BDF-1- mice that received B6 BM and spleen cells showed typical manifestations of aGVHD such as hunched posture, lowered mobility, hair ruffling, ascites and diarrhea (data not shown). Thirteen out of 20 mice died of aGVHD by day 28 of HSCT (Figure 1). On the other hand, i.p. administration of rTM five times a week for 4 weeks significantly reduced the mortality of BDF-1-recipients; only 6 out of 20 mice died by day 28 of HSCT ($P = 0.023$, Figure 1a). Clinical signs of GVHD including lowered mobility, hunched posture and diarrhea were mitigated in BDF-1- recipients who received rTM (data not shown).

We next compared the expression levels of cytokines in PBMCs isolated from BDF-1- recipient mice before and after HSCT. mRNA

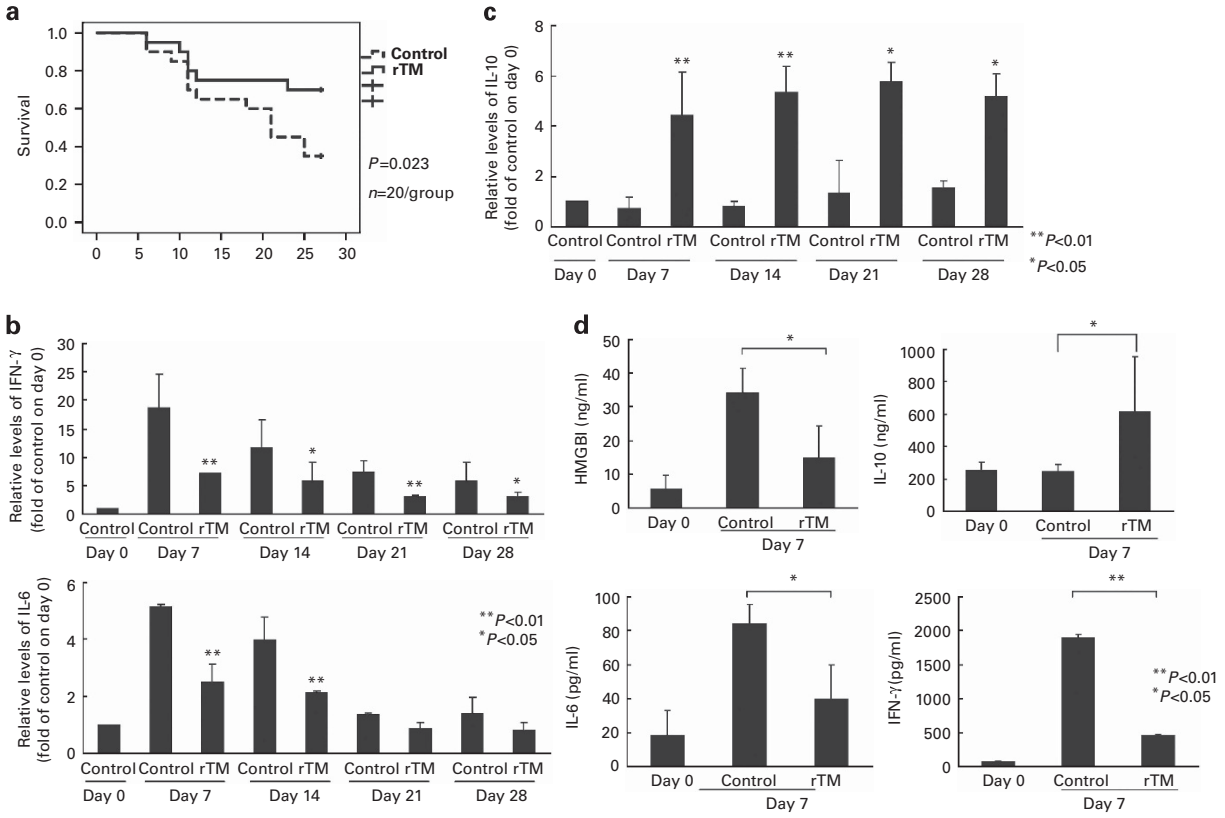

Figure 1. rTM alleviates GVHD. (**a**) Kaplan–Meier curves for the survival of HSCT-recipient mice. BDF-1-recipient mice received BM cells (5×10^6) plus spleen cells (2×10^7) from C57BL/6 donors by tail vein injection following 300 cGy of TBI. Either rTM (1 mg/kg in 100 μL RPMI-1640) or control diluent (100 μL RPMI-1640) was given to mice by i.p. injection five times a week for 4 weeks. The statistical significance of the differences between the two groups was assessed by the log-rank test. rTM decreases plasma levels of inflammatory cytokines and HMGB1 in HSCT-recipient mice. (**b**, **c**) RT-PCR. Peripheral blood was withdrawn from mice ($n = 10$) at the indicated time points after HSCT. RNAs were extracted from mononuclear cells and subjected to real-time RT-PCR to quantify the expression levels of the indicated genes. Expression of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was measured as an internal control. The statistical significance of differences between the two groups was assessed by Student's *t*-test. (**d**) ELISA. Plasma was isolated from mice ($n = 10$) at the indicated time points after HSCT and subjected to ELISA to measure the indicated proteins. Results represent the means ± s.d. of duplicate experiments. The statistical significance of the differences between the two groups was assessed by Student's *t*-test.

levels of IL-6 and IFN-γ increased by ~ 5- and 17-fold, respectively, in PBMCs isolated from BD-1-recipient mice on day 7 of HSCT compared with those in PBMCs isolated before HSCT (Figure 1b). The levels of these cytokines decreased gradually by day 28 of HSCT. Interestingly, the use of rTM potently decreased the expression of these cytokines at all time points (Figure 1b). On the other hand, there was no increase in the levels of the immunosuppressive cytokine IL-10 in PBMCs isolated from control diluent-treated mice after HSCT. However, strikingly elevated levels of IL-10 were noted in PBMCs isolated from rTM-treated mice on day 7 of HCST and afterward (Figure 1c). We next measured the production of cytokine proteins in HSCT-recipient mice by ELISA. Recipients of B6 BM and spleen cells had exaggerated production of IL-6 and IFN-γ on day 7 of HSCT. The use of rTM significantly decreased the production of these cytokines (Figure 1d). Plasma levels of HMGB-1 were also increased on day 7 in HSCT-recipient mice, and the use of rTM decreased these levels by approximately half (Figure 1d). On the other hand, plasma levels of IL-10 were significantly elevated on day 7 of HSCT in rTM-treated mice compared with those in control diluent-treated mice (Figure 1d).

rTM alleviates coagulopathy

Plasma levels of both TM and FDP were elevated in HSCT recipients on day 7 of HSCT, suggesting endothelial cell insult and resulting coagulopathy caused by aGVHD. Intriguingly, the use of rTM significantly lowered the plasma levels of TM and FDP in HSCT-recipient mice (Figure 2).

rTM inhibits alloreactive proliferation of PBMCs but stimulates proliferation of $CD4^+/CD25^+$ T cells *in vitro*

To verify the mechanisms by which rTM alleviated aGVHD in the murine model, we examined the effects of rTM on alloreactive PBMCs isolated from healthy volunteers by utilizing thymidine uptake assay. As expected, rTM potently inhibited allo-reactive proliferation of PBMCs in a dose-dependent manner (Figure 3a). Surprisingly, non-alloreactive resting PBMCs were forced to proliferate in the presence of rTM (300–1000 ng/mL) (Figure 3a).

We next examined the effects of rTM on the proliferation of dif ferent subsets of T cells. $CD4^+/CD25^+$ T cells that contain Treg cells, $CD4^+/CD25^-$ T cells and $CD4^-/CD8^+$ cytotoxic T cells were freshly isolated from healthy volunteers ($n = 3$) by sorting and were cultured in the presence of various concentrations of rTM. Exposure of $CD4^+/CD25^+$ T cells to rTM (1000 ng/mL) for 48 h stimulated their proliferation by ~ 1.8-fold compared with control diluent-treated samples of this subset of T cells (Figure 3b). rTM also significantly simulated the proliferation of $CD4^+/CD25^-$ T cells and $CD4^-/CD8^+$ cytotoxic T cells, although the effect of rTM on these subsets of T cells was less potent than on $CD4^+/CD25^+$ T cells (Figure 3b).

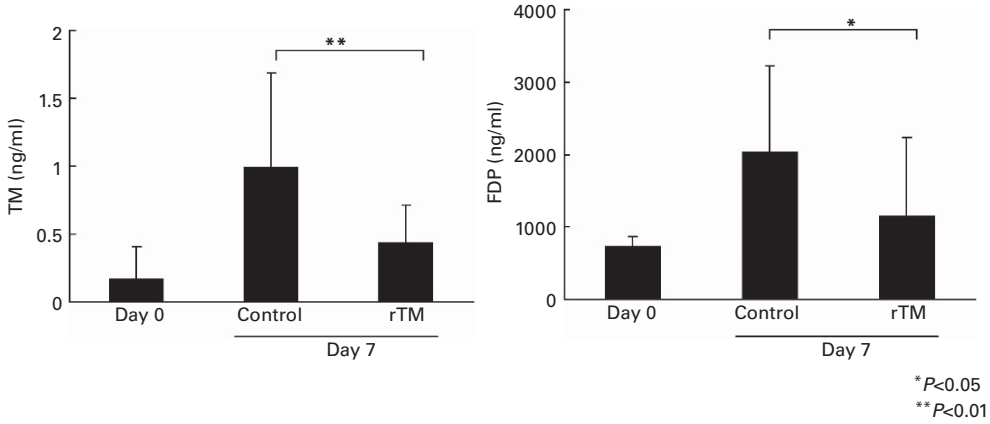

Figure 2. rTM decreases the plasma levels of TM and FDP in HSCT-recipient mice. ELISA. Plasma was isolated from mice ($n = 10$) at the indicated time points after HSCT and subjected to ELISA to measure the levels of TM and FDP. Results represent the means ± s.d. of duplicate experiments. The statistical significance of differences between the two groups was assessed by Student's *t*-test.

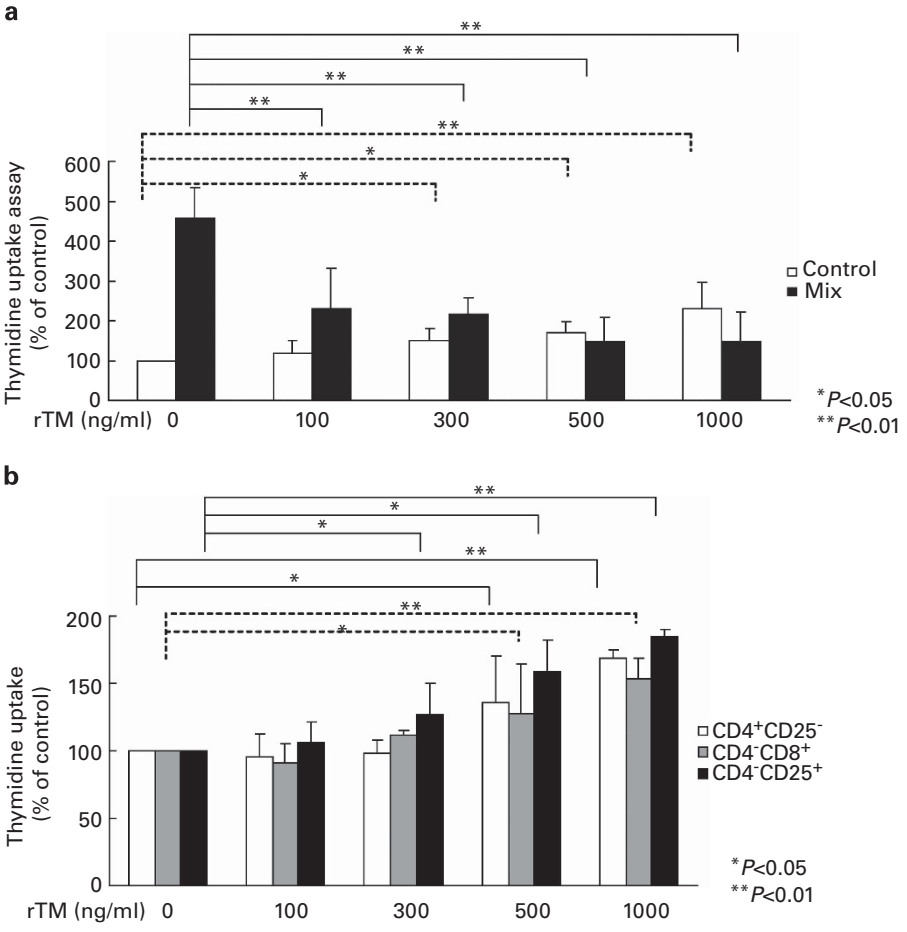

Figure 3. (**a**) rTM inhibits alloreactive proliferation of PBMCs. PBMCs were isolated from healthy mice ($n = 3$) and were cultured with irradiated (300 cGy) allogeneic PBMCs. These cells were exposed to various concentrations of rTM (100 – 1000 ng/mL). Their proliferation was assessed by a thymidine uptake assay on day 3 of culture. Control wells contained irradiated PBMCs alone either with or without rTM. Results represent the means ± s.d. for three experiments performed in triplicate plates. Statistical significance was assessed by one-way Analysis of variance followed by Dunnett's test. A dashed line indicates the comparison between proliferations of non-alloreactie resting PBMCs cultured with or without rTM. A solid line indicates the comparison between alloreactive proliferations of PBMCs cultured with or without rTM rTM, recombinant human soluble thrombomodulin. (**b**) rTM stimulates proliferation of T cells. $CD4^-/CD8^+$, $CD4^+/CD25^-$ and $CD4^+/CD25^+$ lymphocytes were isolated from healthy donors ($n = 3$) by cell sorting. These cells were cultured in the presence of various concentrations of rTM, and their proliferation was measured by thymidine uptake assay on day 3 of culture. Results represent the means ± s.d. for three experiments performed in triplicate plates. Statistical significance was assessed by one-way ANOVA followed by Dunnett's test.

rTM increases the proportion of Treg cells in a murine aGVHD model

We next examined whether the use of rTM would increase the proportion of $CD4^+/CD25^+/Foxp3^+$ Treg cells in the murine aGVHD model. Spleens were removed from BDF-1-recipient mice that received either rTM or control diluent five times a week on day 7 of HSCT. The harvested spleen cells were applied for FACS to quantify the proportion of $CD4^+/CD25^+/Foxp3^+$ Treg cells. The proportion of Treg cells increased in spleens isolated from rTM-treated mice by ~1.5-fold compared with that from the control diluent-treated mice (Figures 4a and b). We previously showed that rTM stimulated the proliferation of endothelial cells in association with the upregulation of the anti-apoptotic protein Mcl-1.[6] We therefore measured the levels of Mcl-1 in $CD4^+/CD25^+/Foxp3^+$ Treg cells isolated from BDF-1-recipient mice that received either rTM or control diluent. Real-time RT-PCR found

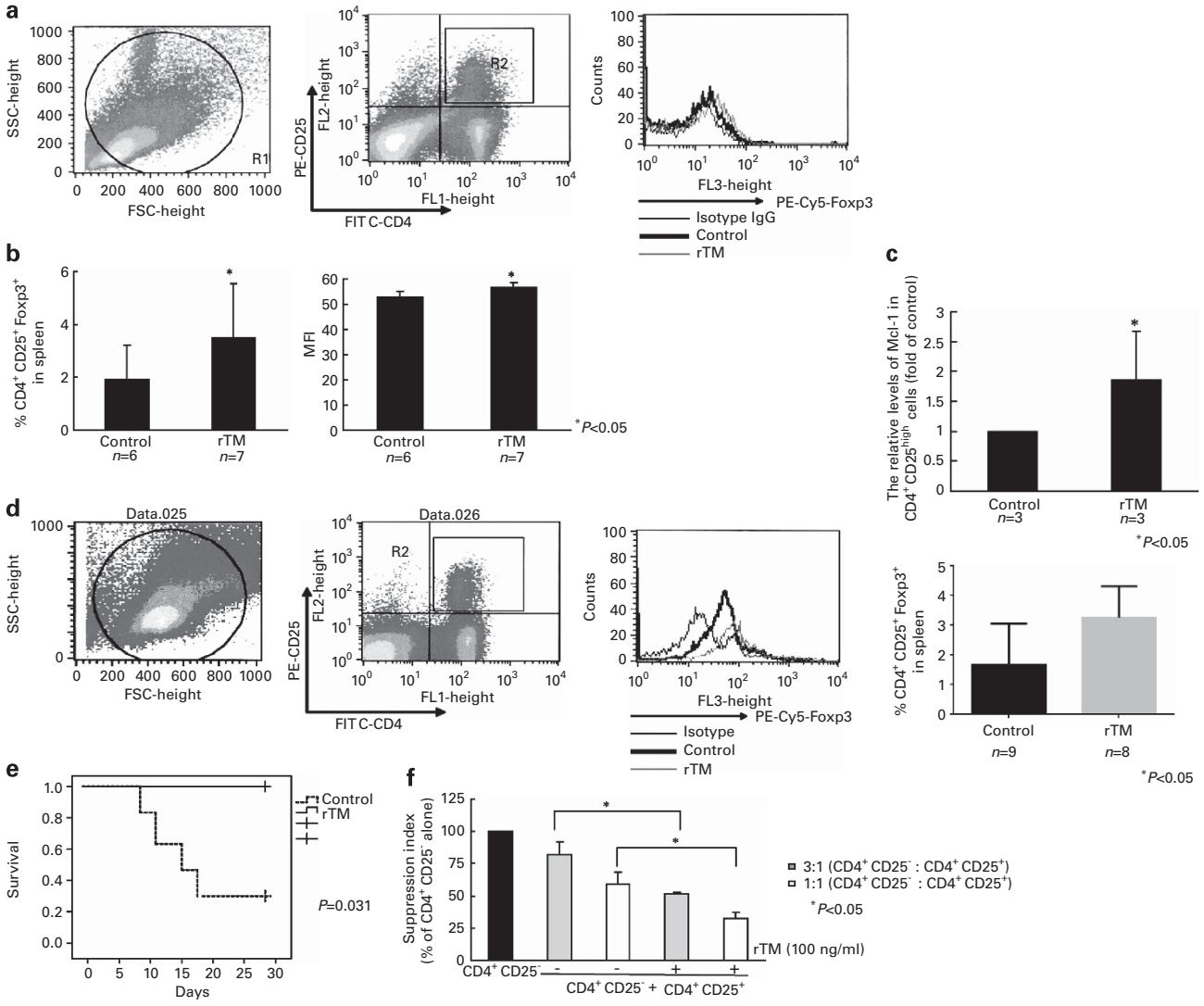

Figure 4. rTM increases the proportion of Treg cells in murine spleens after HSCT in association with an increase in levels of Mcl-1. Spleens were removed from BDF-1 recipient mice on day 7 of HSCT after receiving either rTM ($n=7$) or control diluent ($n=6$) five times a week. Spleen cells were subjected to FACS to quantify the proportion of $CD4^+/CD25^+/Foxp3^+$ cells. (**a**) representative histogram results. (**b**) The bar graph on the left panel represents the means ± s.d. of the proportion of $CD4^+/CD25^+/Foxp3^+$ cells in spleens isolated from either six control diluent- or seven rTM-treated mice. The bar graph on the right panel represents the means ± s.d. of the mean fluorescence intensity of Foxp3 in the $CD4^+/CD25^+$ cells. Statistical significance was assessed by one-way Analysis of variance. (**c**) Reverse transcription PCR. RNA was extracted from $CD4^+/CD25^+/Foxp3^+$ cells and subjected to real-time RT-PCT to measure the levels of Mcl-1. Results represent the means ± s.d. for three experiments performed in duplicate plates. Statistical significance was assessed by one-way Analysis of variance. Transplantation of grafts isolated from rTM-treated mice prolongs the survival of recipient mice. (**d**) FACS. C57BL/6 mice ($n=6$) were treated with either rTM (1 mg/kg) or control diluent for 7 days. Spleens were removed from mice and subjected to FACS to quantify the population of $CD4^+/CD25^+/Foxp3^+$ cells. (**e**) Kaplan–Meier curves for the survival of HSCT-recipient mice. BDF-1-recipient mice received BM cells (5×10^6) plus spleen cells (2×10^7) from C57BL/6 donors that received either rTM ($n=6$, 1 mg/kg) or control diluent ($n=6$) for 7 days by tail vein injection following 300 cGy of TBI. The statistical significance of the differences between the two groups was assessed by the log-rank test. (**f**) rTM enhances the function of $CD4^+/CD25^+$ T cells. Fixed numbers of C57BL/6 $CD4^+CD25^-$ T cells (responder cells) and irradiated allogeneic BDF-1-stimulator cells were mixed with variable numbers of C57BL/6 $CD4^+CD25^+$ T cells that were pretreated with either rTM (100 ng/mL, 48 h) or control diluent to obtain the ratios indicated in the figures. Proliferation was assessed after 5 days by pulsing the cells with 1 μCi/well [^{3}H] thymidine for the last 16 h. The amount of incorporated [^{3}H] thymidine was measured. Statistical significance was assessed by Student's *t*-test.

that the levels of Mcl-1 were greater in Treg cells isolated from rTM-treated mice than those isolated from control diluent-treated mice (Figure 4c).

We next attempted to examine whether transplantation of rTM-treated grafts would affect the severity of aGVHD *in vivo*. Treatment of C57BL/6 mice with rTM (1 mg/kg) for 7 days increased the proportion of $CD4^+/CD25^+/Foxp3^+$ cells in their spleens compared with control diluent - treated mice (Figure 4d). Transplantation of these spleen cells together with BM cells to BDF-1-recipient mice mitigated the severity of GVHD and prolonged their survival compared with the mice that received grafts obtained from control diluent-treated mice (Figure 4e).

Furthermore, we explored whether rTM affected the function of $CD4^+/CD25^+$ T cells. As reported previously,[18] $CD4^+/CD25^+$ T cells mitigated the proliferation of alloreactive $CD4^+/CD25^-$ T cells. This immunosuppressive effect of $CD4^+/CD25^+$ T cells was potentiated in the presence of rTM (Figure 4f).

rTM does not hamper the cytotoxic T-cell activity

We also examined whether rTM affected the cytotoxic activity of T cells. Cytotoxic T cells were generated either in the presence or absence of rTM at 100 ng/mL for 7 days and subjected to a CTL killing assay in which THP-1 acute monocytic leukemia cells were used as a target. Importantly, CTLs generated in the presence of rTM did not lose their cytotoxic activity against THP-1 cells when these cells were cocultured with THP-1 cells at a variable effector: target ratio (10:1–1:1) compared with CTLs generated without rTM (Figure 5).

rTM stimulates the engraftment of human hematopoietic stem/progenitor cells in immunodeficient mice

We finally assessed whether rTM affected the engraftment of transplanted hematopoietic stem/progenitor cells. G-CSF-mobilized $CD34^+$ hematopoietic cells were harvested from a healthy donor and transplanted into severe immunodeficient mice. These mice were treated with either rTM or control diluent. Approximately twice the number of human $CD45^+$ cells was noted in the PB of rTM-recipient mice after 6 weeks of HSCT compared with control diluent-recipient mice (Figure 6a). Also, the proportions of human $CD33^+$ myeloid and $CD19^+$ lymphoid cells in the BM and spleens were greater in rTM-treated mice than in control diluent-treated mice (Figure 6b).

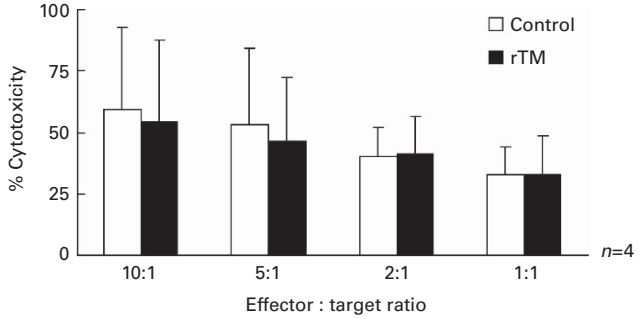

Figure 5. rTM does not hamper cytotoxic T-cell activity. Lymphocytes isolated from healthy mice were cocultured with irradiated (300 cGy) allogeneic PBMCs in the presence of IL-2 (50 ng/mL) either in combination with or without rTM (100 ng/mL) for 7 days. These cells (effector cells) were cocultured with [^{3}H]-thymidine-labeled target cells (THP-1, 1.2×10^4cells per well) at the ratios described in 96-well plates. After a 4 -h culture at 37 °C, plates were harvested and radioactivity was determined in a β-counter. Cytotoxicity is presented as % cytotoxicity = 100 × (counts in control well – counts in test well)/(total counts per well). Results represent the means ± s. d. for four experiments performed in triplicate plates. Statistical significance was assessed by Student's *t*-test.

DISCUSSION

The present study demonstrated that the use of rTM alleviated aGVHD in association with a decrease in the plasma levels of inflammatory cytokines and HMGB1 in a murine aGVHD model (Figure 1). Recently, other investigators have found that serum levels of HMGB1 and proinflammatory cytokines, including IL-6, are increased in patients on day 0 of HSCT, and the use of rTM, but not heparin, decreases their levels.[20] Given that the lectin-like domain of rTM possesses an anti-inflammatory function, it is not surprising that rTM sequestrated HMGB1, resulting in the blockade of proinflammatory signal pathways and the mitigation of aGVHD. These observations encourage the prophylactic use of rTM for the prevention of the development of aGVHD after HSCT. The therapeutic potentials of rTM in different inflammatory diseases have been noted in various animal models such as lipopolysaccharide-challenged sepsis and arthritis.[14,21] Importantly, the use of rTM significantly improves the survival of mechanically ventilated patients with severe sepsis when compared with patients who have not received rTM, further supporting the clinical benefit of rTM as an anti-inflammatory agent.[22]

Interestingly, the use of rTM increased the proportion of $CD4^+/CD25^+/Foxp3^+$ Treg cells in the spleens isolated from HSCT-recipient mice in association with an increase in the production of IL-10 compared with control diluent-treated mice (Figures 1c, d and 4), suggesting a novel mechanism by which rTM acts as an immunosuppressive agent. Similarly, the use of rTM alleviates acute respiratory distress syndrome in association with a decrease in the levels of HMGB1 and an increase in the proportion and levels of Treg and IL-10 in the lungs, respectively, in an α-galactosylceramide- and lipopolysaccharide-induced severe acute respiratory distress syndrome in murine model.[23] The molecular mechanisms by which rTM stimulated the proliferation of Treg remains unknown at the present time; however, elevated levels of Mcl-1 were noted in Treg cells isolated from the spleens of rTM-treated mice compared with those isolated from control diluent-treated mice (Figure 4c). This could be one explanation, as we previously showed that rTM stimulates angiogenesis in association with ERK-mediated upregulation of Mcl-1 in human umbilical vessel cells.[6]

Notably, treatment of donor B6 mice with rTM for 7 days increased the proportion of $CD4^+/CD25^+/Foxp3^+$ cells in their spleens and transplantation of these spleen cells together with BM cells to BDF-1-recipient mice mitigated the severity of GVHD and significantly prolonged their survival compared with mice that received grafts from control diluent-treated mice (Figures 4d and e). These observations suggested that alleviation of GVHD by rTM may be dependent on the effects of rTM on donor hematopoietic cells including regulatory T cells.

We, for the first time, demonstrated that immunological reactions in an allogeneic setting caused endothelial damage and coagulopathy, as evidenced by elevated plasma levels of TM and FDP, respectively, in the aGVHD murine model (Figure 2). TM expressed on endothelial cell surface is cleaved by various proteases produced by activated immune cells under the process of endothelial injury.[24,25] Plasma levels of TM were correlated with the severity of endothelial damage.[21] Elevation of plasma levels of TM and FDP were successfully improved by the use of rTM (Figure 2). The EGF-like domain of rTM possesses a cytoprotective effect that is independent of APC.[6] Life-threatening complications after HSCT include SOS, TAM and ES. The pathogenesis of these complications is related to endothelial injury.[26] A prophylactic dose of rTM might be useful to prevent the development of these potentially lethal complications.

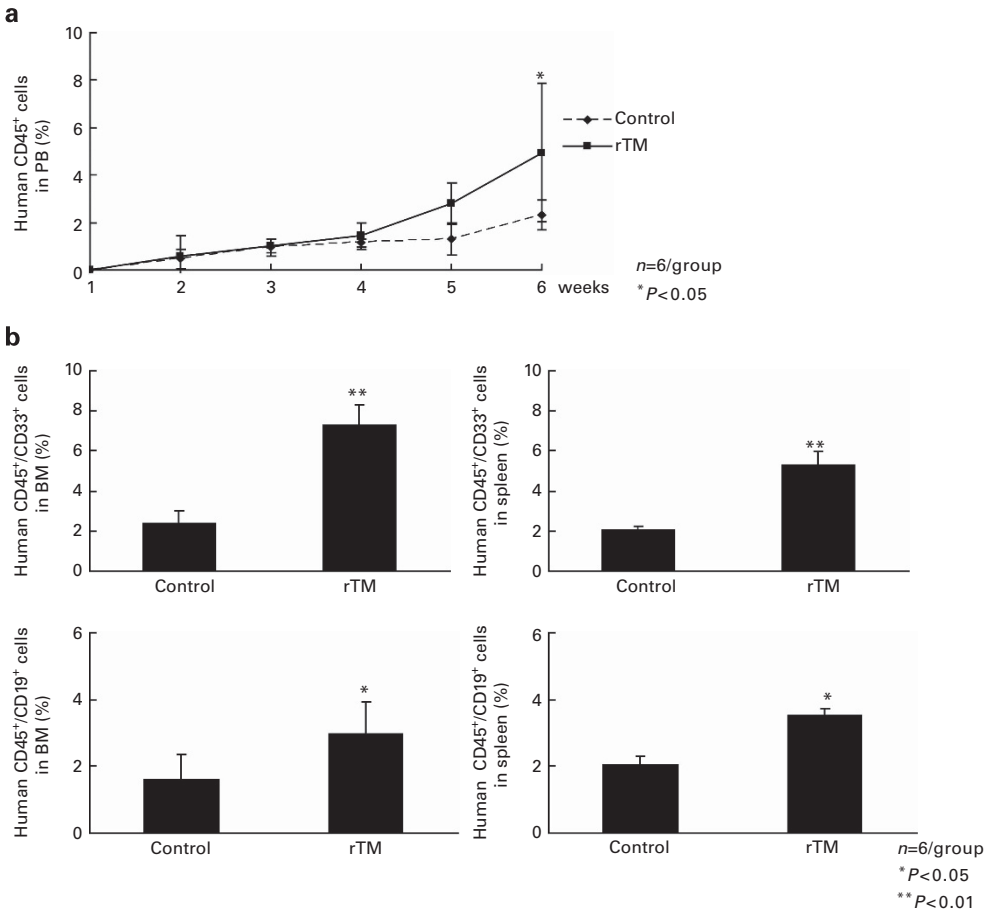

Figure 6. rTM stimulates engraftment of human hematopoietic stem/progenitor cells in immunodeficient mice. (**a**) 6-week-old NOD.Cg-Rag1tm1Mom Il2rgtm1Wjl/SzJ mice were transplanted with G-CSF-mobilized $CD34^+$ hematopoietic stem/progenitor cells (5×10^4 cells) isolated from the PB of a healthy donor via tail vein injection and assigned into two groups. Mice were treated with either rTM ($n = 6$, 1 mg/kg) or control diluent ($n = 6$, RPMI-1640) five times a week for six weeks. Engraftment was examined by quantifying the proportion of human $CD45^+$ cells in PB every week up to six weeks. (**b**) At the end of the experiments, mice were euthanized and the BM and spleens were removed and subjected to FACS to quantify the proportion of human $CD45^+/CD19^+$ and $CD45^+/CD33^+$ cells. Statistical significance was assessed by one-way ANOVA.

Importantly, rTM inhibited the alloreactive proliferation of PBMCs while sparing CTL activity *in vitro* (Figures 3 and 5). In fact, clinical use of rTM for the treatment of DIC that develops after HSCT does not significantly increase the incidence of relapse-related mortality.[10]

Surprisingly, the use of rTM significantly augmented the engraftment of $CD34^+$ human hematopoietic stem/progenitor cells in severe immunodeficient mice (Figure 6). Other investigators have also demonstrated that recombinant TM rescues mice from radiation-induced lethality in an APC-dependent manner.[27] These authors surmised that TM does not directly stimulate the proliferation of hematopoietic cells *in vitro*, although they did not demonstrate this empirically, and hypothesized that TM could protect the BM microenvironment from radiation injury and support the survival of hematopoietic stem cells. We were not able to distinguish BM microenvironment in rTM-treated mice from that in control diluent-treated mice (Figure not shown). Future study is clearly required to explore whether rTM affects the BM microenvironment and hematopoietic stem cells.

Taken together, the prophylactic use of anticoagulant rTM, which has additional activities against exaggerated inflammation and endothelial damage, may be useful to prevent the development of aGVHD. Further studies are warranted to verify the efficacy and safety of rTM in HSCT recipients in a clinical setting.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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