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Regenerating islet-derived 3-alpha is a biomarker of gastrointestinal graft-versus-host disease

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ABSTRACT

There are no plasma biomarkers specific for graft versus host disease (GVHD) of the gastrointestinal (GI) tract, the GVHD target organ most associated with non-relapse mortality (NRM) following hematopoietic cell transplantation (HCT). Using an unbiased, large-scale, quantitative proteomic discovery approach to identify candidate biomarkers that were increased in plasma from HCT patients with GI GVHD, 74 proteins were increased at least 2-fold; 5 were of GI origin. We validated the lead candidate, REG3 α , by ELISA in samples from 1014 HCT patients from three transplant centers. Plasma REG3 α concentrations were 3-fold higher in patients at GI GVHD onset than in all other patients and correlated most closely with lower GI GVHD. REG3 α concentrations at GVHD onset predicted response to therapy at 4 weeks, 1-year NRM, and 1-year survival ($p \leq 0.001$). In a multivariate analysis, advanced clinical stage, severe histologic damage and high REG3 α concentrations at GVHD diagnosis independently predicted 1-year NRM, which progressively increased with higher numbers of onset risk factors present: 25% for patients with 0 risk factors to 86% with 3 risk factors present ($p < 0.001$). REG3 α is a plasma biomarker of GI GVHD that can be combined with clinical stage and histologic grade to improve risk stratification of patients.

INTRODUCTION

Acute graft-versus-host disease (GVHD), a leading cause of non-relapse mortality (NRM) after allogeneic hematopoietic cell transplantation (HCT), is measured by dysfunction in three organ systems: the skin, liver and gastrointestinal (GI) tract.¹⁻⁴ Acute GVHD of the GI tract affects up to 60% of patients receiving allogeneic HCT,^{5,6} causing nausea, vomiting, anorexia, secretory diarrhea and, in more severe cases, abdominal pain and/or hemorrhage.⁷ Acute GVHD typically occurs between two and

eight weeks after transplant, but may occur later,⁴ and is often clinically indistinguishable from other causes of GI dysfunction such as conditioning regimen toxicity, infection or medication. Endoscopic biopsy is often used to confirm the diagnosis,^{1,8} but histologic severity on biopsy has not consistently correlated with clinical outcome.^{3,8-10} Clinical stage two or greater (more than one liter of diarrhea per day) is associated with reduced survival,^{5,6} but daily stool volume can vary considerably. Lower GI GVHD responds poorly to treatment compared to other target organs,⁶ and treatment with high-dose systemic steroid therapy carries significant risks, especially infectious complications in profoundly immunosuppressed patients.^{11,12} A non-invasive, reliable blood biomarker specific for GVHD of the GI tract would thus significantly aid in the management of patients with this disorder.

Here, we report the discovery and validation of a plasma biomarker of acute GI GVHD: regenerating islet-derived 3-alpha (REG3 α), a C-type lectin secreted by Paneth cells.^{13,14}

METHODS

Proteomic analysis

Methods for sample preparation, protein fractionation, mass spectrometry (MS) analysis, protein identification, and quantitative analysis of protein concentrations during the intact protein analysis system (IPAS) have been previously reported.¹⁵⁻¹⁷

Patients and samples

Heparinized blood samples were collected weekly for four weeks after allogeneic HCT, then monthly for two months, and also at the time of key clinical events, including the development of symptoms consistent with GVHD (e.g. the onset of diarrhea). Plasma samples were collected prospectively under protocols approved

by the University of Michigan Institutional Review Board and stored at the University of Michigan. GVHD assessments, sample processing and storage were performed as previously described.^{7,17} In Regensburg, Germany, and Kyushu, Japan, serum samples were collected weekly and at the onset of GVHD symptoms, prepared, frozen and stored per institutional guidelines. Samples were shipped and received frozen on dry ice and no sample was thawed more than twice before analysis. REG3 α concentrations were stable in samples frozen for at least five years. REG3 α concentrations of 12 paired healthy donors plasma and serum were similar (mean \pm SEM: 20 \pm 3 versus 24 \pm 3 ng/ml, respectively).

All patients received pharmacologic GVHD prophylaxis with at least two agents, including a calcineurin inhibitor. No donor grafts were depleted of T cells. All patients with available samples were analyzed, including patients who developed other complications of HCT, such as sinusoidal obstruction syndrome (SOS), idiopathic pneumonia syndrome (IPS) and sepsis/bacteremia. Patients were excluded from analysis only if a plasma sample at the time of GVHD onset was not available, or if methylprednisolone >1 mg/kg (or equivalent) had been administered for more than 48 hours at the time of sample acquisition. One sample was analyzed per patient; patients who developed GVHD had samples selected at the time of initial GVHD diagnosis.

The discovery set consisted of plasma samples from ten HCT patients at the onset of biopsy-proven GI GVHD (clinical stage 1-3) and ten HCT patients who never developed GVHD and who were matched for key transplant characteristics (Table S1). Patient samples in the discovery set were not included in the validation set.

The University of Michigan validation set consisted of four groups: patients with newly diagnosed GVHD involving the GI tract [with or without other organ

involvement] (GI GVHD); patients at similar time points who never developed GVHD symptoms (no GVHD); patients with GI distress that was inconsistent with GVHD either by clinical or histologic criteria (non-GVHD enteritis); and patients who presented with isolated skin GVHD (skin GVHD). Patient numbers and characteristics are shown in Table 1. Enteritis was determined to be inconsistent with GVHD on clinical grounds by documentation of infected stool and by resolution of symptoms without steroid treatment. The etiologies of non-GVHD enteritis are listed in Table 2.

Patients from the Regensburg/Kyushu validation set were divided into four groups as above; patient characteristics are detailed in Table S2, with causes of non-GVHD enteritis listed in Table S3.

Histopathology

GI biopsies were obtained and prepared per institutional guidelines. GVHD was histologically confirmed by duodenal/colonic biopsy in 183 of 197 GI GVHD patients and by skin biopsy in an additional five patients with both rash and GI symptoms.⁹ Skin GVHD was confirmed by biopsy in 272 of 341 patients with rashes and by biopsy of another target organ later affected by GVHD in an additional eight patients. 162 patients of 197 patients with GVHD had diarrhea. 140 of these 162 patients had biopsies (duodenal = 87, colonic = 53) available for formal grading as described by Lerner.¹⁸ If both duodenal and colonic biopsies were available, colonic biopsies were graded only if duodenal biopsies were negative. We did not impute values for unavailable biopsies.

ELISA assays

REG3 α ELISA kits were purchased from MBL International (Ab-Match Assembly Human PAP1 kit and Ab-Match Universal kit), and measurements were performed

according to the manufacturer's protocol. Samples (diluted 1:10) and standards were run in duplicate, absorbance was measured with a SpectraMax M2 (Molecular Devices), and results were calculated with SoftMax Pro v5.4 (Molecular Devices). Elafin, IL2R α , HGF, TNFR1, and IL-8 ELISAs were performed in duplicate as previously reported.^{17,19} Measurements of samples from 66 patients (6.5% of the total population) were repeated in a second ELISA at random intervals and were comparable; correlation coefficient $r=0.82$, $p<0.0001$. Details of the assay parameters are provided in Table S4.

Statistical analysis

The statistical methods used for the IPAS are as previously described.¹⁵⁻¹⁷ REG3 α and albumin concentrations from individual samples in the discovery and validation sets were compared using two-sample t -tests applied to log-transformed concentrations. Differences in characteristics between patient groups were assessed with a Kruskal-Wallis test for continuous values and chi-squared tests of association for categorical values. Receiver operating characteristic (ROC) area under the curves (AUC) were estimated nonparametrically. NRM and relapse mortality were modeled with cumulative incidence regression methods as described by Fine and Gray.²⁰ 1-year overall survival (OS) was modeled with Cox regression methods and probability of response was modeled with logistic regression.

FINDINGS

Discovery Study

We used a proteomics approach to identify candidate biomarkers in a discovery set of pooled plasma samples taken at similar times after HCT from ten patients with biopsy-proven GI GVHD and ten patients without GVHD as previously described (Table S1).¹⁵⁻¹⁷ We identified and quantified 562 proteins of which 74 were increased

at least two-fold in patients with GVHD (Table S5). Five proteins (carboxypeptidase N catalytic chain precursor, pancreatic secretory trypsin inhibitor precursor, palladin, lithostathine 1-alpha precursor, and regenerating Islet-derived 3-alpha) were preferentially expressed in the GI tract based on the relevant literature²¹⁻²⁵ and the Human Protein Atlas (<http://www.proteinatlas.org/>). Commercially available antibodies suitable for quantification of plasma concentrations by ELISA were available for only one of these five proteins, regenerating Islet-derived 3-alpha (REG3 α , Table S5). The MS characteristics of the identified REG3 α peptides are shown in Figure S1 and Table S6. The plasma concentrations of REG3 α in the individual plasma samples in the discovery set were four times higher in the patients with GI GVHD than in asymptomatic controls (Figure S2, $p=0.01$).

Validation study

We next evaluated REG3 α plasma concentration as a biomarker of GI GVHD in samples from a validation set of 871 allogeneic HCT recipients from the University of Michigan (Table 1). Older transplant recipients, an underlying diagnosis of malignant disease, graft sources from unrelated and HLA-mismatched donors were over-represented in the groups with GVHD. The median day of sample acquisition for patients with non-GVHD enteritis was closer to the day of transplant than for all other groups.

Plasma REG3 α concentrations were three times higher in patients at the onset of GI GVHD than in all other patients, including those with non-GVHD enteritis (Figure 1A). There was no specific cause of non-GVHD diarrhea associated with higher REG3 α concentrations. Serum REG3 α concentrations were also higher in GI GVHD in an independent validation set of 143 HCT patients from Regensburg, Germany, and

Kyushu, Japan, although the absolute values were lower (Figure 1B). This difference may be due to a center effect that depends on several factors, including variations in transplant conditioning regimens and supportive care; patients receiving high intensity conditioning regimens had REG3 α concentrations that were twice as high as those receiving moderate intensity conditioning, but this difference did not reach statistical significance (Figure 1C). In addition, all patients in Regensburg and Kyushu received oral antibiotics as GVHD prophylaxis, whereas Michigan patients did not and thus increased GI flora might account for greater REG3 α secretion.²⁶ Neither TBI-based conditioning nor GVHD prophylaxis regimen significantly impacted REG3 α concentrations (data not shown).

We next analyzed REG3 α concentrations according to diagnosis and type of GI symptom. In patients with diarrhea caused by GVHD, REG3 α concentrations at the onset of GVHD were five time higher than in patients with diarrhea from other causes (Figure 1D). In patients without diarrhea, REG3 α concentrations were 25% higher when attributable to GVHD compared to other causes, a difference that was not statistically significant.

We measured concentrations of four previously reported diagnostic markers of systemic acute GVHD (IL2R α , TNFR1, IL-8, and HGF),¹⁹ and of elafin, a biomarker for GVHD of the skin,¹⁷ in all patients with diarrhea (Figure 1C, N=204). ROC curves for these biomarkers distinguished GVHD from non-GVHD with an AUC of 0.80 for REG3 α alone and an AUC of 0.81 for a composite panel of all six biomarkers (Figure 2). In this analysis, 52% of patients with lower GI GVHD also had skin involvement at onset, and thus the AUC for elafin, which is specific for GVHD of the skin,¹⁷ was greater than expected (Table S7). ROC curves of REG3 α concentrations in patients with diarrhea had similar AUCs in both validation sets (Figure S3). REG3 α was

therefore the best single diagnostic biomarker at the onset of symptoms of lower GI GVHD, and additional biomarkers provided no further increased sensitivity or specificity. Using REG3 α at the median concentration provided a positive predictive value (PPV) of 95% and a negative predictive value (NPV) of 32% for GVHD as the etiology of diarrhea. Additional predictive values at other REG3 α concentrations are provided in Table S8.

When we categorized patients by the volume of diarrhea, REG3 α concentrations at the onset of symptoms continued to distinguish between GVHD and non-GVHD etiologies (Figure 3A, $p < 0.001$) but did not correlate with the clinical stage of GVHD. 23 of 26 patients with clinical stage 4 GI GVHD at onset received full intensity conditioning, and these patients showed a trend toward higher REG3 α concentrations than those with stage 1-3 GI GVHD ($p = 0.07$; data not shown). When comparing patients who had less than 1 liter of stool per day due to GVHD versus other causes, the AUC for REG3 α was 0.81 (Figure S4). Plasma REG3 α concentrations at the onset of GVHD were significantly higher in patients whose GI biopsies showed evidence of severe GVHD with mucosal denudation (histologic grade 4) compared to less severe GVHD (Figure 3B; $p = 0.03$). Hypoalbuminemia is associated with the protein-losing enteropathy in GI GVHD,²⁷ and we analyzed the serum albumin level as a potential marker for loss of intravascular proteins into the intestinal lumen. Albumin levels at the onset of GI GVHD also correlated with both the clinical GI GVHD severity (Figure S5A) and histopathologic severity (Figure S5B).

Prognostic value of REG3 α concentrations in patients with lower GI GVHD

The clinical utility of any biomarker is greatly enhanced when it provides prognostic information regarding the future status of a disease and/or patient, e.g. the likelihood of response to treatment. We therefore evaluated the prognostic significance of REG3 α plasma levels in 162 patients taken at the time of diagnosis of lower GI GVHD. REG3 α concentrations were three-fold higher at the time of GVHD diagnosis in patients who had no response to therapy at four weeks^{28,29} than in patients who experienced a complete or partial response (Figure 4A; $p < 0.001$);^{28,29} patients responding to therapy still exhibited REG3 α concentrations more than twice that of non-GVHD controls. REG3 α concentrations at diagnosis also correlated with eventual maximal clinical stage of GI GVHD (Figure S6); patients presenting with isolated skin GVHD who later developed GI GVHD had concentrations comparable to those with skin GVHD who never developed GI GVHD ($p = 0.2$; data not shown). Because maximal GVHD grade correlates with NRM,¹¹ we hypothesized that the REG3 α concentration at GVHD diagnosis would also correlate with NRM. We therefore divided the 162 patients into two equal groups based upon the median REG3 α concentration: high (> 151 ng/ml, $n = 81$) and low (≤ 151 ng/ml, $N = 81$). NRM was twice as high in patients with high REG3 α concentrations, and this difference remained significant after adjusting for known risk factors of donor type, degree of HLA match, conditioning intensity, age and baseline disease severity (59% [95% CI 48-69%] vs. 34% [95% CI 24-46%], $p < 0.001$, Figure 4B). The incidence of relapse mortality was comparable for both groups (14% [95% CI 8-24] vs. 17% [95% CI 8-24], $p = 0.5$; Figure 4C), and thus patients with high REG3 α concentrations at the time of GVHD diagnosis experienced significantly inferior one-year OS (27% [95% CI 19-

39%] vs. 48% [95% CI 38-61%], $p=0.001$; Figure 4D). Causes of one-year mortality for these patients are listed in Table S9.

Of the 162 patients with diarrhea at the onset of GVHD, we possessed all four data points of clinical stage, histologic grade, REG3 α concentration and serum albumin level in 140 patients. As shown in Table 3, the plasma concentration of REG3 α , the clinical severity of GVHD, the histologic severity and serum albumin level at GVHD diagnosis independently predicted lack of response to GVHD therapy four weeks following treatment after adjustment for the aforementioned risk factors (odds ratios: 4.8, 3.9, 18.9, and 2.5, respectively). When lack of response to therapy and NRM were modeled simultaneously on all four parameters, all but albumin remained statistically significant. When only advanced clinical stage and severe histologic grade were considered, NRM was 71% (Figure 4E). The inclusion of high REG3 α concentration further risk-stratified patients who had either advanced clinical stage or histologic severity (Figure 4F; 34% versus 66% for 1 or 2 risk factors, respectively, $p<0.001$), and patients who had all 3 risk factors experienced significantly greater NRM than those with any 2 of the risk factors (86% versus 66%, $p<0.001$). Details of patient risk factors are listed in Table S10; NRM by all other risk factor combinations are shown in Figure S7.

DISCUSSION

The etiology of diarrhea following HCT presents a common diagnostic dilemma.^{30,31} We identified REG3 α as a candidate biomarker specific for lower GI GVHD through an unbiased, in-depth tandem MS-based discovery approach that can quantify proteins at low concentrations and that we previously used successfully to identify elafin as a plasma biomarker specific for GVHD of the skin.¹⁷ Our discovery

approach identified 74 proteins that were increased at least two-fold in the plasma from patients with GI GVHD. Of note, the list did not include cytokeratin-18 (KRT18), which has been reported to be specific for both liver and intestinal GVHD.³² This discrepancy may be explained by limitations in proteomics technology and the significantly later acquisition times of samples in the earlier report.

REG proteins act downstream of IL-22 to protect the epithelial barrier function of the intestinal mucosa^{33,34} through the binding of bacterial peptidoglycans.¹³ Intestinal stem cells (ISCs) are principal cellular targets of GVHD in the GI tract,^{3,35} where intestinal flora are critical for amplification of GVHD damage.^{36,37} A leading hypothesis is that ISCs are protected by anti-bacterial proteins such as REG3 α secreted by neighboring Paneth cells into the crypt microenvironment.³⁸ If death of an intestinal stem cell eventually manifests itself as denudation of the mucosa, the patchy nature of GVHD histologic damage may be explained as the lack of mucosal regeneration following the dropout of individual ISCs.^{3,35} REG3 α reduces the inflammation of human intestinal crypts *in vitro*,^{14,39} and its administration protects ISCs and prevents GI epithelial damage *in vivo*,³⁴ raising interesting therapeutic possibilities for this molecule.

REG3 α plasma concentrations correlate with disease activity in inflammatory bowel disease, and can distinguish infectious and autoimmune causes of diarrhea.¹⁴ The correlation of mucosal denudation (histologic grade 4) with high REG3 α concentrations suggests that microscopic breaches in the mucosal epithelial barrier caused by severe GVHD permit REG3 α to traverse into the systemic circulation. The tight proximity of Paneth cells with ISCs concentrates their secretory contents in that vicinity, so that mucosal barrier disruption caused by stem cell dropout may preferentially allow Paneth cell secretions, including REG3 α , to traverse into the

bloodstream. We hypothesize that plasma levels of REG3 α may therefore serve as a surrogate marker for the cumulative area of these breaches to GI mucosal barrier integrity, a parameter impossible to measure by individual tissue biopsies. Such an estimate of total damage to the mucosal barrier may also help explain the prognostic value of REG3 α with respect to therapy responsiveness and NRM.

In this study, three high-risk parameters each independently correlated with lack of response to treatment and to higher NRM: elevated plasma REG3 α concentration, higher clinical stage of GVHD at diagnosis and grade 4 histologic severity. All three of these values thus provided important prognostic information prior to the initiation of therapy rather than at the time of maximum grade of GVHD, which by definition includes responsiveness to therapy.^{5,6,11} This study confirms earlier reports where higher clinical stage of GI GVHD^{5,6} and more severe histology correlated with worse survival.¹⁰ In our study the 1-year NRM was 33% (22/67 patients) in patients with clinical stage 1 lower GI GVHD when considering clinical severity alone. Seven of 8 patients (88%) who had the 2 other high risk factors present experienced 1-year NRM while 25% (15/59) of patients with 1 or no risk factors experienced 1-year NRM. In this regard it should be noted that REG3 α levels did not obviate the need for biopsy. If the prognostic value of REG3 α is confirmed in additional patients, we believe the integration of clinical stage, histologic grade and REG3 α plasma concentrations into a single grading system will permit better risk stratification and rapid identification of those patients with severe GI damage in whom standard treatment is likely to be insufficient.

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AUTHOR CONTRIBUTIONS

J.L.M.F. planned the study, interpreted the data and wrote the paper.

A.C.H. designed and planned the experiments, performed research, performed data collection and quality assurance, analyzed data, and wrote the paper; J.K.G. and E.Hu. performed pathology evaluations and wrote the paper.

T.M.B. was the study statistician and wrote the paper; E.Ho., T.T., J.E.L., S.W.C, K. L., K.A., and P.R. contributed to patient accrual, clinical data collection and quality assurance, research discussion, and wrote the paper; M.T.VL. performed experiments and wrote the paper; A.C., Q.Z., and S.H. performed the proteomics experiments, interpreted data and wrote the paper; and S.P. conceived and planned the study design, performed experiments, interpreted data and wrote the paper.

References:

1. Cutler C, Antin JH. Manifestation and Treatment of Acute Graft-Versus-Host-Disease. In: *Appelbaum F, Forman SJ, Negrin RS, Blume KG, eds. Thomas' Hematopoietic Cell Transplantation*: Blackwell Publishing Ltd; 2009:1287-1303.
2. Welniak LA, Blazar BR, Murphy WJ. Immunobiology of allogeneic hematopoietic stem cell transplantation. *Annu Rev Immunol*. 2007;25:139-170.
3. Mowat A, Socie G. Intestinal Graft-vs.-Host Disease. In: *Ferrara JLM, Cooke KR, Deeg HJ, eds. Graft-vs-Host Disease*. New York: Marcel Dekker; 2004:279-327.
4. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet*. 2009;373(9674):1550-1561.
5. Martin PJ, McDonald GB, Sanders JE, et al. Increasingly frequent diagnosis of acute gastrointestinal graft-versus-host disease after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transpl*. 2004;10(5):320-327.
6. MacMillan ML, Weisdorf DJ, Wagner JE, et al. Response of 443 patients to steroids as primary therapy for acute graft-versus-host disease: comparison of grading systems. *Biol Blood Marrow Transpl*. 2002;8(7):387-394.
7. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transpl*. 1995;15(6):825-828.
8. Shulman HM, Kleiner D, Lee SJ, et al. Histopathologic diagnosis of chronic graft-versus-host disease: National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: II. Pathology Working Group Report. *Biol Blood Marrow Transpl*. 2006;12(1):31-47.
9. Washington K, Jagasia M. Pathology of graft-versus-host disease in the gastrointestinal tract. *Hum Pathol*. 2009;40(7):909-917. Prepublished on 2009/06/16 as DOI S0046-8177(09)00118-X.
10. Ertault-Daneshpouy M, Leboeuf C, Lemann M, et al. Pericapillary hemorrhage as criterion of severe human digestive graft-versus-host disease. *Blood*. 2004;103(12):4681-4684.
11. Weisdorf D, Haake R, Blazar B, et al. Treatment of moderate/severe acute graft-versus-host disease after allogeneic bone marrow transplantation: an analysis of clinical risk features and outcome. *Blood*. 1990;75(4):1024-1030.
12. Deeg HJ. How I treat refractory acute GVHD. *Blood*. 2007;109(10):4119-4126.
13. Cash HL, Whitham CV, Behrendt CL, Hooper LV. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science*. 2006;313(5790):1126-1130.
14. Gironella M, Iovanna JL, Sans M, et al. Anti-inflammatory effects of pancreatitis associated protein in inflammatory bowel disease. *Gut*. 2005;54(9):1244-1253.
15. Faca V, Coram M, Phanstiel D, et al. Quantitative analysis of acrylamide labeled serum proteins by LC-MS/MS. *J Proteome Res*. 2006;5(8):2009-2018.

16. Faca V, Pitteri SJ, Newcomb L, et al. Contribution of protein fractionation to depth of analysis of the serum and plasma proteomes. *J Proteome Res.* 2007;6(9):3558-3565.
17. Paczesny S, Braun T, Levine JE, et al. Elafin is a biomarker of graft-versus-host disease of the skin. *Science Translational Medicine.* 2010;2(13):50-57.
18. Lerner KG, Kao GF, Storb R, Buckner CD, Clift RA, Thomas ED. Histopathology of graft-vs.-host reaction (GvHR) in human recipients of marrow from HL-A-matched sibling donors. *Transplant Proc.* 1974;6(4):367-371.
19. Paczesny S, Krijanovski OI, Braun TM, et al. A biomarker panel for acute graft-versus-host disease. *Blood.* 2009;113(2):273-278.
20. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. *J Am Stat Assoc.* 1999;94:496-509.
21. Skidgel RA, Erdos EG. Structure and function of human plasma carboxypeptidase N, the anaphylatoxin inactivator. *Int Immunopharmacol.* 2007;7(14):1888-1899.
22. Marchbank T, Freeman TC, Playford RJ. Human pancreatic secretory trypsin inhibitor. Distribution, actions and possible role in mucosal integrity and repair. *Digestion.* 1998;59(3):167-174.
23. Mykkanen OM, Gronholm M, Ronty M, et al. Characterization of human palladin, a microfilament-associated protein. *Mol Biol Cell.* 2001;12(10):3060-3073.
24. Watanabe T, Yonekura H, Terazono K, Yamamoto H, Okamoto H. Complete nucleotide sequence of human reg gene and its expression in normal and tumoral tissues. The reg protein, pancreatic stone protein, and pancreatic thread protein are one and the same product of the gene. *J Biol Chem.* 1990;265(13):7432-7439.
25. Christa L, Carnot F, Simon MT, et al. HIP/PAP is an adhesive protein expressed in hepatocarcinoma, normal Paneth, and pancreatic cells. *Am J Physiol.* 1996;271(6 Pt 1):G993-1002.
26. Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. *Nat Immunol.* 2000;1(2):113-118.
27. Weisdorf SA, Salati LM, Longsdorf JA, Ramsay NK, Sharp HL. Graft-versus-host disease of the intestine: a protein losing enteropathy characterized by fecal alpha 1-antitrypsin. *Gastroenterology.* 1983;85(5):1076-1081.
28. MacMillan ML, DeFor TE, Weisdorf DJ. The best endpoint for acute GVHD treatment trials. *Blood.* 2010;115(26):5412-5417.
29. Levine JE, Logan B, Wu J, et al. Graft-versus-host disease treatment: predictors of survival. *Biol Blood Marrow Transplant.* 2010;16(12):1693-1699.
30. Cox GJ, Matsui SM, Lo RS, et al. Etiology and outcome of diarrhea after marrow transplantation: a prospective study. *Gastroenterology.* 1994;107(5):1398-1407.
31. Barker CC, Anderson RA, Sauve RS, Butzner JD. GI complications in pediatric patients post-BMT. *Bone Marrow Transplant.* 2005;36(1):51-58.

32. Luft T, Conzelmann M, Benner A, et al. Serum cytokeratin-18 fragments as quantitative markers of epithelial apoptosis in liver and intestinal graft-versus-host disease. *Blood*. 2007;110(13):4535-4542.
33. Sanos SL, Vonarbourg C, Mortha A, Diefenbach A. Control of epithelial cell function by interleukin-22-producing RORgammat(+) innate lymphoid cells. *Immunology*;132(4):453-465.
34. Zheng Y, Valdez PA, Danilenko DM, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med*. 2008;14(3):282-289.
35. Takashima S, Kadowaki M, Aoyama K, et al. The Wnt agonist R-spondin1 regulates systemic graft-versus-host disease by protecting intestinal stem cells. *J Exp Med*. 2011;208(2):285-294.
36. van Bakkum DW, Roodenburg J, Heidt PJ, van der Waaij D. Mitigation of secondary disease of allogeneic mouse radiation chimeras by modification of the intestinal microflora. *J Natl Cancer Inst*. 1974;52(2):401-404.
37. Gerbitz A, Schultz M, Wilke A, et al. Probiotic effects on experimental graft-versus-host disease: let them eat yogurt. *Blood*. 2004;103(11):4365-4367.
38. Elphick DA, Mahida YR. Paneth cells: their role in innate immunity and inflammatory disease. *Gut*. 2005;54(12):1802-1809.
39. Closa D, Motoo Y, Iovanna JL. Pancreatitis-associated protein: from a lectin to an anti-inflammatory cytokine. *World J Gastroenterol*. 2007;13(2):170-174.

Table 1. Patient characteristics of the University of Michigan validation set.

	GI GVHD^{†,‡}	No GVHD	Non- GVHD Enteritis[§]	Skin GVHD	p-value
Total N=871	N=167	N=362	N=52	N=290	
Age (years)					0.003
Median	50	46	48	49	
(range)	(0-67)	(0-68)	(3-66)	(0-70)	
Disease (%)					0.002
Malignant	99%	92%	96%	97%	
	(N=165)	(N=334)	(N=50)	(N=282)	
Other	1%	8%	4%	3%	
	(N=2)	(N=28)	(N=2)	(N=8)	
Disease status at transplant* (%)					0.63
Other/low/ intermediate risk	64%	69%	68%	68%	
	(N=105)	(N=232)	(N=34)	(N=192)	
High risk	36%	31%	32%	32%	
	(N=60)	(N=102)	(N=16)	(N=90)	
Donor type (%)					<0.001
Related donor	45%	64%	54%	40%	
	(N=75)	(N=233)	(N=28)	(N=115)	
Unrelated donor	55%	36%	46%	60%	
	(N=92)	(N=129)	(N=24)	(N=175)	
Donor match (%)					<0.001
Matched donor	70%	90%	92%	73%	
	(N=117)	(N=325)	(N=48)	(N=212)	
Mismatched donor	30%	10%	8%	27%	
	(N=50)	(N=37)	(N=4)	(N=78)	
Conditioning regimen intensity					0.06

(%)

High intensity	57%	67%	63%	57%
	(N=95)	(N=243)	(N=33)	(N=165)
Moderate intensity	43%	33%	37%	43%
	(N=72)	(N=119)	(N=19)	(N=125)
Grade of GVHD at onset (%)				
0	0%	100%	100%	0%
	(N=0)	(N=362)	(N=52)	(N=0)
I	0%	0%	0%	69%
	(N=0)	(N=0)	(N=0)	(N=201)
<i>Skin Stage 1</i>	0%	0%	0%	41%
	(N=0)	(N=0)	(N=0)	(N=118)
<i>Skin Stage 2</i>	0%	0%	0%	29%
	(N=0)	(N=0)	(N=0)	(N=83)
II	57%	0%	0%	30%
	(N=96)	(N=0)	(N=0)	(N=88)
<i>Isolated Skin Stage 3</i>	0%	0%	0%	30%
	(N=0)	(N=0)	(N=0)	(N=88)
<i>Isolated Upper GI Stage</i>	17%	0%	0%	0%
^{1†}	(N=29)	(N=0)	(N=0)	(N=0)
<i>Lower GI Stage 1[†]</i>	40%	0%	0%	0%
	(N=67)	(N=0)	(N=0)	(N=0)
III-IV	43%	0%	0%	1%
	(N=71)	(N=0)	(N=0)	(N=1)
<i>Isolated Skin Stage 4</i>	0%	0%	0%	1%
	(N=0)	(N=0)	(N=0)	(N=1)
<i>GI Stage 2[†]</i>	13%	0%	0%	0%
	(N=22)	(N=0)	(N=0)	(N=0)
<i>GI Stage 3[†]</i>	16%	0%	0%	0%
	(N=27)	(N=0)	(N=0)	(N=0)

	<i>GI Stage 4[†]</i>	13%	0%	0%	0%	
		(N=22)	(N=0)	(N=0)	(N=0)	
Day after HCT						<0.001
	Median	33	31	24	28	
	(range)	(11-216)	(7-185)	(7-93)	(5-175)	

*High risk of disease status at HCT is according to Center for International Blood and Marrow Transplant Research (CIBMTR) guidelines.

[†]Including 29 patients with isolated upper GI GVHD and 138 with lower ± upper GI GVHD.

[‡]With or without other GVHD target organ involvement.

[§] Including 13 patients with isolated upper GI non-GVHD enteritis and 39 patients with lower ± upper GI non-GVHD enteritis.

Table 2. Causes of non-GVHD enteritis in the University of Michigan validation set

Non-GVHD lower GI enteritis +/- upper GI symptoms: N=39

C. difficile infection	54% (N=21)
Diarrhea w/ negative biopsy	15% (N=6)
N/V and diarrhea w/ negative biopsies	28% (N=11)
Ulcerative esophagitis and diarrhea (negative biopsies)	3% (N=1)

Non-GVHD upper GI enteritis without diarrhea (all biopsy negative): N=13

Nausea/vomiting	54% (N=7)
Anorexia	15% (N=2)
Chemical gastropathy	23% (N=3)
H. pylori gastritis	8% (N=1)

Table 3. REG3 α concentrations and characteristics at onset of GVHD diarrhea predict 4-week response to GVHD therapy and 1-year NRM.

	Independent		Simultaneous	
	Odds Ratio	<i>p</i> -value*	Odds Ratio	<i>p</i> -value*
No response to treatment (at 4 weeks)				
REG3 α (high vs. low)	4.8	<0.001	5.7	0.001
GVHD GI onset stage (2-4 vs. 1)	3.9	0.001	3.0	0.027
Histologic grade (4 vs. 1-3)	18.9	<0.001	16.7	<0.001
Albumin (low vs. high)	2.5	0.02	1.4	0.5
1-Year NRM				
	Independent		Simultaneous	
	Hazard Ratio	<i>p</i> -value*	Hazard Ratio	<i>p</i> -value*
REG3 α (high vs. low)	2.2	0.003	2.4	0.002
GVHD GI onset stage (2-4 vs. 1)	3.0	<0.001	3.1	<0.001
Histologic grade (4 vs. 1-3)	3.6	<0.001	2.9	<0.001
Albumin (low vs. high)	2.3	0.004	1.6	0.2

*Adjusted for age, donor type, HLA match, conditioning intensity and disease status at transplant.

FIGURE LEGENDS

Figure 1. REG3 α concentrations in plasma samples from HCT patients of two independent validation sets. (A) University of Michigan patients (n =871) **(B)** Regensburg, Germany, and Kyushu, Japan (n = 143). **(C)** Plasma REG3 α concentrations in patients classified by GI symptoms and histologic diagnosis and categorized by conditioning regimen intensity. High intensity regimens included: cyclophosphamide \pm cytarabine, thiotepa, fludarabine and/or TBI; cyclophosphamide/VP-16/BCNU; busulfan + cytarabine, clofarabine, melphalan, cyclophosphamide/anasacrin or cytarabine/cyclophosphamide; BCNU/VP-16/cytarabine/melphalan; TBI \pm VP-16; melphalan. Moderate intensity regimens included: fludarabine + busulfan or treosulfan \pm TBI, melphalan, zevalin or anasacrin/cytarabine; fludarabine \pm TBI, melphalan, or cyclophosphamide; fludarabine/BCNU/melphalan; TBI. **(D)** Patients classified by symptoms and etiology (n = 675).

Figure 2. ROC curves for patients with post-HCT diarrhea. ROC curves comparing REG3 α concentrations for patients with diarrhea caused by GVHD (n = 162) and not caused by GVHD (N = 42). REG α alone (thick blue): AUC=0.80; IL2R α (thick brown): AUC=0.69; Elafin (thick red): AUC=0.68; IL-8 (thin blue): AUC=0.61; HGF (thin brown): AUC=0.61; TNFR1 (thin red): AUC=0.60; Composite of all 6 biomarkers (solid black): AUC=0.81.

Figure 3. REG3 α expression according to severity of GVHD at diagnosis.

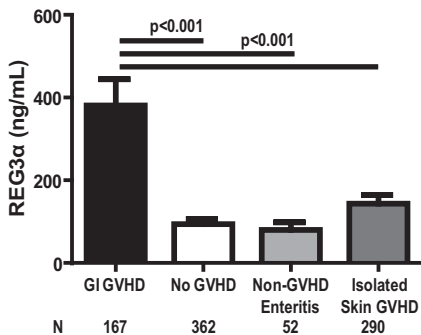
Patients were classified by volume of diarrhea (**A**) and histologic grade (**B**).

Figure 4. Prognostic value of REG3 α concentrations at onset of GVHD. (A)

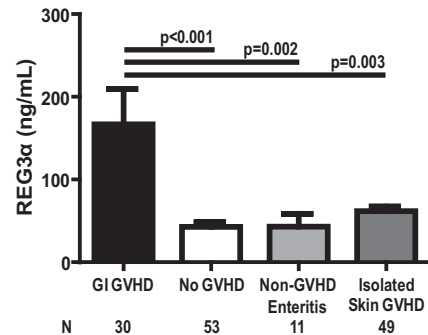
Patients were classified by response to GVHD therapy after 4 weeks (N=160). (**B** to **D**) Patients were classified by REG3 α concentration: low (\leq 151 ng/ml, n=81; thin line) and high ($>$ 151 ng/ml, n=81; thick line). (**B**) NRM (34% versus 59%, $p<0.001$) (**C**) Relapse mortality (17% versus 14%, $p=0.59$). (**D**) 1-year survival (48% versus 27%, $p=0.001$). All p-values are adjusted for donor source, HLA-match, conditioning intensity, recipient age and baseline disease severity according to the Center for International Blood and Marrow Transplant Research (CIBMTR) guidelines. (**E**) 1 year NRM for patients classified by number of risk factors at GVHD onset, using clinical stage (high risk = stage 2-4) and histologic grade (high risk = grade 4). 0 (purple, NRM=26%); 1 (red, NRM=60%); 2 (blue, NRM=71%). 0 vs. 1, $p<0.001$; 1 vs. 2, $p=0.006$. (**F**) 1 year NRM for patients classified by number of risk factors at the time of GVHD diagnosis as in E and including REG3 α concentration (high risk $>$ 151ng/ml). 0 (purple, NRM=25%); 1 (red, NRM=34%); 2 (purple, NRM=66%); 3 (brown, NRM=86%). 0 vs. 1, $p=0.2$; 1 vs. 2, $p<0.001$; 2 vs. 3, $p<0.001$.

Figure 1

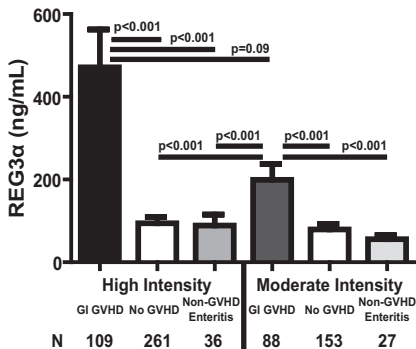
A



B



C



D

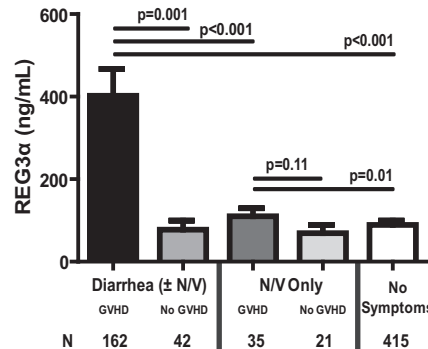


Figure 2

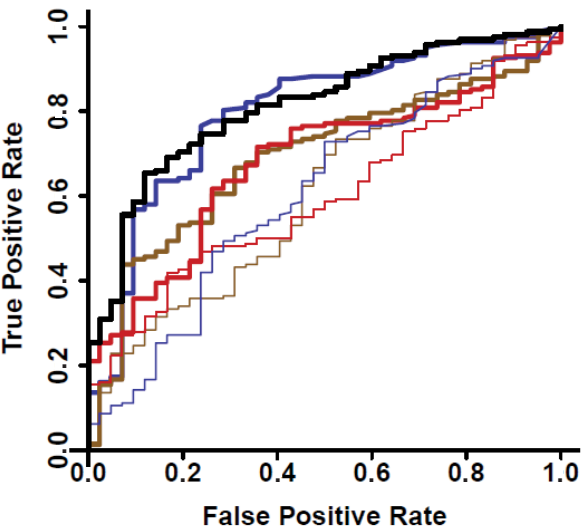


Figure 3

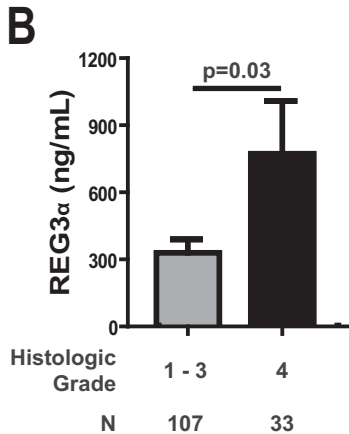
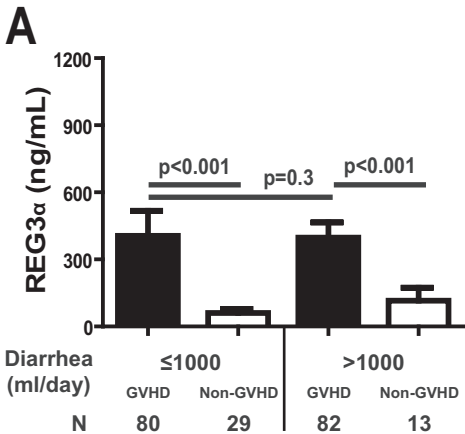
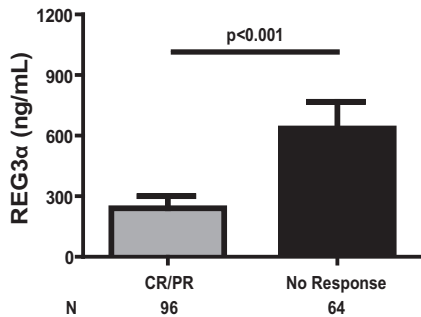
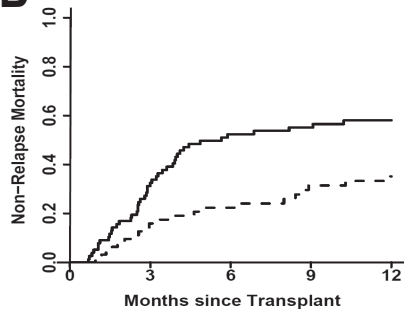


Figure 4

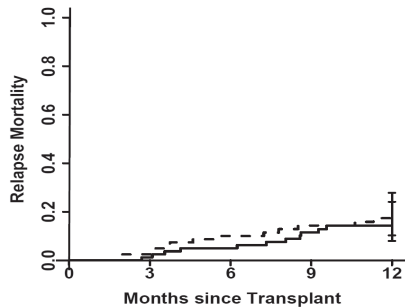
A



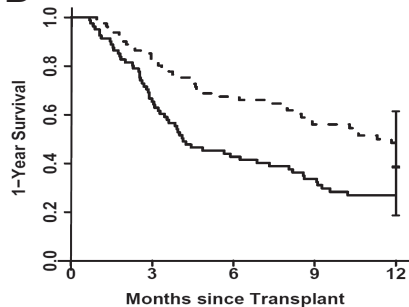
B



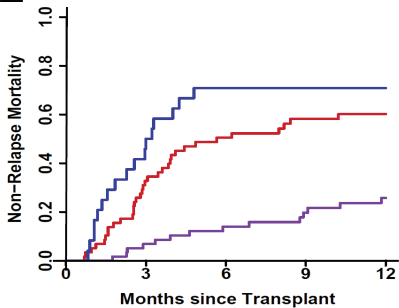
C



D



E



F

