STIMULUS REPORT

blood advances

Modulating endothelial cells with EGFL7 to diminish aGVHD after allogeneic bone marrow transplantation in mice

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Key Points

- Treatment of GVHD with EGFL7 protein results in decreased disease severity and prolonged survival.
- EGFL7 treatment improved immune reconstitution and did not inhibit graft-versus-leukemia effect.

Acute graft-versus-host disease (aGVHD) is the second most common cause of death after allogeneic hematopoietic stem cell transplantation (allo-HSCT), underscoring the need for novel therapies. Based on previous work that endothelial cell dysfunction is present in aGVHD and that epidermal growth factor-like domain 7 (EGFL7) plays a significant role in decreasing inflammation by repressing endothelial cell activation and T-cell migration, we hypothesized that increasing EGFL7 levels after allo-HSCT will diminish the severity of aGVHD. Here, we show that treatment with recombinant EGFL7 (rEGFL7) in 2 different murine models of aGVHD decreases aGVHD severity and improves survival in recipient mice after allogeneic transplantation with respect to controls without affecting graft-versus-leukemia effect. Furthermore, we showed that rEGFL7 treatment results in higher thymocytes, T, B, and dendritic cell counts in recipient mice after allo-HSCT. This study constitutes a proof of concept of the ability of rEGFL7 therapy to reduce GHVD severity and mortality after allo-HSCT.

Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is used to treat patients with high-risk/refractory hematological malignancies and/or blood disorders. Unfortunately, acute graft-versus-host disease (aGVHD) is the principal complication of allo-HSCT.[1] aGVHD is mediated by alloreactive donor T lymphocytes and occurs in response to differences in major and/or minor histocompatibility antigens expressed by recipient cells.[2,3] Despite the use of standard GVHD prophylaxis regimens such as calcineurin inhibitors and other agents, 30% to 75% of allo-HSCT patients eventually develop aGVHD.[4,5] Patients with severe and/or steroid-refractory aGVHD have a poor long-term prognosis and a mortality rate reaching 70% to 80%.[6-9] Thus, alternative treatments for controlling excessive aGVHD are needed.

Epidermal growth factor-like domain 7 (EGFL7) is an ~30-kDa secreted protein important for angiogenesis and neurogenesis.[9-11] EGFL7 inhibits endothelial cell (EC) activation by proinflammatory cytokines through a negative feedback loop.[12] In a murine model of multiple sclerosis, EGFL7 reduced neuroinflammation by binding avβ3 integrins and preventing T-cell infiltration.[13] Thus, unlike immunosuppressive therapies that target immune cell function, EGFL7 acts primarily on blood vessels to reduce immune cell infiltration. Given the importance of lymphocyte homing in aGVHD pathology,[14,15] we hypothesized that

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Requests for data sharing may be submitted to Martin Guimond (martin.guimond@umontreal.ca or martin.guimond@gmail.com).

The full-text version of this article contains a data supplement.

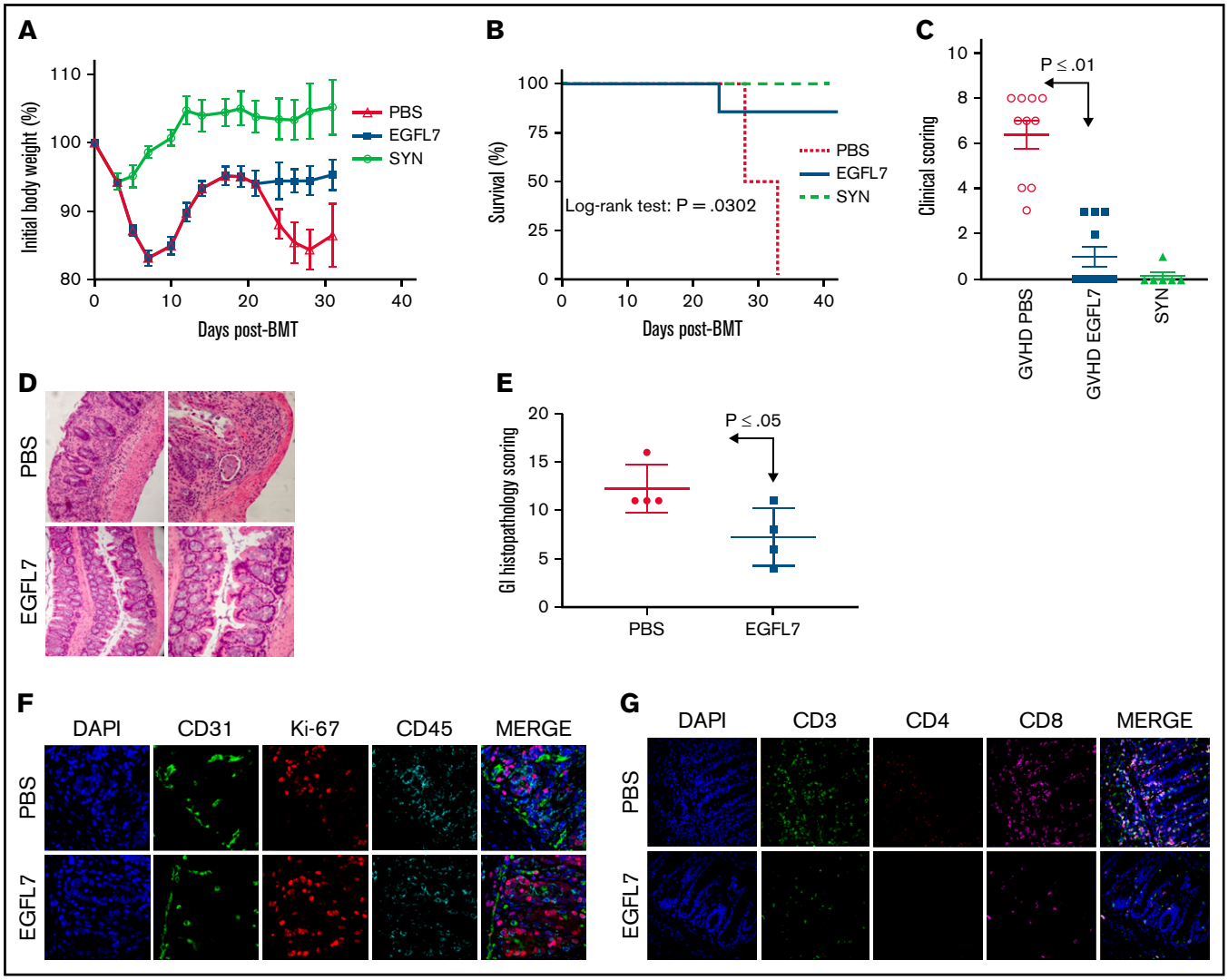

Figure 1. Effect of EGFL7 on GVHD severity in B6→B6D2F1 mice. (A) Weights of animals that had undergone transplantation were measured daily and averaged for the group (green circles: syngeneic T cells [no GVHD]; red triangles: allogeneic treated with PBS; blue squares: allogeneic treated with rEGFL7). Daily EGFL7 treatment was initiated at day +21 post-BMT. Data were pooled from 3 experiments with 6 to 13 mice per group. (B) Survival curve of transplanted mice (dotted green line: syngeneic control [SYN], blue line: allogeneic treated with rEGFL7, and thin dotted red line: allogeneic treated with PBS). Data were pooled from 3 experiments with 6 to 13 mice per group. (C) Clinical scores of GVHD + PBS, GVHD + rEGFL7, and syngeneic mice at day +28 post-BMT. (D) Histopathology of the gut 28 days after allo-HSCT. Left: magnification ×200 and right: magnification ×400. (E) Gastrointestinal (GI) histopathology score of the gut of GVHD + rEGFL7 and GVHD + PBS-treated mice. Results show mean ± standard error of the mean (SEM). (F) Immunofluorescence analysis of the intestine from PBS or rEGFL7-treated mice transplanted with allogenic splenocytes that were stained for immunofluorescence. Cells were stained with CD31 (secondary antibody: donkey anti-goat alexa fluor 488), CD45 (secondary antibody: donkey anti-rabbit alexa fluor 647), and Ki-67 antibodies (secondary antibody donkey anti-rat alexa fluor 594). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Magnification ×400. (G) Cells were stained with CD3 (secondary antibody donkey anti-goat alexa fluor 488), CD4 (secondary antibody donkey anti-rabbit alexa fluor 647), and CD8 antibodies (secondary antibody donkey anti-rat alexa fluor 594). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Magnification ×400. Data show 1 representative sample from each experimental group, which consists of 3 to 4 mice per group.

blocking EC activation after allo-HSCT could reduce GVHD severity. This study is the first to demonstrate EGFL7 as a potential novel treatment of allo-HSCT patients that develop aGVHD.

Methods

Mice and reagents

C57BL/6.SJL (cat. no. 002014), B6D2F1 (cat. no. 100006), BALB/c (cat. no. 000651), and C57Bl/6J (cat. no. 000664) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were housed at Maisonneuve-Rosemont Hospital and The Ohio State University animal facilities. Animal studies were performed in accordance with the Maisonneuve-Rosemont Hospital Animal Care Committee and Institutional Animal Care and Use Committee (The Ohio State University). Recombinant human EGFL7 (rEGFL7) was purchased from Peprotech (Rocky Hill, NJ).

BM transplantation and GVHD

B6D2F1 mice were irradiated (2 × 600 rad, 3 hours apart), and 10^7 bone marrow (BM) cells from C57BL/6 donor mice (H-2^b)

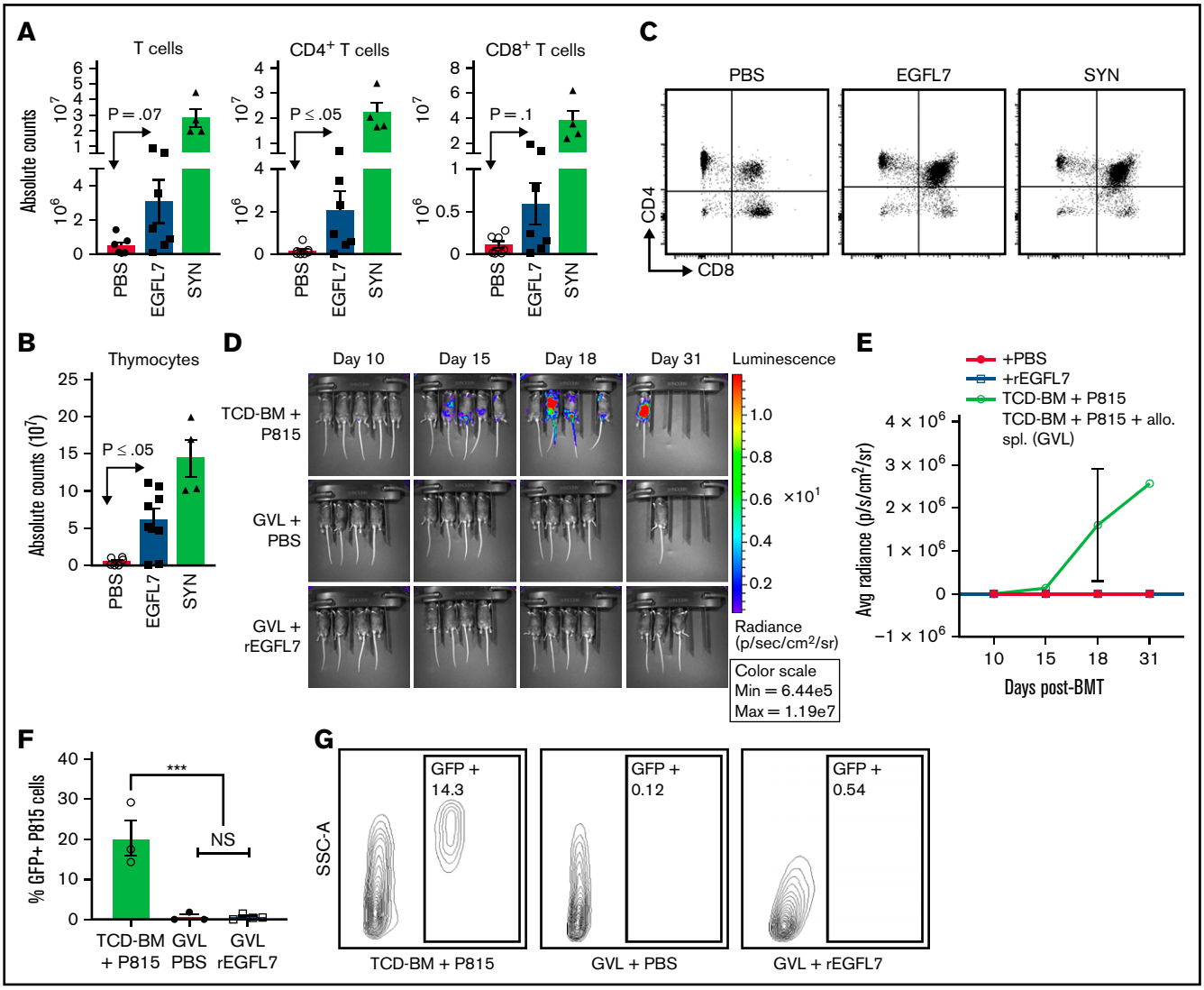

Figure 2. Effect of EGFL7 on immune reconstitution and graft-versus-leukemia effect. (A-C) Splenocytes and thymocytes were collected from PBS-treated mice as they reached their endpoints (day +28 to 35) and from EGFL7-treated mice (day +30 to 35 post-BMT). (A) Absolute counts of TCR$^+$, CD4$^+$, and CD8$^+$ lymphocytes in the spleen of Syn, GVHD + rEGFL7, and GVHD + PBS treated (B6→B6D2F1) mice. Data were pooled from 3 experiments with 4 to 7 mice per group. Gating was performed on CD45.1$^+$ and CD45.2$^+$ cells. (B) Absolute counts of BM-derived thymocytes (CD45.2$^+$) in Syn, GVHD + rEGFL7, and GVHD + PBS treated (B6→B6D2F1) mice. Data were pooled from 3 experiments with 4 to 8 mice per group. Results show mean ± SEM. Statistical analysis compared GVHD + rEGFL7 vs GVHD + PBS treated (B6→B6D2F1) mice, and *P* values were determined by a Mann-Whitney *U* test. (C) Dot-plot analysis of thymocytes (derived from the BM cells [CD45.2$^+$CD45.1$^-$]), based on the expression of CD4, CD8, and CD45.2 antigens, in the thymus of SYN control, GVHD + rEGFL7, and GVHD + PBS mice. These data are representative of 3 or more experiments with 6 to 8 mice per group. Histogram showed mean ± SEM. Statistical analysis compared GVHD + rEGFL7 vs GVHD + PBS treated (B6→B6D2F1) mice, and *P* values were determined by a Mann-Whitney *U* test. Transplant for GVL was performed in BALB/c mice as described in "Methods." (D-E) Whole-body bioluminescent signal intensity of recipient mice (n = 4-5 per cohort). Mice were imaged on indicated days. Average radiance expressed as mean ± SEM. One representative transplant experiment of 2 is shown. (F) Splenocytes were isolated at the time when mice reached their endpoints, days 28 to 33 post-BMT for PBS vehicle and days 30 to 35 post-BMT in rEGFL7-treated mice. Percentage GFP positivity representing P815 leukemic cell infiltration in the spleen. Each dot represents a single mouse. (G) Representative flow cytometric contour plots. Allo. spl., allogeneic splenocytes; GFP, green fluorescent protein; Min, minimum; Max, maximum; SSC-A, side-scatter-area; SYN, syngeneic control; TCD-BM, T cell depleted bone marrow. ***$P < .001$.

were injected IV into lethally irradiated B6D2F1 recipients (H-2$^{b/d}$) along with 3 × 10^6 T cells from B6.SJL (allogeneic) or B6D2F1 (syngeneic) mice. T cells were isolated using the T-cell enrichment kit (Stemcell Technologies). Mice were monitored daily and scored thrice a week for clinical severity of acute GVHD using a scoring system modified from Cooke et al.[16] Vehicle (phosphate-buffered saline [PBS]) or rEGFL7 (10 μg) was administered intraperitoneally daily to mice, starting when signs of GVHD appear (~17 to 21 days post–stem cell transplantation). Mice with a clinical score of 7 or more were considered very sick and euthanized according to approved animal protocols.

Other detailed methods

All other methods are described in the supplemental data.

Results and discussion

Current therapies for aGVHD rely largely on the inhibition of immune cell activation and effector functions.[17] Here, we evaluated how EGFL7, an inhibitor of EC activation, can reduce aGVHD. We used a parent into F1 (B6→B6D2F1) mouse model to evaluate the therapeutic benefit of EGFL7 treatment (supplemental Figure 1A). Mice at day +21 post-HSCT showed evidence of mild hunching posture and dull fur. Because rEGFL7 administration could represent an alternative second-line therapy, we initiated rEGFL7 treatment at day +21 post–bone marrow transplantation (BMT) to determine the effect of rEGFL7 on active, established disease rather than preventing the development of aGVHD. After 7 days of rEGFL7 treatment, mice showed marked improvement in posture, fur appearance, and activity. This was accompanied by a stop or reduction in body weight loss in rEGFL7-treated mice in contrast to PBS-treated mice that continued to lose weight (Figure 1A). Importantly, rEGFL7 treatment results in lower clinical scores and prolonged survival of mice after allo-HSCT compared with PBS controls (Figure 1B-C). We validated this result in a second mouse model of aGVHD, B6→Balb/c, showing increased survival of rEGFL7-treated mice compared with PBS controls (supplemental Figure 2A-C). The gut and liver are typically affected by aGVHD.[18,19] Visual examination of the colon revealed the presence of swelling and a reduction in the length in the PBS group compared with the rEGFL7-treated mice (supplemental Figure 1B-C). Histopathology analysis revealed a higher amount of leukocyte infiltration in both large intestine and liver of the PBS group compared with rEGFL7-treated mice (Figure 1D; supplemental Figure 1D). Large intestine of PBS-treated mice showed focal to diffuse areas of villous atrophy with a dense amount of mixed inflammatory infiltrate in the lamina propria composed predominantly of lymphomononuclear cells and neutrophils. In addition, PBS-treated mice showed a more profound loss of mucous-secreting goblet cells in the mucosal epithelium along with enhanced crypt epithelial cell apoptosis (>1 apoptotic body per crypt), and focal crypt abscess formation. In contrast, rEGFL7-treated mice showed normal integrity of mucosal epithelium with negligible to mild degree of lamina propria inflammation and a lesser number of crypt epithelial cell apoptotic bodies (Figure 1D). In the liver, rEGFL7-treated mice showed a fewer number and limited extent of portal tracts involvement by inflammatory cell infiltrates, smaller degree of bile duct injury, lobular necroinflammatory activity, and vascular endotheliitis (supplemental Figure 1D). Thus, reduction in leukocyte infiltration in the gut and liver is consistent with an overall lower aGVHD histopathological score in rEGFL7-treated mice compared with the PBS-treated group (Figure 1E; supplemental Figure 1E). Finally, we performed immunofluorescence microscopy to assess EC and T-lymphocyte populations in the intestine. We found that rEGFL7 treatment resulted in increased proliferation (Ki-67$^+$) of intestine ECs (CD31$^+$CD45$^-$) (Figure 1F; supplemental Figure 1F) with an associated decrease in infiltrating CD4$^+$ and CD8$^+$ T cells (Figure 1G; supplemental Figure 1G). In addition, we found that rEGFL7 treatment resulted in a decrease in the expression of VCAM1 on CD31$^+$ ECs in the gut, suggesting that EGFL7 can reduce EC activation (supplemental Figure 1H-I). EC damage occurs during GVHD,[20,21] and based on these data, we show that rEGFL7 treatment not only reduces EC activation but also perhaps promotes EC repair, leading to reduction in T-cell infiltration in the gut and liver, resulting in GVHD improvement.

aGVHD insults to primary and secondary lymphoid organs impair immune reconstitution after allo-HSCT.[22-25] Treatment with rEGFL7 tends to increase T-cell (Figure 2A), B-cell, and dendritic cell (DC) counts in the spleen (supplemental Figure 3A-B) and BM (supplemental Figure 3C-E) compared with PBS-treated mice. rEGFL7 therapy also tends to increase thymocyte counts with normalization of double-positive and single-positive CD4$^+$ and CD8$^+$ thymocyte populations in rEGFL7 compared with PBS-treated mice (Figure 2B-C). Variability in immune reconstitution may be related to GVHD insults prior to initiating EGFL7 treatment, but additional studies are needed. Thus, increases in T-cell, B-cell, and dendritic cell counts are consistent with a reduction in aGVHD severity in rEGFL7-treated mice. However, it is also possible that the effect of rEGFL7 on immune reconstitution could result from direct effects on these cells/tissues, thus contributing to decreased disease severity. Part of the success of an allogeneic transplant is the ability to sustain the graft-versus-leukemia (GVL) effect important for eliminating minimal residual disease.[3-5] To study the impact of rEGFL7 treatment on GVL, we transplanted a luciferase-transduced murine mastocytoma P815 cell line along with or without alloreactive-B6 splenocytes. Recipients of allogeneic splenocytes were treated daily with PBS or rEGFL7 starting at day +3 until day +10 post-BMT. Mice transplanted with mastocytoma P815 cells without allogeneic splenocytes all succumbed to leukemia. In mice that received allogeneic splenocytes, treatment with rEGFL7 did not interfere with GVL effects as seen by decreased leukemia burden similar to PBS controls (Figure 2D-E). Splenocytes were isolated from mice at time of death, and flow cytometric analysis confirmed the absence of GFP$^+$ P815 cells (GFP$^+$) in the spleen of rEGFL7-treated mice (Figure 2F-G), further validating the GVL-sparing effect of rEGFL7. Importantly, further investigation is warranted to evaluate the impact of rEGFL7 on relapse post–allo-HSCT, as EGFL7 effect has been documented on human AML cells.

In conclusion, we identified EGFL7 as a potential therapy to reduce/treat aGVHD. Although immunosuppressive therapies typically impair immune cell activation/functions, the effectiveness of EGFL7 therapy to treat GVHD relies largely on the inhibition of EC activation and access of immune cells to inflamed tissues.[11] However, we cannot exclude the possibility that EGFL7 might have a direct effect on alloreactive T lymphocytes. This work represents the first proof of concept that EGFL7 therapy can diminish/treat aGVHD and provides a novel perspective about modulating ECs to reduce GVHD.

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Authorship

Contribution: A.M.D., M.M.M., C.G., N.E.S., S. Kalyan, M. K., R.K., M. Goulard, S. Kolovich, A.R., E.N., C.-E.B., F.D., and A.A. performed GVHD experiments and contributed to data interpretation; R.G. provided scientific input to experiments and reviewed the manuscript; and M. Guimond, A.M.D., and P.R. designed the project, analyzed data, and wrote the manuscript.

Conflict-of-interest disclosure: M. Guimond and A.M.D. hold a revisionary patent for the use of EGFL7 in the treatment of GVHD. The remaining authors declare no competing financial interests.

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References

1. Ferrara JL, Deeg HJ. Graft-versus-host disease. *N Engl J Med.* 1991;324(10):667-674.
2. Teshima T, Ordemann R, Reddy P, et al. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nat Med.* 2002;8(6):575-581.
3. Matte CC, Liu J, Cormier J, et al. Donor APCs are required for maximal GVHD but not for GVL. *Nat Med.* 2004;10(9):987-992.
4. Hill L, Alousi A, Kebriaei P, Mehta R, Rezvani K, Shpall E. New and emerging therapies for acute and chronic graft *versus* host disease. *Ther Adv Hematol.* 2018;9(1):21-46.
5. Holtan SG, Pasquini M, Weisdorf DJ. Acute graft-versus-host disease: a bench-to-bedside update. *Blood.* 2014;124(3):363-373.
6. Castilla-Llorente C, Martin PJ, McDonald GB, et al. Prognostic factors and outcomes of severe gastrointestinal GVHD after allogeneic hematopoietic cell transplantation. *Bone Marrow Transplant.* 2014;49(7):966-971.
7. Martin PJ, Rizzo JD, Wingard JR, et al. First- and second-line systemic treatment of acute graft-versus-host disease: recommendations of the American Society of Blood and Marrow Transplantation. *Biol Blood Marrow Transplant.* 2012;18(8):1150-1163.
8. Pidala J, Kim J, Roman-Diaz J, et al. Pentostatin as rescue therapy for glucocorticoid-refractory acute and chronic graft-versus-host disease. *Ann Transplant.* 2010;15(4):21-29.
9. Oh J, Park SH, Lee TS, Oh HK, Choi JH, Choi YS. High expression of epidermal growth factor-like domain 7 is correlated with poor differentiation and poor prognosis in patients with epithelial ovarian cancer. *J Gynecol Oncol.* 2014;25(4):334-341.
10. Bambino K, Lacko LA, Hajjar KA, Stuhlmann H. Epidermal growth factor-like domain 7 is a marker of the endothelial lineage and active angiogenesis. *Genesis.* 2014;52(7):657-670.
11. Nichol D, Stuhlmann H. EGFL7: a unique angiogenic signaling factor in vascular development and disease. *Blood.* 2012;119(6):1345-1352.
12. Pinte S, Caetano B, Le Bras A, et al. Endothelial cell activation is regulated by epidermal growth factor-like domain 7 (Egfl7) during inflammation. *J Biol Chem.* 2016;291(46):24017-24028.
13. Larochelle C, Uphaus T, Broux B, et al. EGFL7 reduces CNS inflammation in mouse. *Nat Commun.* 2018;9(1):819.
14. Sackstein R. A revision of Billingham's tenets: the central role of lymphocyte migration in acute graft-versus-host disease. *Biol Blood Marrow Transplant.* 2006;12(1 suppl 1):2-8.
15. Nomura S, Ishii K, Fujita S, Nakaya A, Satake A, Ito T. Associations between acute GVHD-related biomarkers and endothelial cell activation after allogeneic hematopoietic stem cell transplantation. *Transpl Immunol.* 2017;43-44:27-32.
16. Cooke KR, Kobzik L, Martin TR, et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. *Blood.* 1996;88(8):3230-3239.
17. Coutinho AE, Chapman KE. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Mol Cell Endocrinol.* 2011;335(1):2-13.
18. Hill GR, Ferrara JL. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood.* 2000;95(9):2754-2759.
19. Shulman HM, Sharma P, Amos D, Fenster LF, McDonald GB. A coded histologic study of hepatic graft-versus-host disease after human bone marrow transplantation. *Hepatology.* 1988;8(3):463-470.
20. Biedermann BC, Sahner S, Gregor M, et al. Endothelial injury mediated by cytotoxic T lymphocytes and loss of microvessels in chronic graft versus host disease. *Lancet.* 2002;359(9323):2078-2083.
21. Mir E, Palomo M, Rovira M, et al. Endothelial damage is aggravated in acute GvHD and could predict its development. *Bone Marrow Transplant.* 2017;52(9):1317-1325.
22. Mackall CL, Fleisher TA, Brown MR, et al. Age, thymopoiesis, and $CD4^+$ T-lymphocyte regeneration after intensive chemotherapy. *N Engl J Med.* 1995;332(3):143-149.

23. Takebayashi M, Amakawa R, Tajima K, et al. Blood dendritic cells are decreased in acute graft-versus-host disease. *Bone Marrow Transplant.* 2004;33(10):989-996.

24. Shono Y, Ueha S, Wang Y, et al. Bone marrow graft-versus-host disease: early destruction of hematopoietic niche after MHC-mismatched hematopoietic stem cell transplantation. *Blood.* 2010;115(26):5401-5411.

25. Gauthier SD, Leboeuf D, Manuguerra-Gagné R, Gaboury L, Guimond M. Stromal-derived factor-1α and interleukin-7 treatment improves homeostatic proliferation of naïve CD4(+) T cells after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant.* 2015;21(10):1721-1731.