GRAFT-VERSUS-HOST DISEASE

Human β-defensin 2 ameliorates acute GVHD by limiting ileal neutrophil infiltration and restraining T cell receptor signaling

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Acute graft-versus-host disease (aGVHD), which is driven by allogeneic T cells, has a high mortality rate and limited treatment options. Human β-defensin 2 (hBD-2) is an endogenous epithelial cell–derived host-defense peptide. In addition to its antimicrobial effects, hBD-2 has immunomodulatory functions thought to be mediated by CCR2 and CCR6 in myeloid cells. In this study, we analyzed the effect of recombinant hBD-2 on aGVHD development. We found that intestinal β-defensin expression was inadequately induced in response to inflammation in two independent cohorts of patients with aGVHD and in a murine aGVHD model. Treatment of mice with hBD-2 reduced GVHD severity and mortality and modulated the intestinal microbiota composition, resulting in reduced neutrophil infiltration in the ileum. Furthermore, hBD-2 treatment decreased proliferation and proinflammatory cytokine production by allogeneic T cells in vivo while preserving the beneficial graft-versus-leukemia effect. Using transcriptome and kinome profiling, we found that hBD-2 directly dampened primary murine and human allogeneic T cell proliferation, activation, and metabolism in a CCR2- and CCR6-independent manner by reducing proximal T cell receptor signaling. Furthermore, hBD-2 treatment diminished alloreactive T cell infiltration and the expression of genes involved in T cell receptor signaling in the ilea of mice with aGVHD. Together, we found that both human and murine aGVHD were characterized by a lack of intestinal β-defensin induction and that recombinant hBD-2 represents a potential therapeutic strategy to counterbalance endogenous hBD-2 deficiency.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HCT) is a curative therapy option for malignant and nonmalignant hematological diseases. However, its success is limited by complications, including acute graft-versus-host disease (aGVHD), which is a major cause of morbidity and mortality in patients undergoing allo-HCT. During aGVHD pathogenesis, allogeneic donor T cells recognize and damage epithelial tissues of the allo-HCT recipient, particularly the skin, liver, and gastrointestinal (GI) tract (*1*). Despite standard GVHD prophylaxis with cyclosporine A (CsA)– or tacrolimus-based regimens, 28 to 45% of allo-HCT recipients develop aGVHD grades II to IV, and 11 to 23% experience severe aGVHD grades III to IV (*2*, *3*).

A central event in the pathogenesis of aGVHD is the activation of donor-derived allogeneic T cells by host antigen-presenting cells (APCs). After stimulation, alloreactive T cells proliferate, differentiate, and act as the main effector cells, inducing cell death in GVHD target organs, such as the GI tract (*1*, *4*). During this process, large amounts of cytokines are released, particularly T helper 1 (T_H1) cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor (TNF), and interleukin-2 (IL-2), contributing to the massive inflammatory response during aGVHD. On the other hand, $CD4^+$ and $CD8^+$ donor T cells are also crucial for the beneficial graft-versus-leukemia (GVL) effect, where they eliminate residual malignant cells in patients with leukemia. Furthermore, neutrophil granulocytes have been shown to be recruited into the ileum by translocating intestinal bacteria and contribute to tissue damage and allogeneic T cell activation during aGVHD (*5*, *6*).

Defensins are endogenous host-defense peptides that can protect epithelial barrier integrity and shape the intestinal microbiota composition, constituting an important component of the innate immune system (*7*). In addition to their antimicrobial function,

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defensins are involved in host immunoregulation, including effects on immune cell migration, cytokine secretion, and regulatory T cell induction (*8*–*10*). Given their multifaceted mode of action at mucosal surfaces, there is an increasing interest in the role of defensins in intestinal inflammation. In humans, the classes of α- and β-defensins can be distinguished on the basis of the position of their intramolecular disulphide bonds (*11*, *12*). α-Defensins comprise the human neutrophil peptides 1 to 4, which are expressed by granulocytes, and human defensins 5 and 6, which are produced by Paneth cells of the small intestine. With four human β-defensins (hBD-1 to hBD-4) described so far, hBD-2 was the first inducible human defensin that has been found (*13*). hBD-2 is produced by epithelial cells, including enterocytes, upon stimulation with microbial products and proinflammatory cytokines (*13*, *14*). hBD-2 expression is strongly increased in the inflamed intestines of patients with ulcerative colitis (UC), whereas defective hBD-2 induction has been described in patients with Crohn's disease (*14*). Functional studies have demonstrated that oral administration of recombinant hBD-2 ameliorates dextran sulfate sodium (DSS)–induced experimental colitis, indicating that hBD-2 can regulate intestinal inflammation (*15*). In line with this, a C-C chemokine receptor 2 (CCR2)–dependent regulatory effect of hBD-2 on myeloid immune cell activation in the context of colitis has been described (*15*). However, the role of hBD-2 during aGVHD pathogenesis and its effect on the T cell response have not yet been elucidated.

Therefore, we explored the effect of β-defensins on murine and human T cell responses and their therapeutic potential in different experimental GVHD and GVL models. We found lack of induction of β-defensins in the inflamed intestines of patients and mice with aGVHD. Treatment with exogenous recombinant hBD-2 strongly reduced aGVHD severity in vivo while sparing the beneficial GVL effect. Changes in the microbiome of hBD-2–treated mice resulted in reduced neutrophil infiltration of the ileum during aGVHD. Furthermore, we found that hBD-2 directly dampened T cell responses through a T cell receptor (TCR)–dependent mechanism. Together, we identified a role of an hBD in regulating T cell responses and evaluated hBD-2 as potential new treatment option modulating aGVHD.

RESULTS

β-Defensin expression is inadequately induced in patients and mice with aGVHD

We first aimed to understand whether β-defensin production is dysregulated during the development of aGVHD in the intestine as a major target organ of aGVHD and a central organ of defensin production. We therefore analyzed hBD-2 expression in intestinal samples from healthy controls, patients with GVHD, and patients with UC. hBD-2 expression is physiologically induced in epithelia in response to microbial and proinflammatory stimuli, which is in line with hBD-2 up-regulation in the inflamed colons of patients with UC (*14*). Using immunohistochemical staining, we confirmed a strong up-regulation of hBD-2 expression in the colons of patients with UC compared with noninflamed colon tissue from healthy controls (Fig. 1, A and B). In contrast to UC, hBD-2 expression was not induced in the inflamed colons of patients with GI-GVHD (Fig. 1, A and B). We confirmed these data in a second independent cohort of patients at the RNA level using quantitative reverse transcription polymerase chain reaction (qRT-PCR) for *HBD2*, again showing deficient induction of hBD-2 in the intestines of patients suffering from aGVHD (Fig. 1C).

We next asked whether we could reproduce these findings in murine aGVHD. Using a mouse major histocompatibility complex (MHC) mismatch GVHD model (C57BL/6 donor➔BALB/c recipient), we studied intestinal expression of *Defb4* (the gene encoding mBD-4), which is the murine ortholog of *HBD2*, at different time points after allo-HCT. As positive control, we found that *Defb4* expression was strongly up-regulated in the colons of mice with DSS-induced colitis (Fig. 1D). In contrast, *Defb4* expression was reduced in the colons and ilea of mice developing aGVHD as compared with naïve mice (Fig. 1, D and E). This down-regulation of hBD-2 was specific for the allo-HCT setting and not observed in control mice receiving irradiation only, allogeneic bone marrow (BM) alone, or syngeneic transplantation (fig. S1). Together, these results indicate a lack of β-defensin induction during aGVHD in patients and mice, potentially contributing to disease pathogenesis.

β-Defensin treatment reduces aGVHD severity and mortality

To investigate whether replacement of the defective β-defensin production during aGVHD could modify the course of disease, we treated mice that had undergone allo-HCT (C57BL/6 donor➔BALB/c recipient) with recombinant hBD-2 by oral administration for 11 days. We determined organ hBD-2 concentrations by liquid chromatography–tandem mass spectrometry and found quantifiable and physiologically relevant amounts of hBD-2 (*16*) in the livers, ilea, and colons of hBD-2–treated mice, with the highest concentrations reached in the intestine (fig. S2). Treatment with hBD-2 significantly prolonged survival after allo-HCT as compared with the vehicle phosphate-buffered saline (PBS)–treated group, with 60% of hBD-2–treated mice showing long-term survival ($P < 0.0001$; Fig. 1F). Furthermore, hBD-2 treatment markedly ameliorated disease severity as shown by reduced histopathological GVHD scores in the colon, ileum, liver, and skin as main target organs of aGVHD (Fig. 1G). Engraftment and donor hematopoiesis were not adversely affected by hBD-2 (fig. S3). The conditioning regimen and aGVHD development are typically associated with weight loss, which was reduced by hBD-2 treatment (fig. S4A). CsA is frequently used for GVHD prophylaxis in Europe. Therefore, we compared the efficiency of hBD-2 and CsA treatment in the C57BL/6➔BALB/c aGVHD model. We found that survival was increased in hBD-2–treated mice as compared with mice treated with CsA or PBS (Fig. 1H). Similarly, the mouse weight recovered faster in hBD-2–treated compared with CsA-treated mice with aGVHD (fig. S4B). Using a second aGVHD model (BALB/c donor➔C57BL/6 recipient), we confirmed the finding that hBD-2 improves survival after allo-HCT, precluding strain-dependent effects of hBD-2 (fig. S4C). Together, our data indicate that hBD-2 treatment ameliorates aGVHD in different murine models.

hBD-2 affects the intestinal microbiome and reduces neutrophil recruitment into the ilea of aGVHD mice

GVHD is characterized by complex changes in the intestinal microbiota, and hBD-2 exerts antimicrobial functions. Therefore, we next assessed the effects of oral hBD-2 treatment on the intestinal microbiome after allo-HCT. Oral hBD-2 treatment had no effect on intestinal bacterial density as assessed by 16*S* ribosomal DNA qPCR (fig.

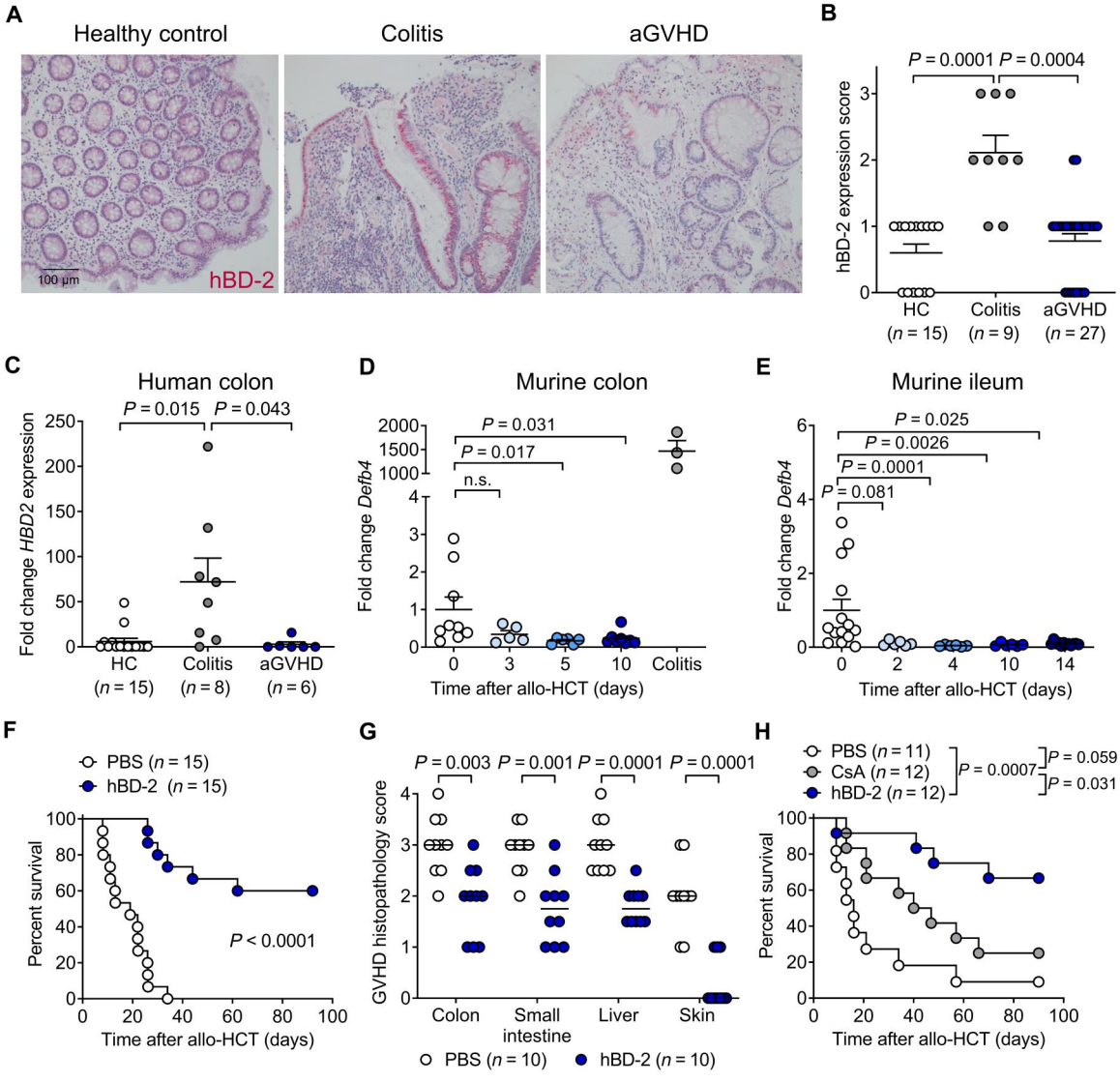

Fig. 1. Induction of β-defensins is defective in human and mouse aGVHD, and hBD-2 treatment reduces aGVHD severity. (**A** to **C**) Expression of hBD-2 in colon biopsies from healthy controls (HC), patients with colitis, or patients with acute GI-GVHD. Representative immunohistochemical hBD-2 staining (dark red) (A) and quantification of hBD-2 expression in colon sections (B) are shown. (C) *HBD2* expression was assessed by qRT-PCR in a second independent HC and patient cohort. (**D** and **E**) Colonic (D) and ileal (E) *Defb4* (mBD-4) expression was quantified in mice at indicated time points after allo-HCT (C57BL/6 donor➔BALB/c-recipient, $n = 5$ to 14 per group) and in mice with DSS-induced colitis ($n = 3$). n.s., not significant. (**F**) Kaplan-Meier survival curves of mice after allo-HCT (C57BL/6➔BALB/c, $n = 15$ per group) treated orally with PBS or hBD-2 from day 0 (d0) until d10. (**G**) Histopathology scores of aGVHD mice ($n = 10$ per group) treated as in (F) and analyzed on d7 after allo-HCT. (**H**) Survival of mice after allo-HCT (C57BL/6➔BALB/c, $n = 11$ or 12 per group) treated orally with PBS, hBD-2, or cyclosporine A (CsA). Groups were compared using Kruskal-Wallis test and Mann Whitney *U* test or log-rank test for survival experiments.

S5A). Shotgun metagenomic sequencing revealed that the overall composition of the intestinal microbiota was not changed by hBD-2 treatment (Fig. 2A and fig. S5, B and C). Furthermore, hBD-2 treatment did not alter intestinal microbial diversity in GVHD mice (Fig. 2B and fig. S5D). We used compositional data analysis to evaluate specific taxa and found changes in specific genera and species in hBD-2–treated mice, including a relative increase in multiple *Bacteroides* species, particularly *Bacteroides vulgatus*, *Bacteroides ovatus*, and *Bacteroides caccae* (Fig. 2C and fig. S5E), whereas *Ruminococcaceae* were reduced (fig. S5E). An increase in *Bacteroides* in the intestinal microbiota has recently been shown to be associated with reduced GVHD severity in mice (*17*). Furthermore, transmigration of intestinal bacteria to the ileal lamina propria was reduced in hBD-2–treated GVHD mice (Fig. 2D).

Because neutrophils are recruited to the ileum upon bacterial translocation (*5*), we next studied neutrophil infiltration of the ileal lamina propria by flow cytometry. We found that the proportion of $CD11b^+Ly6G^+$ neutrophils was reduced in hBD-2–treated mice on day 3 after allo-HCT, which is the peak of neutrophil influx (Fig. 2E). In agreement, the activity of myeloperoxidase (MPO), which is highly expressed in neutrophils, was reduced in

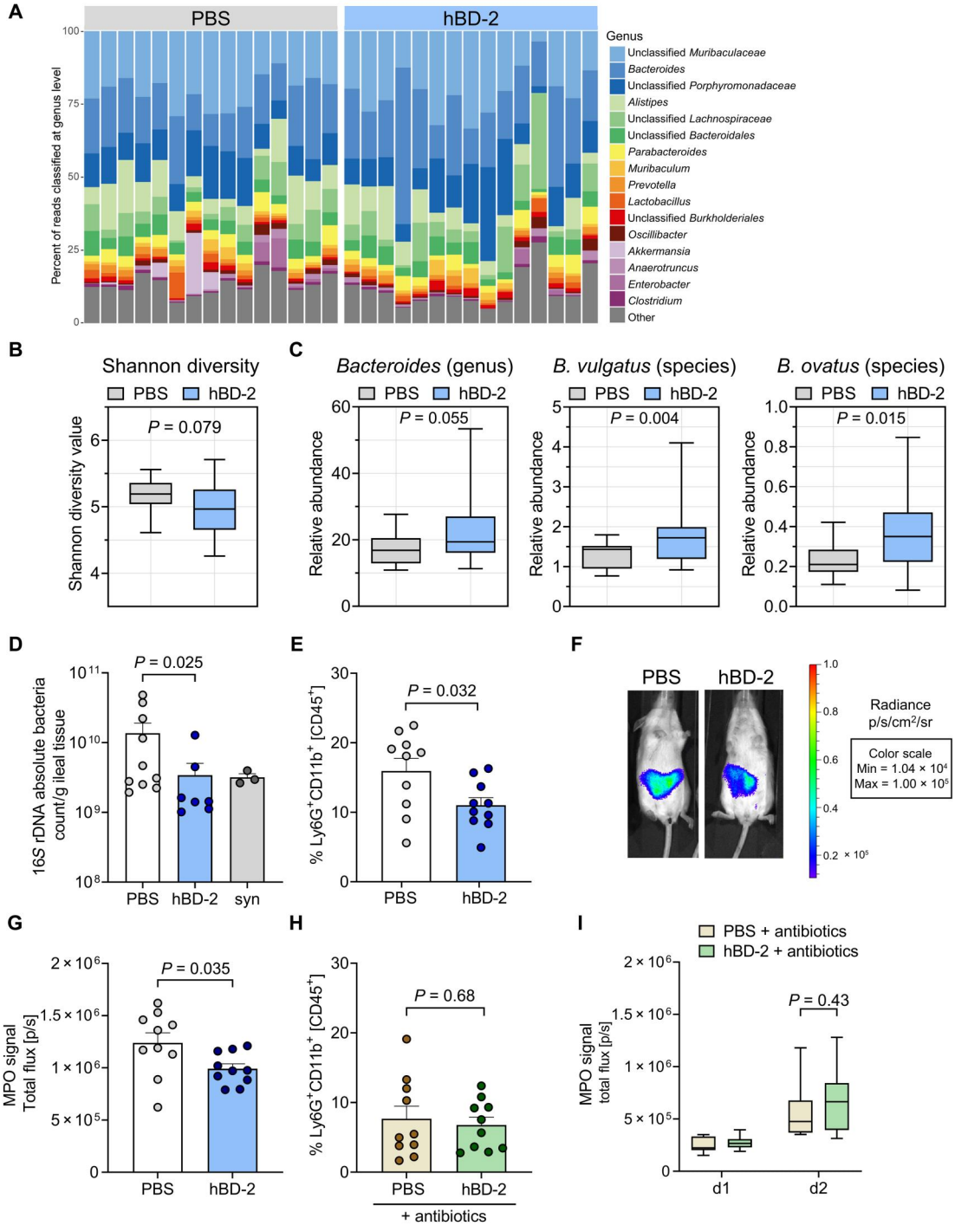

Fig. 2. hBD-2 reduces intestinal neutrophil infiltration in a microbiome-dependent manner. The allo-HCT major mismatch model C57BL/6➔BALB/c was used. Mice were treated once daily with hBD-2 or PBS. Data were pooled from two or three independent experiments. (**A** to **C**) Taxonomic microbial composition (A) and Shannon diversity index (B) were determined in the feces of PBS- and hBD-2–treated mice ($n = 15$ per group) isolated on d7 and d8 after allo-HCT. (C) Relative abundance of specific genera and strains in hBD-2–treated and PBS-treated aGVHD mice. *P* values of Wilcoxon signed-rank tests are shown. (**D**) Bacterial infiltration in the ileal lamina propria on d7 after allo-HCT or after syngeneic HCT (syn; BALB/c➔BALB/c) was studied by 16*S* ribosomal DNA (rDNA) qPCR. Data were analyzed by Mann-Whitney *U* test. (**E**) The percentage of $CD11b^+Ly6G^+$ neutrophils of total $CD45^+$ cells in the ileum on d3 after allo-HCT was assessed by flow cytometry ($n = 10$ per group). Data were analyzed by unpaired two-tailed Student's *t* test. (**F**) Representative images are shown of MPO bioluminescence signal after injection of luminol in PBS- or hBD-2–treated mice on d2 after allo-HCT. (**G**) Quantification of MPO signal in mice from (F) ($n = 10$ per group), analyzed by unpaired two-tailed Student's *t* test. (**H**) Percentage of $CD11b^+Ly6G^+$ neutrophils of total $CD45^+$ cells in the ilea of mice pretreated with antibiotics ($n = 10$ per group) on d3 after allo-HCT as assessed by flow cytometry. Data were analyzed by unpaired two-tailed Student's *t* test. (**I**) Quantification of MPO bioluminescence signal on d1 and d2 after allo-HCT in mice pretreated with antibiotics ($n = 10$ per group). Data were analyzed by repeated measures two-way ANOVA with Sidak's multiple comparisons post hoc test.

hBD-2–treated mice, as assessed by in vivo MPO imaging using luminol (Fig. 2, F and G) (*18*, *19*). Functional characterization revealed reduced TNF, but not reactive oxygen species (ROS), production in splenic neutrophils of hBD-2–treated aGVHD mice (fig. S6, A and B). In contrast to neutrophils, the proportion and activation of intestinal dendritic cells (fig. S6, C to E) and macrophages (fig. S6, F to H) were not affected by hBD-2 treatment. To study whether the reduction in neutrophils was dependent on the observed effects of hBD-2 on the intestinal microbiota, we used an antibiotic cocktail to decontaminate the mice before allo-HCT (fig. S7A). The reduced neutrophil infiltration of the ileum by hBD-2 treatment was abrogated when the mice had been treated with antibiotics (Fig. 2H). Similarly, hBD-2 did not affect the lower abdominal MPO signal in mice treated with antibiotics (Fig. 2I). In line with a microbiome-dependent and durable effect, neutrophil reduction by hBD-2 was still detectable in mice at day 14 after allo-HCT (fig. S7B). Histopathology of acute GVHD target organs revealed that hBD-2 still significantly reduced aGVHD severity in the colon ($P = 0.036$) but not in the ilea of decontaminated mice (fig. S7C). Together, these data indicate that the effects of hBD-2 on intestinal neutrophil infiltration rely on an intact microbiome.

hBD-2 dampens allogeneic T cell responses in vivo and in vitro

Our data suggest that hBD-2–induced aGVHD amelioration does not entirely depend on its influence on the microbiome. Acute GVHD is characterized by an allogeneic T cell response that is mainly T_H1 cell–driven (*1*). To clarify whether hBD-2 influences allogeneic T_H1 responses in aGVHD mice, we analyzed allogeneic (H-$2K^{b+}$) T cells from the spleens of allo-HCT recipient mice for T_H1 signature cytokine production. We found that the proportion of both TNF-producing (Fig. 3, A and B) and IFN-γ–producing (fig. S8, A and B) allogeneic $CD4^+$ T cells were decreased in hBD-2–treated GVHD mice as compared with PBS-treated GVHD mice. Similarly, TNF and IFN-γ production in allogeneic $CD8^+$ T cells was reduced by hBD-2 treatment (fig. S8, C and D). Furthermore, oral hBD-2 treatment decreased in vivo T cell proliferation of $CD4^+$ or $CD8^+$ T cells after allo-HCT (Fig. 3C and fig. S8E), although T cell activation, as measured by CD69 expression, was not reduced after hBD-2 treatment (fig. S8, F and G). In contrast, we did not find changes in the numbers of $FoxP3^+$ regulatory T cells, IL-4^+ T_H2 cells, IL-$17A^+$ T_H17 cells, or the naïve and memory T cell phenotype in hBD-2–treated mice compared with vehicle control mice (fig. S8, H to J).

We next aimed to determine the effects of hBD-2 on the allogeneic T cell response in a well-controllable system in vitro using coculture assays of BALB/c BM-derived dendritic cells (BMDCs) with allogeneic C57BL/6 T cells. In agreement with the in vivo data from the aGVHD model, hBD-2 treatment reduced allogeneic $CD4^+$ and $CD8^+$ T cell proliferation in a dose-dependent manner (Fig. 3D and fig. S9A). Furthermore, T cell activation was decreased by hBD-2 treatment (Fig. 3E and fig. S9B). In contrast, hBD-2 did not affect expression of costimulatory molecules, MHC II, or proinflammatory cytokines in BMDCs (fig. S9, C to F). Therefore, we next analyzed whether hBD-2 directly affected T cells using a BMDC-independent proliferation assay that relies on T cell stimulation by anti-CD3/anti-CD28 microbeads. hBD-2 also reduced $CD4^+$ and $CD8^+$ T cell proliferation in this stimulation model, indicating a BMDC-independent, direct effect of hBD-2 on T cell proliferation (Fig. 3F and fig. S10A). T cell viability was not affected by hBD-2 treatment (fig. S10, B and C). In contrast, we found that hBD-2 reduced cell cycle progression in $CD4^+$ and $CD8^+$ T cells in vitro, with an increased proportion of hBD-2–treated T cells in the G_0 phase (Fig. 3, G and H, and fig. S10D). T cell inhibition by hBD-2 was independent of CCR2 and CCR6, which have been previously described as interaction partners for hBD-2 (fig. S11, A to H) (*15*, *20*–*22*).

We next investigated whether hBD-2 could also restrain T cell responses of defined specificity by using OT-1 mice, which carry a transgenic TCR recognizing ovalbumin peptide residues 257 to 264 (SIINFEKL; referred to as OVA) in the context of H-$2K^b$, resulting in MHC I–restricted, OVA-specific $CD8^+$ T cells (OT-1 cells) (*23*). In line with our previous data, hBD-2 treatment reduced OT-1 cell proliferation (Fig. 3I), activation (fig. S11I), and IFN-γ secretion (fig. S11J) after stimulation with OVA–MHC I tetramers.

Because hBD-2 is a human peptide, we also studied the effect of its murine ortholog mBD-4 on murine T cell responses in vitro. Comparable to hBD-2, mBD-4 treatment reduced $CD8^+$ T cell proliferation and $CD4^+$ T cell activation without affecting T cell viability (Fig. 3J and fig. S12). Together, these data indicate that hBD-2 and its murine ortholog have direct inhibitory effects on T cells.

hBD-2 treatment does not interfere with the GVL effect in vivo

The therapeutic efficiency of allo-HCT considerably depends on the GVL activity of donor T cells. On the basis of the inhibitory effects hBD-2 exerted on the T cell response, we next studied whether hBD-2 treatment interferes with the GVL effect using a cell line–based leukemia model [Ba/F3 FMS-like tyrosine kinase 3–internal tandem duplication (FLT3-ITD)-luciferase (luc)$^+$] and a genetic acute myeloid leukemia (AML) model [FLT3-ITD/mixed lineage leukemia–partial tandem duplication (MLL-PTD)]. To mimic the clinical situation, mice received leukemia cells of the same genetic background (BALB/c) as the recipient mice, together with allogeneic (C57BL/6) BM cells and allogeneic T cells. Mice receiving only BM cells without T cells served as BM controls. In both GVL models, mice only developed mild to moderate aGVHD and primarily died of leukemia (fig. S13, A and B, and table S1). In the Ba/F3 FLT3-ITD-luc^+ model, mice receiving allogeneic T cells showed reduced leukemia cell expansion compared with the BM controls as assessed by bioluminescence imaging (Fig. 4, A and B), demonstrating a functional GVL effect and resulting in improved survival (Fig. 4C). Treatment with hBD-2 did not reduce the GVL activity of allogeneic T cells compared with PBS treatment (Fig. 4, A to C). We confirmed these findings in the FLT3-ITD/MLL-PTD AML model. Leukemia burden was reduced and survival prolonged in mice that received allogeneic T cells, and this GVL effect was preserved in mice treated with hBD-2 (Fig. 4, D and E, and fig. S13, C and D). In line with these findings, hBD-2 treatment did not affect the expression of the cytotoxic markers CD107A, granzyme B, and perforin in $CD8^+$ T cells after allo-HCT in vivo (fig. S13, E to G). Our data indicate that T cell cytotoxicity and the GVL effect after allo-HCT are not impaired by hBD-2 therapy.

hBD-2 regulates the intestinal T cell response during aGVHD in mice

Because hBD-2 did not interfere with the GVL effect in vivo, we next asked whether it locally affects the T cell response in the GI

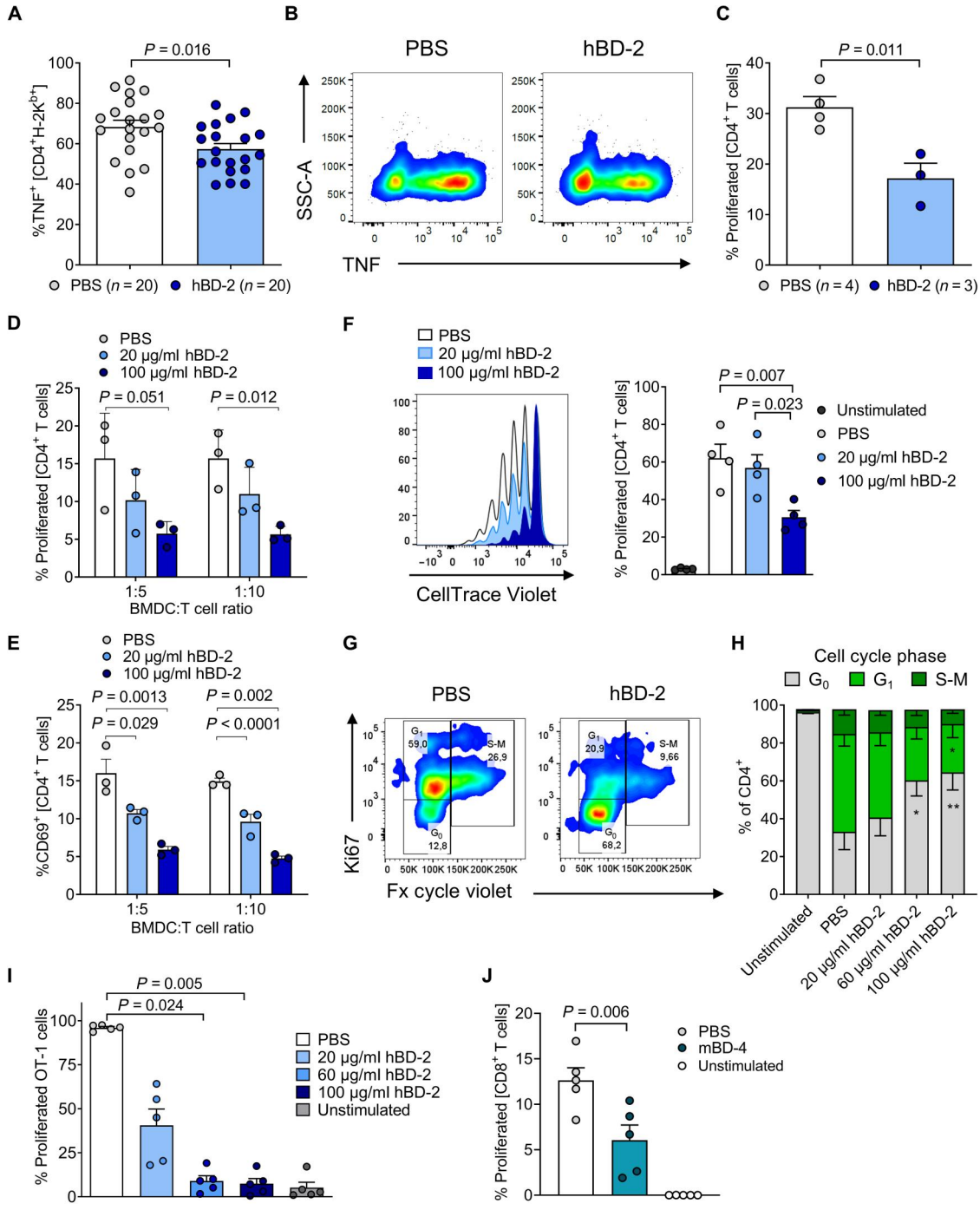

Fig. 3. hBD-2 dampens T cell responses in vivo and in vitro. (**A**) TNF expression in allogeneic splenic CD4$^+$H-2K^{b+} T cells from PBS- or hBD-2–treated mice on d8 after allo-HCT (C57BL/6➔BALB/c; $n = 20$ per group) was assessed by flow cytometry. Groups were compared using unpaired two-tailed Student's t test. (**B**) Representative flow cytometry plots of TNF expression gated on CD4$^+$H-2K^{b+} T cells. SSC-A, side scatter area. (**C**) In vivo splenic CD4$^+$ T cell proliferation was measured 48 hours after transplantation of CellTrace Violet–stained allogeneic (C57BL/6) T cells in BALB/c recipient mice treated with PBS or hBD-2. (**D** and **E**) Murine BMDCs (BALB/c) were cocultured with allogeneic T cells (C57BL/6) in the indicated ratios and exposed to PBS or hBD-2. CD4$^+$ T cell proliferation (D) and CD69 expression (E) are shown; data were pooled from three independent experiments and analyzed using one-way ANOVA. (**F**) CD4$^+$ T cell proliferation after stimulation with anti-CD3/anti-CD28 microbeads and treatment with PBS or hBD-2 was assessed by CellTrace Violet staining. Representative flow cytometry plot (left) and pooled data from four independent experiments analyzed by one-way ANOVA (right). (**G** and **H**) Cell cycle analysis of murine splenic CD4$^+$ T cells after stimulation with anti-CD3/anti-CD28 microbeads and treatment with hBD-2. Representative flow cytometry plot of cells treated with PBS or hBD-2 (60 µg/ml) (G) and pooled data from four independent experiments analyzed by two-way ANOVA (H). (**I**) OT-1 cells were stimulated with OVA-MHC tetramers in the presence of PBS or hBD-2, and cell proliferation was assessed by CellTrace Violet dye dilution. Pooled data are shown from five independent experiments analyzed by Kruskal-Wallis test. (**J**) Proliferation of CD8$^+$ T cells stimulated with anti-CD3/anti-CD28 microbeads and treated with mBD-4 (10 µg/ml) or PBS was assessed by CellTrace Violet dye dilution. Data were pooled from five independent experiments and analyzed by one-way ANOVA.

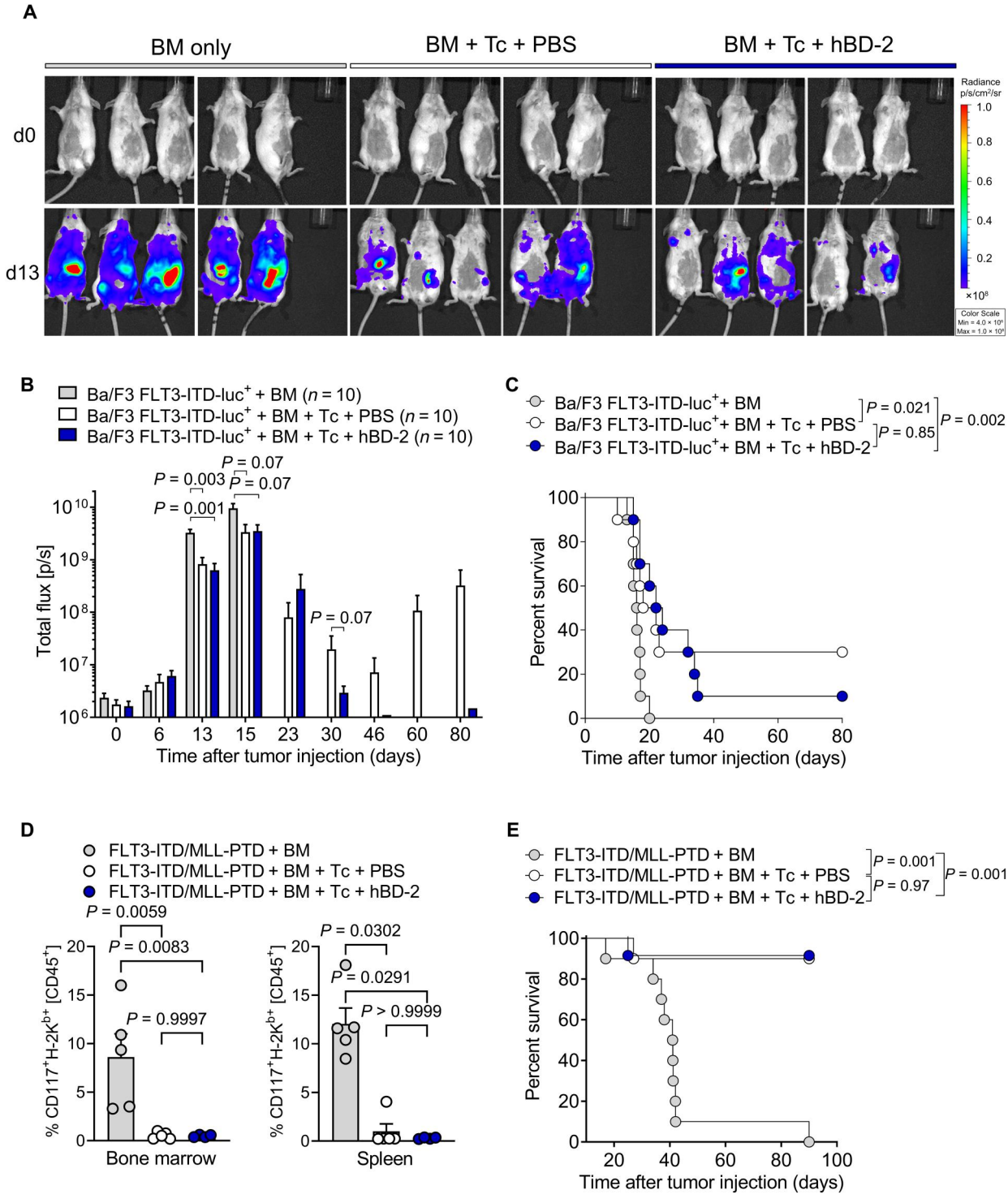

Fig. 4. hBD-2 treatment does not interfere with the GVL effect. (**A** to **C**) BALB/c recipient mice ($n = 9$ or 10 per group) received allogeneic C57BL/6 BM cells and Ba/F3 FLT3-ITD-luc$^+$ leukemia cells with or without additional C57BL/6 T cells (Tc) and were orally treated with PBS or hBD-2 from d2 until d12. Leukemia cell expansion was assessed by bioluminescence imaging (BLI). Representative images from d0 and d13 (A) and quantification of BLI signal (B) from two independent experiments are shown. *P* value was calculated using mixed-effects model (repeated measures two-way ANOVA for missing values). (C) Percentage survival of mice. Pooled data from two independent experiments analyzed by log-rank test. (**D** and **E**) C57BL/6 recipient mice ($n = 10$ per group) received allogeneic BALB/c BM cells and FLT3-ITD/MLL-PTD AML cells with or without additional BALB/c Tc. Leukemia cells (CD117$^+$H-2K^{b+}) in BM (left) and spleen (right) were quantified by flow cytometry on d24 after allo-HCT (D). Statistics by one-way ANOVA with Tukey's multiple comparisons post hoc test [(D) BM] or by Kruskal-Wallis test with Dunn's multiple comparisons post hoc test [(D) spleen] in case of nonparametric distribution. (E) Percentage survival from two independent mouse experiments analyzed by log-rank test.

tract as a major target of aGVHD (*24*) and physiological production site of hBD-2 (*25*). Using flow cytometry, we found reduced immune infiltration of $CD45^+$ leukocytes in the colons and ilea of GVHD mice treated with hBD-2 as compared with vehicle-treated mice (Fig. 5A). With respect to the T cell response, we found reduced proportions of allogeneic $CD4^+H\text{-}2K^{b+}$ T cells infiltrating the ileum epithelium after hBD-2 treatment (Fig. 5B). These effects on the epithelium were durable and still present on day 14 after allo-HCT (fig. S14, A and B). Similarly, hBD-2 reduced immune cell infiltration in the liver as another major target organ of aGVHD, although the allogeneic $CD4^+$ T cell phenotype in the liver remained largely unchanged (fig. S14, C to H). Using three-dimensional intestinal organoid cultures, we next studied the effect of hBD-2 on intestinal tissue destruction by allogeneic T cells ex vivo. PBS- and hBD-2–treated organoids did not display any gross morphological differences (fig. S15A). However, coculture of organoids with allogeneic T cells caused organoid destruction, which could be prevented by exposing the cultures to hBD-2, resulting in an improved organoid disintegration score (Fig. 5, C and D).

To dissect the gene networks regulated by hBD-2 during aGVHD in the gut in vivo, we performed microarray-based gene expression analysis from the intestine of GVHD mice treated with PBS or hBD-2. We found that genes involved in the inflammatory response and cell migration were down-regulated in the intestine of hBD-2–treated as compared with PBS-treated GVHD mice (Fig. 5E and fig. S15B), although T cell migration toward CXCL12 in vitro was unaffected (fig. S15C). To decipher molecular pathways affected by hBD-2 treatment in the gut, we performed gene set enrichment analysis, which revealed that the TCR and TCR signaling pathway gene sets were among the top 10 down-regulated pathways in the intestines of hBD-2–treated GVHD mice (Fig. 5F). Our data suggest that hBD-2 treatment reduces allogeneic T cell infiltration and migration in the GI tract after allo-HCT.

hBD-2 dampens TCR signaling and T cell metabolism

Given the observation that hBD-2 treatment reduced expression of genes involved in TCR signaling in the guts of aGVHD mice, we asked whether hBD-2 was able to directly regulate TCR signaling. Microarray analysis from primary murine T cells exposed to PBS or hBD-2 in vitro revealed that transcripts belonging to the TCR signaling gene set were down-regulated in T cells treated with hBD-2 (Fig. 6A). Because TCR ligation triggers a chain of phosphorylation events, we next performed kinome profiling to corroborate our gene expression data. PamChip tyrosine kinase activity arrays were used to assess differences in kinase activity in T cells stimulated through the TCR and CD28 in the presence of hBD-2 or PBS. Combinatorial analysis of phosphorylation of 147 peptides that passed quality control revealed a hierarchical list of predicted tyrosine kinases that were inhibited by hBD-2 treatment (Fig. 6B). In agreement with our previous findings, many kinases involved in proximal TCR signaling were among the top 20 kinases down-regulated in hBD-2–exposed T cells. These included ZAP70, Syk, and the Src family kinase Yes (Fig. 6B). Phosphoflow-based analysis revealed reduced phosphorylation of the CD3ζ chain, indicating a very early block in TCR signaling by hBD-2 (Fig. 6, C and D); however, Syk phosphorylation was not significantly different (fig. S16, A and B; $P = 0.09$).

TCR ligation triggers an increase in intracellular Ca^{2+} concentration, which is crucial for nuclear factor of activated T cell (NFAT) translocation to the nucleus and T cell activation. In agreement, Ca^{2+} influx and NFAT activity were reduced in hBD-2–treated compared with PBS-treated T cells upon stimulation (fig. S16, C and D). Furthermore, hBD-2 reduced extracellular signal–regulated kinase phosphorylation (fig. S16E) and IL-2 production (Fig. 6E) as functional readout for TCR signaling. Surface staining confirmed that the hBD-2 effects were not caused by a down-regulation of TCR, CD3, or CD28 surface expression or up-regulation of programmed cell death protein 1 surface expression (fig. S17). Because Syk and different Src kinases are also involved in B cell receptor signaling, we next studied the effect of hBD-2 on B cells in vitro. hBD-2 did not affect anti–immunoglobulin M antibody–induced B cell proliferation, activation, or Syk phosphorylation, pointing toward a TCR-specific effect (fig. S18).

T cell activation and effector function require a metabolic shift from the previously quiescent state, and TCR signaling is involved in this metabolic reprogramming (*26*, *27*). Microarray analysis revealed that hBD-2 reduced expression of glycolysis and oxidative phosphorylation (OXPHOS)–related genes in primary murine T cells (fig. S19A). Functional metabolic assays confirmed that hBD-2 treatment reduced the extracellular acidification rate as a parameter of glycolytic activity (Fig. 6, F and G, and fig. S19, B and C). Furthermore, we found a reduction in the maximal respiration rate in hBD-2–treated T cells as assessed by a mitochondrial stress test (Fig. 6, H and I). Together, our findings indicate that hBD-2 regulates proximal TCR signaling in murine T cells, which is associated with a reduction in metabolic activity in these cells, including glycolysis and OXPHOS.

Primary human T cell responses are constrained by hBD-2 treatment

To validate our findings made in the mouse model in human T cells, we next analyzed how treatment with hBD-2 affects allogeneic T cell proliferation in coculture assays using human monocyte-derived dendritic cells (moDCs) and allogeneic primary human T cells isolated from peripheral blood mononuclear cells (PBMCs) of a distinct donor. In line with our data from the murine system, exposure to hBD-2 reduced allogeneic $CD4^+$ and $CD8^+$ T cell proliferation (Fig. 7, A and B). To test whether the effect of hBD-2 was directly on the human T cells or indirectly mediated through regulating moDC function, we assessed the effect of hBD-2 on human T cells stimulated with anti-CD3/anti-CD28 microbeads. hBD-2 also reduced T cell proliferation in this moDC-independent proliferation assay (Fig. 7, C and D). Cell viability was not affected by hBD-2 treatment (fig. S20).

We next performed kinome profiling from human primary T cells. In agreement with the data from murine T cells (Fig. 6B), multiple tyrosine kinases involved in proximal TCR signaling were among the top 20 kinases down-regulated in hBD-2–treated human T cells (Fig. 7E), including ZAP70, SYK, and the Src family members Yes, Src, Lyn, Fyn, and Lck (Fig. 7E). Western blot and phosphoflow analysis confirmed reduced ZAP70 phosphorylation in both $CD4^+$ and $CD8^+$ T cells (Fig. 7, F and G) and reduced Src family phosphorylation in $CD8^+$ T cells (Fig. 7, H and I). As a whole, these data indicate that hBD-2 is able to dampen proliferation and proximal TCR signaling in both human and murine T cells.

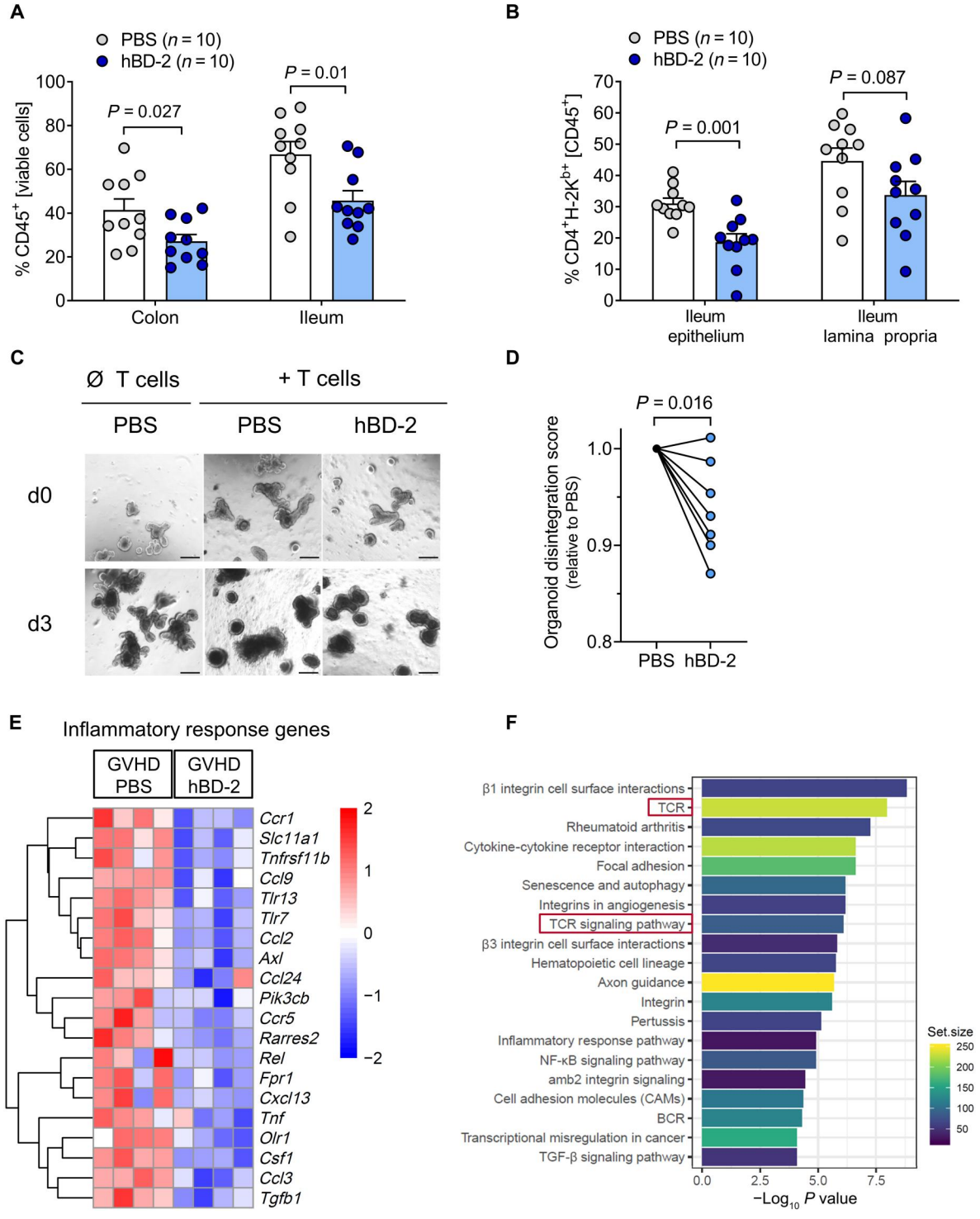

Fig. 5. hBD-2 regulates intestinal aGVHD. (**A** and **B**) Intestinal cells from recipient mice (C57BL/6➔BALB/c, *n* = 10 per group) treated daily with PBS or hBD-2 were analyzed by flow cytometry on d7 after allo-HCT. Infiltration of $CD45^+$ cells in the colon and ileum (A) and infiltration of allogeneic $CD4^+H\text{-}2K^{b+}$ T cells in the ilea of recipient mice (B) are shown. Data were pooled from two independent experiments and analyzed using unpaired two-tailed Student's *t* test. (**C**) Representative images of small intestinal organoids cultured with or without allogeneic T cells in the presence of PBS or hBD-2 on d0 and d3 of coculture; scale bars, 200 μm. (**D**) Quantification of organoid disintegration score 3 days after coculture with allogeneic T cells. Data were pooled from seven independent experiments and analyzed by one-sample *t* test. (**E** and **F**) Microarray results are shown for colon samples of recipient mice (C57BL/6➔BALB/c, *n* = 4 per group). (E) The top 20 down-regulated genes by hBD-2 treatment in the inflammatory response pathway gene set are depicted. (F) The top 20 gene sets down-regulated in the hBD-2–treated group as assessed by gene set enrichment analysis are shown. *X* axis depicts $-\log_{10}$ of the adjusted *P* value, and color code represents the gene set size. The red boxed gene sets highlight TCR- and TCR signaling–associated pathways. BCR, B cell receptor; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TGF-β, transforming growth factor–β.

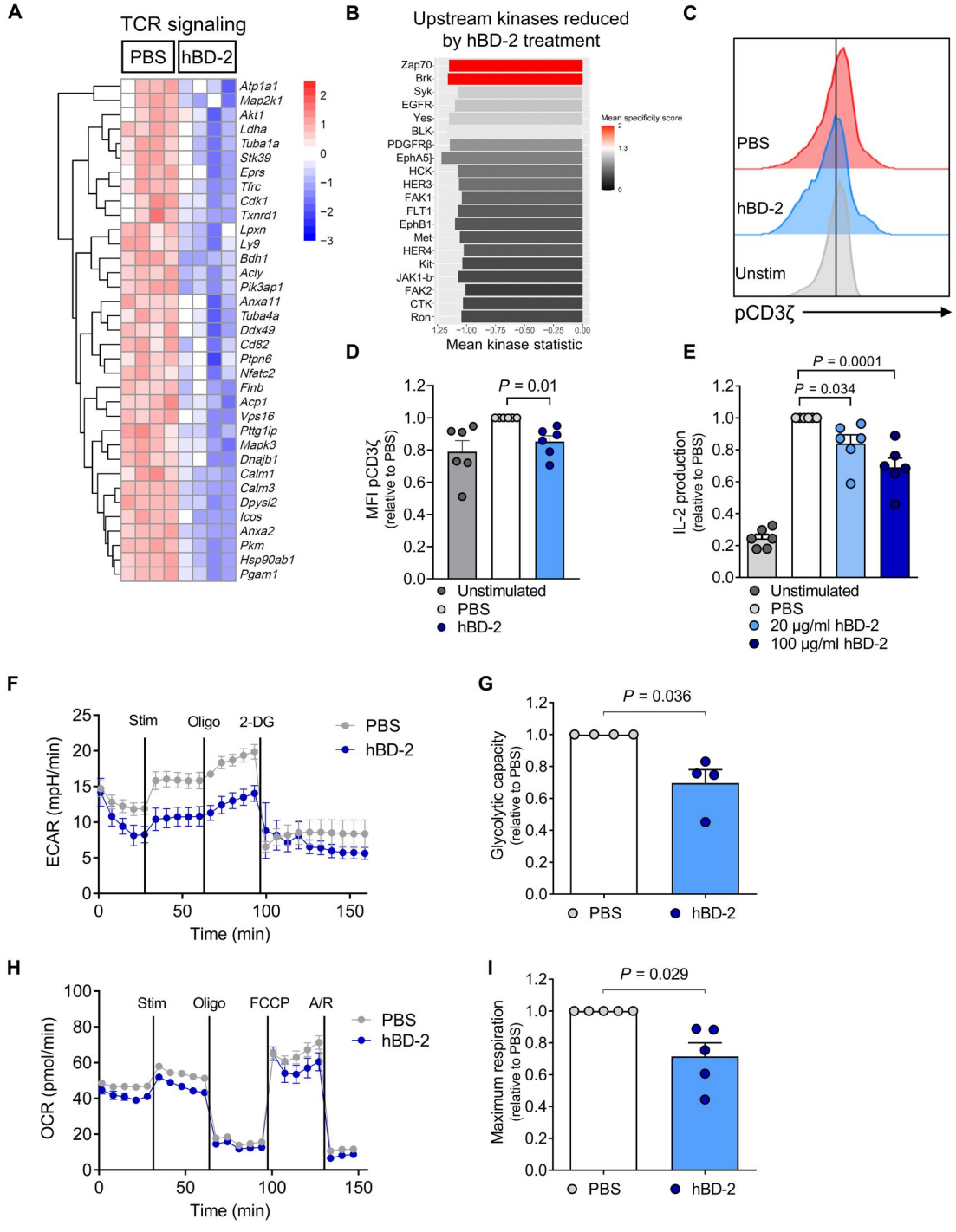

Fig. 6. hBD-2 reduces proximal TCR signaling and metabolic activity in murine T cells. (**A**) Microarray of murine splenic T cells after stimulation with anti-CD3/anti-CD28 microbeads and treatment with PBS or hBD-2 (60 μg/ml) ($n = 4$ per group). The top 35 down-regulated genes by hBD-2 treatment in the TCR gene set are depicted. (**B**) Kinase activity array of murine splenic T cells pretreated with PBS or hBD-2 (60 μg/ml) and stimulated with anti-CD3/anti-CD28 microbeads ($n = 2$ per group). Upstream kinases with reduced activity after hBD-2 treatment are shown. (**C** and **D**) Murine $CD4^+$ and $CD8^+$ T cells were pretreated with PBS or hBD-2 (20 μg/ml) followed by stimulation with anti-CD3/anti-CD28 microbeads for 5 min followed by phosphoflow analysis of pCD3ζ. Representative image of pCD3ζ signal (C) and quantification of pCD3ζ median fluorescence activity (MFI) (D). Data were pooled from six independent experiments and analyzed using one-sample *t* test. (**E**) IL-2 enzyme-linked immunosorbent assay of cell culture supernatant from T cells stimulated with anti-CD3/anti-CD28 microbeads and treated with PBS or hBD-2 for 24 hours. Data were pooled from six independent experiments and analyzed using one-way ANOVA. (**F** to **I**) Murine splenic T cells treated with PBS or hBD-2 (60 μg/ml) and stimulated with anti-CD3ε antibody were analyzed using Seahorse assay. Data were pooled from four or five independent experiments and analyzed by one-sample *t* test. Representative image of extracellular acidification rate (ECAR) (F), quantification of glycolytic capacity (G), representative image of oxygen consumption rate (OCR) (H), and quantification of maximum respiration (I) are shown. Oligo, oligomycin; 2-DG, 2-deoxy-D-glucose; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; A/R, antimycin/rotenone; Stim, stimulated; Unstim, unstimulated.

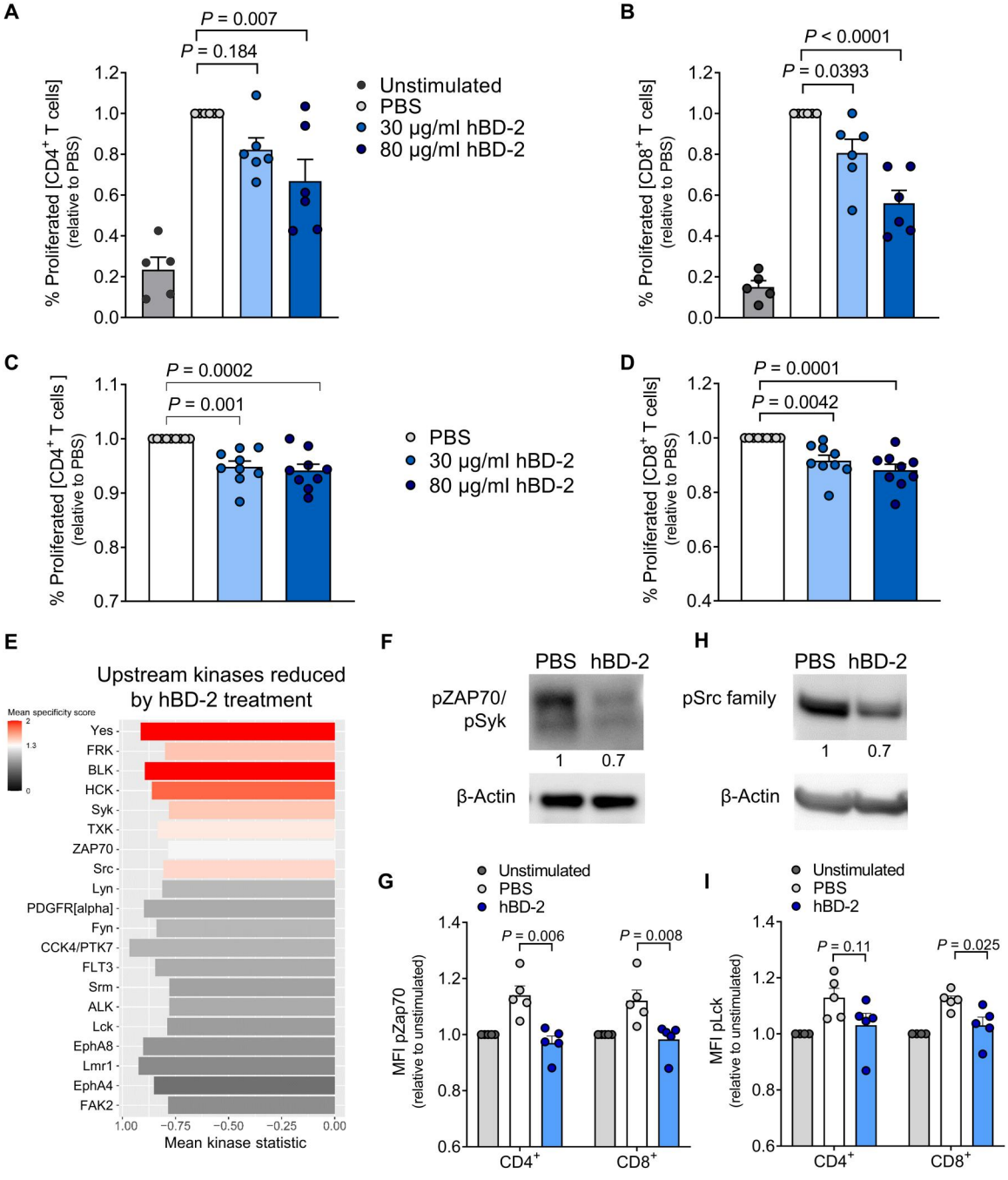

Fig. 7. hBD-2 reduces proximal TCR signaling in human T cells. (**A** and **B**) In vitro coculture of human T cells and human allogeneic moDCs in the presence of PBS or hBD-2. Proliferation of $CD4^+$ T cells (A) or $CD8^+$ T cells (B) was assessed using CellTrace Violet staining. Data were pooled from five independent experiments and analyzed by one-way ANOVA. (**C** and **D**) Proliferation of human $CD4^+$ T cells (C) or $CD8^+$ T cells (D) stimulated with anti-CD3/anti-CD28 microbeads was quantified by CellTrace Violet dye dilution and analyzed by one-way ANOVA. (**E**) Kinase activity array of human T cells pretreated with PBS or hBD-2 (60 µg/ml) and stimulated with anti-CD3/anti-CD28 microbeads for 15 min ($n = 3$ per group). Upstream kinases with reduced activity after hBD-2 treatment are shown. (**F**) Human T cells were treated as in (E), and pZAP70/pSyk expression was assessed by Western blot. (**G**) Human T cells were pretreated with PBS or hBD-2 (60 µg/ml) followed by stimulation with anti-CD3/anti-CD28 microbeads for 5 min followed by phosphoflow analysis of pZap70. (**H**) Human T cells were treated as in (E), and pSrc family expression was assessed by Western blot. In (F) and (H), representative blots and mean band intensity of biological triplicates normalized to PBS control are shown. (**I**) Human T cells were treated as in (G), followed by phosphoflow analysis of pLck. Pooled data from five independent experiments analyzed by unpaired two-tailed Student's *t* test [(I) and CD4 (G)] or Mann-Whitney *U* test [CD8 (G)].

DISCUSSION

β-Defensins are endogenous peptides produced in epithelial cells that have both antimicrobial and immunomodulatory functions. Using human samples and murine models, we observed that aGVHD is characterized by a lack of β-defensin induction in the intestine. We found that hBD-2 has promising therapeutic activity in aGVHD and is able to limit neutrophil recruitment to the ileum and regulate T cell responses through blockade of proximal TCR signaling.

hBD-2 expression is physiologically up-regulated in the skin and intestine in response to bacterial stimuli and proinflammatory cytokines (*13*, *14*). In line with this, hBD-2 concentrations are strongly increased in the inflamed intestines of patients with UC (*14*, *28*). In contrast, hBD-2 expression is not induced in the intestines of patients with Crohn's disease (*14*). We observed a similar lack of hBD-2 induction in two independent patient cohorts with acute GI-GVHD in this study. Nucleotide-binding oligomerization domain containing 2 (NOD2) mediates induction of hBD-2, and NOD2 variants are associated with transplantation-related mortality and severe GVHD after allo-HCT and with Crohn's disease (*29*, *30*). Despite the strong intestinal inflammation in patients with Crohn's disease or acute GI-GVHD, hBD-2 seems to be inappropriately up-regulated in these diseases, potentially contributing to enhanced inflammation and dysbiosis.

To date, little is known about the role of defensins during aGVHD. Eriguchi *et al.* (*31*, *32*) described that Paneth cells are targeted and markedly reduced during murine GVHD, resulting in decreased α-defensin production. In patients, the expression of defensins during GVHD has been analyzed in only a few studies with limited patient numbers and with controversial results regarding the correlation of defensin expression and GVHD patient outcomes (*33*–*35*). The therapeutic effect of the α-defensin cryptdin-4 (Crp-4) has been investigated in an aGVHD mouse model. Oral Crp-4 administration was able to prevent GVHD-related dysbiosis but failed to reduce GVHD-related mortality (*36*). Although these studies mostly focus on α-defensins, the role and therapeutic potential of β-defensins during GVHD has not been studied.

Using different mouse models, we found that hBD-2 treatment is highly effective in reducing aGVHD severity and mortality in mice while preserving the GVL effect. Mechanistically, hBD-2 affected the allogeneic T cell response, the intestinal microbiota, and intestinal neutrophil infiltration. Previous work has revealed a role for neutrophils in the pathogenesis of aGVHD. Neutrophils infiltrate the murine ileum early after allo-HCT in response to bacterial translocation across the intestinal mucosal barrier and contribute to GVHD pathogenesis through ROS production (*5*, *6*). Furthermore, they are activated to express MHC II and present allo-antigens to donor T cells in the mesenteric lymph nodes, further amplifying the graft-versus-host reaction (*6*). Depleting neutrophils using monoclonal antibodies or treatment with antimicrobial antibodies improved survival of aGVHD mice (*5*, *6*). However, neutrophil depletion is not a feasible strategy in the clinic, and a strategy to specifically limit neutrophil recruitment to the ileum would be preferred.

We found that hBD-2 reduced the infiltration of the intestinal lamina propria with bacteria in aGVHD mice, indicating decreased bacterial translocation across the intestinal mucosal barrier. Furthermore, hBD-2 treatment diminished ileal neutrophil infiltration in vivo, which was dependent on the microbial flora and not observed in antibiotic-treated mice. Although previous studies have reported an association between intestinal microbial diversity after allo-HCT and aGVHD-related mortality (*37*, *38*), we did not observe increased microbial diversity in hBD-2–treated mice compared with PBS-treated mice using shotgun metagenomic sequencing. Moreover, hBD-2 treatment did not result in the classical shift toward *Enterococcus* and loss of *Clostridia* species observed in patients and mice with aGVHD (*38*–*40*); rather, we observed changes in specific genera and species, including an increase in *Bacteroides* species and a decrease in *Ruminococcaeae* species. These changes might directly contribute to reduced intestinal inflammation in hBD-2–treated aGVHD mice. Several studies have indicated a beneficial role of *Bacteroides* in the context of GVHD development (*41*). In mice, reduced abundance of *Bacteroides* in the intestinal microbiota was associated with more severe GVHD (*17*, *31*). Similarly, the abundance of *Bacteroides* was lower in patients undergoing allo-HCT who developed aGVHD than in patients without GVHD (*42*–*44*). Furthermore, the microbial shift caused by hBD-2 might affect the recruitment of neutrophils to the ileum, thereby indirectly contributing to reduced GVHD severity. Our finding that hBD-2 only reduced neutrophil recruitment and lower abdominal MPO signal in GVHD mice with an intact microbial flora supports this hypothesis.

Prior studies have suggested that, in addition to its antimicrobial function, hBD-2 can also modulate myeloid immune cell migration and function (*15*, *20*). Koeninger *et al.* (*15*) have recently described an immunoregulatory function of hBD-2 by reducing proinflammatory cytokine production in dendritic cells. Here, we found that hBD-2 is able to regulate T cell proliferation, activation, migration, and cytokine production in APC-independent stimulation models, supporting the concept that hBD-2 has a direct impact on T cells that is independent of APCs. The two best-described receptors through which hBD-2 influences myeloid immune cell migration and function are CCR2 and CCR6 (*15*, *20*–*22*). However, our data suggest that hBD-2 dampens T cell responses in a TCR-dependent manner and independently of the presence of CCR2 and CCR6. Transcriptome and kinome profiling revealed a reduction of genes and activity of kinases involved in TCR signaling in hBD-2–treated murine and human T cells. Intriguingly, activation, proliferation, and Syk phosphorylation in B cells were not affected by hBD-2 treatment. These findings suggest that hBD-2 might not affect Syk/Src kinase signaling in all cell types but may potentially interact specifically with the TCR/CD3 complex. A link between TCR activation and metabolic reprogramming, resulting in an induction of aerobic glycolysis and OXPHOS, has been previously reported (*27*, *45*–*47*). In line with these studies, we found that reduced TCR signaling translated into decreased glycolysis, OXPHOS, and IL-2 production in hBD-2–exposed T cells. This T cell inhibitory function of hBD-2 could potentially be a physiological regulation mechanism to dampen and terminate exuberant T cell responses against self-antigens or commensal microbiota at epithelial surfaces, which necessitates further investigation.

There are several limitations to the current study that should be addressed in the future. First, although we found a beneficial effect of hBD-2 on disease severity in aGVHD mouse models, the effect should furthermore be assessed in combined aGVHD/GVL models, where modest to severe aGVHD develops and leukemia still relapses in some of the recipients. Second, further investigations are required

to identify the receptor on T cells to which hBD-2 binds and potential intracellular interaction partners. Third, future in vivo imaging studies using luc$^+$ donor T cells would further substantiate our finding that hBD-2 reduced donor T cell migration to the intestine. Last, although we found that hBD-2 treatment induced an increase in *Bacteroides* species and reduced neutrophil infiltration of the ileum only in mice with an intact microbiome, identifying the specific bacteria, potential microbial products, and mechanisms linking these two findings will be an important aim of future investigations.

In summary, our study revealed that human and murine aGVHD are associated with a lack of intestinal hBD-2 induction. Recombinant hBD-2 replacement therapy ameliorated aGVHD and decreases allogeneic T cell proliferation by dampening of TCR signaling while sparing the GVL effect. We found that hBD-2 did not affect expression of T cell cytotoxic markers, was most highly concentrated in the ileum and colon, and primarily affected intestinal T cells in vivo, which might be reasons for preservation of the GVL effect. The recombinant hBD-2 used in this study is now being optimized for industrial scale production. Pilot activities for good manufacturing practice production are ongoing, and first in human studies are expected in 2024. Our study suggests that early prophylactic treatment after allo-HCT is required to compensate for the loss of endogenous hBD-2. Because we found that hBD-2 has an effect on the early recruitment of neutrophils to the gut, we think that it is a reasonable clinical strategy to administer hBD-2 orally early during the transplantation procedure, specifically from days 0 to 14 relative to allo-HCT. Our data provide a rationale for the further clinical development of hBD-2–based prophylactic strategies for patients with aGVHD.

MATERIALS AND METHODS

Study design

To investigate the functional role of hBD-2 after allo-HCT, we used established murine aGVHD and GVL models and analyzed the allogeneic T cell response using transcriptome and kinome profiling. Shotgun metagenomic sequencing was used to examine the effect of hBD-2 on the intestinal microbiome. Only age- and sex-matched mice that were 6 to 12 weeks old were used for transplantation experiments. Mice were randomized to different experimental groups before the start of each experiment. All mice were housed under specific pathogen–free conditions in individually ventilated cages at the Zentrale Klinische Forschung (ZKF) mouse facility of the University of Freiburg. Furthermore, we studied β-defensin expression in mice with aGVHD and in two independent aGVHD patient cohorts from the Medical Center – University of Freiburg. The expression of hBD-2 in intestinal biopsies from patients with colitis, acute GI-GVHD, and uninflamed intestine (patients who underwent diagnostic endoscopy and received a diagnosis of no intestinal inflammation) was analyzed retrospectively. Because of the noninterventional character of the study, no randomization or blinding was performed. The study was registered at clinicaltrials.gov (NCT04522843).

Murine aGVHD models

Most mouse allo-HCT experiments were performed using the C57BL/6 into BALB/c transplantation model as previously described (*48*). To induce aGVHD, we lethally irradiated BALB/c recipient mice with 10 gray (Gy) split into two equal doses at an interval of at least 4 hours on day 0. Directly after the second irradiation, 5×10^6 allogeneic (C57BL/6) BM cells and 4×10^5 allogeneic (C57BL/6) CD4$^+$ and CD8$^+$ T cells isolated by magnetic-activated cell sorting (MACS) were injected intravenously. Where indicated, we used a BALB/c into C57BL/6 aGVHD model. In this model, C57BL/6 recipient mice were irradiated with 11 Gy split into two equal doses at an interval of at least 4 hours on day 0. Directly after the second irradiation, 5×10^6 allogeneic (BALB/c) BM cells and 8×10^5 allogeneic (BALB/c) CD4$^+$ and CD8$^+$ T cells isolated by MACS were transplanted.

hBD-2 treatment in vivo

Recombinant hBD-2 (Defensin Therapeutics) was dissolved in PBS at a concentration of 0.3 μg/μl. Mice were treated with hBD-2 (1.2 mg/kg) in 100 μl of PBS through oral gavage once daily from days 0 until 10 after allo-HCT. Control mice received 100 μl of PBS at the same schedule.

GVHD histopathological score

To assess histopathological GVHD severity, we collected sections of the liver, small intestine, colon, and skin from the recipient mice. Samples were stained with hematoxylin and eosin. Samples were scored on the basis of a published histopathology scoring system (*49*) by an experienced pathologist blinded to the experimental groups.

Murine allogeneic T cell proliferation assay

T cell proliferation was assessed as previously described (*48*). In brief, splenic T cells were MACS-purified using the Pan T cell isolation kit (Miltenyi Biotec) labeled with 1 μM CellTrace Violet (Thermo Fisher Scientific) and cocultured with lipopolysaccharide (LPS)–stimulated, allogeneic BMDCs at a cell ratio of BMDCs:T cells of 1:5 or 1:10 in 96-well format. T cell proliferation was determined by the extent of CellTrace Violet dye dilution as measured by flow cytometry using the BD LSRFortessa cell analyzer and analyzed using FlowJo v.10 (Tree Star).

Human allogeneic T cell proliferation assay

MoDCs were derived from PBMCs isolated using Ficoll (Merck) gradient centrifugation. The PBMCs were cultured in a dish for 2 hours before washing off the nonadherent cells. The adherent cells were cultured for 6 days in RPMI 1640 containing 10% fetal calf serum (FCS), 25 μM β-mercaptoethanol, IL-4 (20 ng/ml), and granulocyte-macrophage colony-stimulating factor (100 ng/ml). For maturation of the moDCs, LPS (200 ng/ml) was added on day 6. The mature moDCs were used for coculture with allogeneic T cells (isolated from a different healthy donor) on day 7. CellTrace Violet–stained T cells (stained as above) and moDCs were cocultured at a cell ratio of moDCs:T cells of 1:2 for 72 hours.

APC-independent T cell proliferation assays

Splenic CD4$^+$ and CD8$^+$ T cells were isolated from C57BL/6 mice using the Pan T cell isolation kit (Miltenyi Biotec). The cells were labeled with CellTrace Violet as above; activated with anti-CD3/anti-CD28 microbeads (Thermo Fisher Scientific); and treated with either PBS, hBD-2, or mBD-4 at the indicated concentrations. Cells were harvested after 48 to 72 hours and analyzed by flow cytometry using the BD LSRFortessa cell analyzer and FlowJo v.10

software (Tree Star). For human APC-independent proliferation assays, PBMC-derived T cells were labeled with CellTrace Violet as above and cultured in RPMI 1640 containing 10% FCS and 25 μM β-mercaptoethanol. They were activated with anti-CD3/anti-CD28 microbeads and treated with either PBS or hBD-2 for 72 hours, followed by flow cytometry–based analysis as above.

Statistical analysis

Raw, individual data are presented in data files S1 and S2. GraphPad Prism v9 was used for statistical analysis. For normally distributed data, two-tailed unpaired Student's *t* test or one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons post hoc test was used. The Mann-Whitney *U* test or Kruskal-Wallis test with Dunn's multiple comparisons post hoc test was used when the data did not conform to a normal distribution as assessed by Shapiro-Wilk test. Differences in survival (Kaplan-Meier survival curves) were analyzed by log-rank (Mantel-Cox) test. All in vitro data were validated in at least three independent experiments. Uncertainty is represented in the figures as the SEM, except where otherwise indicated. All comparisons used a two-sided $\alpha = 0.05$ for significance testing.

Supplementary Materials

This PDF file includes:
Materials and Methods
Figs. S1 to S20
Tables S1 to S4
References (*50*–*78*)

Other Supplementary Material for this manuscript includes the following:
Data files S1 to S4
MDAR Reproducibility Checklist

View/request a protocol for this paper from *Bio-protocol*.

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Acknowledgments: We would like to acknowledge the Lighthouse Core Facility for assistance with flow cytometry, P. Henneke for provision of *Ccr2*-deficient mice, and K. Gräwe for performing immunohistochemical staining. **Funding:** The research reported in this publication was supported by grants from the DKMS Foundation for Giving Life grant DKMS-SLS-MHG-2019-02 (to N.K.); German Research Foundation (DFG), SFB-1479 (project ID: 441891347; to N.K., R.Z., S.M., M.B., and J.D.), SFB1160 (project no. 256073931; to R.Z., K.A., M.B., S.M. and C.S.), SFB1453 (project ID: 431984000-S1; to M.B.), and CRC/TRR167 (project ID: 259373024/Z01; to M.B.); DFG under Germany's Excellence Strategy, CIBSS – EXC 2189 (project ID: 390939984; to N.K., R.Z., and S.M.); EQUIP - Funding for Medical Scientists, Faculty of Medicine, University of Freiburg (to N.K. and J.K.); German Federal Ministry of Education and Research (BMBF) within the Medical Informatics Funding Scheme - MIRACUM-FKZ 01ZZ1801B (to M.B.) and EkoEstMed-FKZ 01ZZ2015 (to G.A.), the Else Kröner-Fresenius-Stiftung (2019_A74; to N.K.); and the German Cancer Consortium (DKTK) (to M.B.). **Author contributions:** T.R., N.K., R.Z., J.D., M.B., B.A.H.J., P.N., J.W., S.M., and A.S.B. conceptualized the study. T.R., N.K., R.Z., K.H.-M., B.A.S., A.S.B., and J.K. developed the methodology. T.R., N.K., K.H.-M., N.M.W., S.D., K.A., C.S., A.S.-G., M.S., L.M.B., E.H., S.K., and B.A.S. performed experiments. G.A., M.B., A.S.-G., K.A., C.S., B.A.S., A.S.B., T.R., and N.K. analyzed the data. T.R., N.K., G.A., K.H.-M., N.M.W., and B.A.S. visualized the data. J.K. and P.N. provided important resources. N.K., T.R., and R.Z. wrote the original draft of the manuscript. All authors reviewed and edited the manuscript. **Competing interests:** P.N. is co-owner and Chief Executive Officer of Defensin Therapeutics ApS, Denmark. B.A.H.J. is, in addition to his associate professorship at University of Copenhagen, Chief Scientific Officer of Defensin Therapeutics. J.W. is co-owner of Defensin Therapeutics. Defensin Therapeutics has supported this work by providing hBD-2. The company had no share in the data analysis, integration, and presentation. The views expressed in this manuscript are those of the authors and not necessarily those of Defensin Therapeutics or other funding bodies that could be construed as a potential conflict of interest. P.N. is the inventor on a patent entitled "Prevention and treatment of graft-versus-host-disease with defensins" (no. 17203527.1). Unrelated to the submitted work, R.Z. has received honoraria from Novartis, Incyte, and Mallinckrodt. The other authors declare that they have no competing interests. **Data and materials availability:** Microarray data are available at GEO under accession number GSE190855 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE190855). Kinase array data are available at GEO under accession number GSE198405 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE198405). Microbiome data have been deposited in the NCBI BioProject database under BioProject accession number PRJNA790131 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA790131?reviewer=utfv397f1s638pkve88f17j1ds). All other data associated with this study are present in the paper or the Supplementary Materials. HBD-2 for this study was provided by Defensin Therapeutics and is covered by a material transfer agreement. For data or material requests, please contact the corresponding author.

Submitted 17 March 2022
Resubmitted 15 August 2022
Accepted 22 November 2022
Published 21 December 2022
10.1126/scitranslmed.abp9675

Science Translational Medicine

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Sci. Transl. Med., **14** (676), eabp9675.
DOI: 10.1126/scitranslmed.abp9675

Science Translational Medicine (ISSN) is published by the American Association for the Advancement of Science. 1200 New York Avenue NW, Washington, DC 20005. The title *Science Translational Medicine* is a registered trademark of AAAS.