Transcriptome analysis in acute gastrointestinal graft-*versus* host disease reveals a unique signature in children and shared biology with pediatric inflammatory bowel disease

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Clinical Stage of acute GI GVHD	Age (years) at endoscopy	Underlying diagnosis	Preparative Regimen	HLA match	Relation	Stem cell source	Acute GVHD prophylaxis	Day post BMT of endoscopy	Organs involved in addition to gut
1	15.5	ALL	Cy/TBI/ATG	10/10	URD	BM	CSA/MTX	53	None
1	7.3	AML	Bu/Cy/ATG	10/10	URD	BM	CSA/MTX	703	None
2	9.9	ALL	Cy/TBI	9/10	URD	BM	CSA/MTX	168	Skin, Liver
2	16.5	ALL	Cy/TBI/ATG	9/10	URD	PBSC	CSA/MTX/ Abatacept	26	None
2	21.5	PNH	Alemtuzumab/ Flu/Melphalan	10/10	URD	BM	CSA/MMF	71	None
2	7.8	ALL	Cy/TBI	10/10	URD	Cord	CSA/MMF	84	Skin
3	4.6	Aplastic anemia	Alemtuzumab/ Flu/Melphalan	9/10	URD	BM	CSA/MMF	130	Skin
4	16.6	AML	Bu/Cy/ATG	10/10	URD	PBSC	CSA/MTX	35	Skin
4	16.8	Aplastic Anemia	Flu/ Melphalan	9/10	URD	PBSC	CSA/MMF	283	Skin, Liver
0	25	AML	Bu/FLU/ATG	10/10	URD	BM	CSA/MTX	32	None
0	9.4	AML	Bu/CY/ATG	10/10	URD	BM	CSA/MTX	48	None
0	6.5	ALL	Cy/TBI	9/10	URD	Cord	CSA/MMF	130	None
0	11.9	CVID	Alemtuzumab/ Flu/Melphalan	9/10	URD	BM	CSA/ Prednisone	562	None
0	12.5	Hyper IGM	Bu/Flu/ Alemtuzumab	10/10	URD	BM	CSA/MMF	Pre- BMT	None
0	27.2	DC	No BMT	-	-	-	-	Pre- BMT	NA
0	16.9	XMEN	Alemtuzumab/ Flu/Melphalan	9/10	URD	PBSC	T-Cell Dep	Pre- BMT	None
0	14	Beta Thalassemia Major	Bu/Flu/Thiotepa	10/10	Uncle	BM	CSA/ Maraviroc/ Prednisone	Pre- BMT	None

AML= Acute Myeloid Leukemia ALL= Acute Lymphocytic Leukemia CVID= Combined variable immune deficiency DC= Dyskeratosis Congenita CGD= Chronic Granulomatous Disease XIAP= X-linked inhibitor of apoptosis protein deficiency XMEN=X-linked immunodeficiency with magnesium defect, Epstein Barr virus infection and neoplasia, PNH= Paroxysmal nocturnal hemoglobinuria Bu= Busulfan, Cy= Cyclophosphamide, Flu= Fludarabine, TBI= Total Body Irradiation, ATG= Antithymocyte Globulin URD= Unrelated donor BM= Bone Marrow PBSC= Peripheral Blood Stem Cells CSA= Cyclosporine, MTX= Methotrexate, MMF= Mycophenolate mofetil

Supplement Table 1. Demographics of patients who are included in freshly frozen GI analyses.

Clinical Stage of acute GI GVHD	Age (years) at endoscopy	Underlying diagnosis	Preparative Regimen	HLA match	Relation	Stem cell source	Acute GVHD prophylaxis	Day post BMT of endoscopy	Organs involved in addition to gut
3	10	Aplastic anemia	Bu/Cy/ATG	9/10	URD	BM	CSA/MTX	53	Skin
3	12	HLH	Alemtuzumab/ Flu/Melphalan	10/10	URD	BM	CSA/ Prednisone	97	Skin
3	8	Beta Thalassemia	Bu/Flu/Thiotepa	9/10	URD	BM	CSA/ Prednisone/ Maraviroc	18	Skin
3	10	Beta Thalassemia	Bu/Flu/Thiotepa	10/10	Related	BM	CSA/ Prednisone	23	None
3	8	CML	Cy/TBI/ATG	10/10	URD	BM	CSA/ Prednisone	83	None
3	4	Dyskeratosis Congenita	Fludarabine/ Melphalan	10/10	URD	BM	CSA/MMF/ Maraviroc	23	None
3	19	GATA2 deficiency	Busulfan/ Fludarabine	10/10	Related	BM	CSA/MTX	29	None
3	11	Fanconi Anemia	ATG/BU/CY/FLU	9/10	URD	BM	CSA/ Prednisone	27	Skin
3	2	HLH	Alemtuzumab/ Flu/Melphalan	10/10	URD	BM	CSA/ Prednisone	17	None
4	14	XIAP	Alemtuzumab/ Flu/Melphalan	10/10	Related	BM	CSA/ Prednisone	95	Skin, liver
3	17	AML	Bu/Cy/ATG	10/10	URD	BM	CSA/MTX	39	Skin
3	29	Wiskott Aldrich syndrome	Bu/Cy/ATG	10/10	Related	BM	CSA/ Prednisone	45	Skin
3	20	AML	Bu/Cy/ATG	10/10	URD	PBSC	CSA/MTX	46	None
3	5	AML	Bu/Cy/ATG	10/10	URD	BM	CSA/MTX	29	Skin
3	26	Aplastic Anemia	Cyclophosphamide/ ATG	10/10	Related	BM	CSA/ Prednisone	57	Skin
4	16	EBV-LPD	Flu/Mel/Thio/ ATG	10/10	URD	BM	Tacro/ MMF	38	Skin
3	8	Sickle Cell Disease	Alemtuzumab/ Flu/Melphalan	10/10	Related	BM/ Cord	CSA/ Prednisone	24	None
3	16	MDS	Bu/Cy	9/10	URD	PBSC	Aβ TCR CD19 selection	48	Skin
3	23	AML/SDS*	Fludarabine/ Melphalan	10/10	URD	PBSC	CSA/MMF/ Maraviroc	30	None
3	9	ALL	Cy/TBI	4/6	URD	Cord	CSA/MMF	67	Skin

0	1.4	Wiskott Aldrich Syndrome	Bu/Flu/ Alemtuzumab	10/10	URD	BM	CSA/MMF	98	None
0	15	ALL	Cy/TBI	10/10	Related	BM	CSA/MTX	28	None
0	12	Sickle Cell Disease	Alemtuzumab/ Flu/Melphalan	10/10	Related	BM	CSA/MTX	183	None
0	16	JIA/MAS	Alemtuzumab/ Flu/Melphalan	9/10	URD	PBSC	CSA/ Prednisone/ Maraviroc	73	None
0	0.9	Diamond Blackfan Anemia	Bu/Cy	10/10	URD	Cord	CSA/MTX	84	None
0	0.4	Hurler's Syndrome	Bu/Cy	10/10	URD	Cord	CSA/MMF	55	None
0	1	Griscelli Syndrome	Alemtuzumab/ Flu/Melphalan	8/10	URD	BM	CSA/ Prednisone	49	None
0	12	Beta Thalassemia	Bu/Flu/Thiotepa	9/10	URD	BM	CSA/MMF/ Abatacept	116	None
0	25	MDS	Bu/Cy	9/10	URD	PBSC	CSA/MMF/ Abatacept	103	None
0	10	ALL	Cy/TBI	10/10	URD	BM	CSA/MTX	41	None
0	2.5	Evans Syndrome	Alemtuzumab/ Flu/Melphalan	10/10	Related	BM	CSA/ Prednisone	39	None
0	1.5	Hurler's Syndrome	Alemtuzumab/ Flu/Melphalan	9/10	URD	BM	CSA/MTX	59	None
0	6	Wiskott Aldrich syndrome	Bu/Flu/ Alemtuzumab	10/10	URD	BM	CSA/MMF/ Maraviroc	78	None
0	19.	CML	Bu/Cy	10/10	URD	BM	CSA/MMF/ Abatacept	23	None
0	12	Sickle Cell Disease	Alemtuzumab/ Flu/Melphalan	10/10	Related	BM	CSA/MTX/Abatacept	28	None

JIA/MAS= Juvenile idiopathic arthritis/macrophage activation syndrome CML= Chronic myeloid leukemia DC= Dyskeratosis Congenita CGD= Chronic Granulomatous Disease XIAP= X-linked inhibitor of apoptosis protein deficiency, EBV-LPD Epstein Barr virus lymphoproliferative disease SDS= Schwachman Diamond Syndrome HLH= Hemophagocytic lymphohistiocytosis MDS= Myelodysplastic syndrome Bu= Busulfan, Cy= Cyclophosphamide, Flu= Fludarabine, TBI= Total Body Irradiation, ATG= Antithymocyte Globulin URD= Unrelated donor BM= Bone Marrow PBSC= Peripheral Blood Stem Cells CSA= Cyclosporine, MTX= Methotrexate, MMF= Mycophenolate mofetil

Supplement Table 2. Demographics of 35 patients included in the paraffin embedded GI biopsy analyses

Upregulated GO term Biological Process from freshly frozen biopsies	FDR B&H P value freshly frozen biopsies	Comparable or similar GO term biological processes from Paraffin embedded biopsies	FDR B&H P value paraffin embedded biopsies
GO:0006952_Defense response	3.47E-25	GO:0006968_Cellular defense response	p=0.002
GO:0043207_Response to external biotic stimulus	1.13E-21	GO:0071216_Cellular response to biotic stimulus	p=0.002
GO:0002252_Immune effector process	6.48E-17	GO:0002697_Regulation of immune effector process	1.39E-06
GO:0034097 Response to cytokine	6.91E-16	GO:0050663_ Cytokine secretion	1.25E-07
GO:0045087_Innate immune response	6.07E-15	GO:0045088_Regulation of innate immune response	0.011909
GO:0006954_Inflammatory response	2.67E-13	GO:0050727_Regulation of inflammatory response	3.54E-10
GO:0009617_Response to bacterium	2.05E-11	GO:0042742_Defense response to bacterium	0.007037
GO:0034341_Response to interferon-gamma	2.55E-11	GO:0034341_Response to interferon-gamma	0.02
GO:0019221_Cytokine-mediated signaling pathway	5.06E-10	GO:0002718_Regulation of cytokine production involved in immune response	0.017352
GO:0001775_Cell activation	1.14E-09	GO:0001906_Cell killing	0.000463

Supplement Table 3. Top 10 upregulated GO term biological processes in acute GI GVHD from freshly frozen GI biopsies and similar biological processes from paraffin embedded biopsies.

Upregulated Gene	Name	Function
MTRNR2L8	MT-RNR2 Like 8	Involved in Bare Lymphocyte Syndrome. Plays a role as a neuroprotective and antiapoptotic factor.
MARCO	Macrophage Receptor with Collagenous Structure	Part of the innate antimicrobial immune system. Involved in pattern recognition receptor, which binds Gram-positive and Gram-negative bacteria
TM4SF4	Transmembrane 4 L Six Family Member 4	Regulates the adhesive and proliferative status of intestinal epithelial cells
CHIT1	Chitinase 1	Secreted by activated macrophages, plays a role in the degradation of chitin-containing pathogens
HBB	Hemoglobin Subunit Beta	Transport of oxygen
FHAD1	Forkhead phosphopeptide associated domain 1	Protein coding gene, related to DNA transcription, associated with Char Syndrome, a developmental disorder of heart, face and limbs
LYAR	Ly1 Antibody Reactive	Negatively regulates the antiviral innate immune response by targeting IRF3 and impairing its DNA-binding activity
CA8	Carbonic Anhydrase 8	Involved in metabolic pathways
CXCL16	C-X-C Motif Chemokine Ligand 16	Acts as a scavenger receptor on macrophages
COTL1	Coactosin Like F-Actin Binding Protein 1	Binds F-actin and interacts with 5-lipoxygenase, which is the first enzyme in leukotriene biosynthesis
FAM195B	Family with Sequence Similarity 195, Member B	MAP Kinase signaling
DNAJA1	DnaJ Heat Shock Protein Family (Hsp40) Member A1	Ubiquitin protein ligase binding and chaperone binding
Downregulated Genes		
ZBTB38	Zinc Finger and BTB Domain Containing 38	Transcriptional activator
ADAL	Adenosine Deaminase Like	Metabolism of nucleotides
FSD1L	Fibronectin Type III and SPRY Domain Containing 1 Like	Protein coding, exact function unknown
MTRNR2L12	MT-RNR2 Like 12	Associated with Hirschsprung's disease Neuroprotective and antiapoptotic factor
YBX2	Y-Box Binding Protein 2	Regulation of the stability and/or translation of germ cell mRNAs
GPR15	G Protein-Coupled Receptor 15	Chemokine receptor

Supplement Table 4. Genes upregulated and downregulated in acute GI GVHD from freshly frozen GI biopsies but not in ulcerative colitis.

	GI GVHD (n=30)	No GVHD (n=30)	Skin GVHD (n=25)
Median Age (Years)	13(range 1-32 years)	7 (range 0.6-23 years)	7 (range 0.7-37 years)
Diagnosis			
Malignancy	11	4	13
Immune Deficiency	6	11	5
Bone Marrow Failure	9	2	2
Hemoglobinopathy	4	13	3
Metabolic Disorders	0	0	2
Preparative regimen			
Myeloablative	21	22	23
Reduced Intensity	9	8	2
HLA Match			
7/8	9	5	7
8/8	21	25	18
Relation			
Related	8	15	2
Unrelated	22	15	23
Stem cell source			
Bone marrow	24	28	18
Peripheral Blood Stem Cells	6	2	5
Umbilical Cord Blood	0	0	2
GVHD Prophylaxis			
CNI + methotrexate	9	7	14
CNI+ Mycophenolate mofetil	5	5	4
CNI + Methylprednisolone	16	18	4
Ex-vivo T-cell depletion	0	0	2
CNI	0	0	1

Supplement Table 5. Demographics of patients with isolated skin GVHD (n=25), GI GVHD (n=30) and HSCT patients without GVHD (n=30) on whom plasma CD64 was measured.

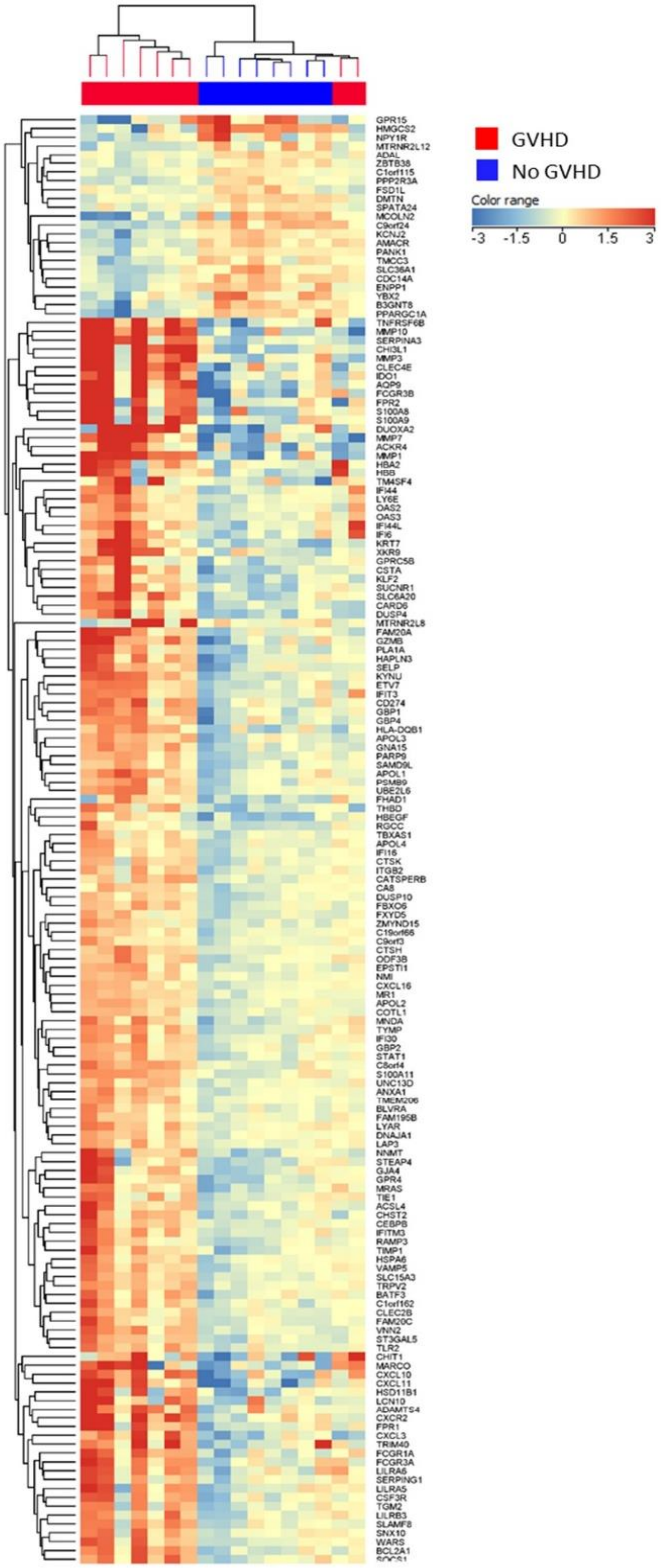
	Inflammatory Bowel Disease (n=47)	**Non-Inflammatory Bowel Disease Controls** (n=42)
Median age at endoscopy	14 years (range 5-21 years)	14 years (8-18 years)
Gender Male Female	 28 19	 17 25
Underlying Diagnosis	Crohn's Disease (n=47)	Irritable Bowel Disease (n=19) Constipation (n=10) Dyspepsia (n=6) Functional abdominal pain (n=6) Food allergy (n=1)

Supplement Table 6. Demographics of patients with Inflammatory Bowel Disease and non- IBD controls on whom plasma CD64 data was available for comparison.

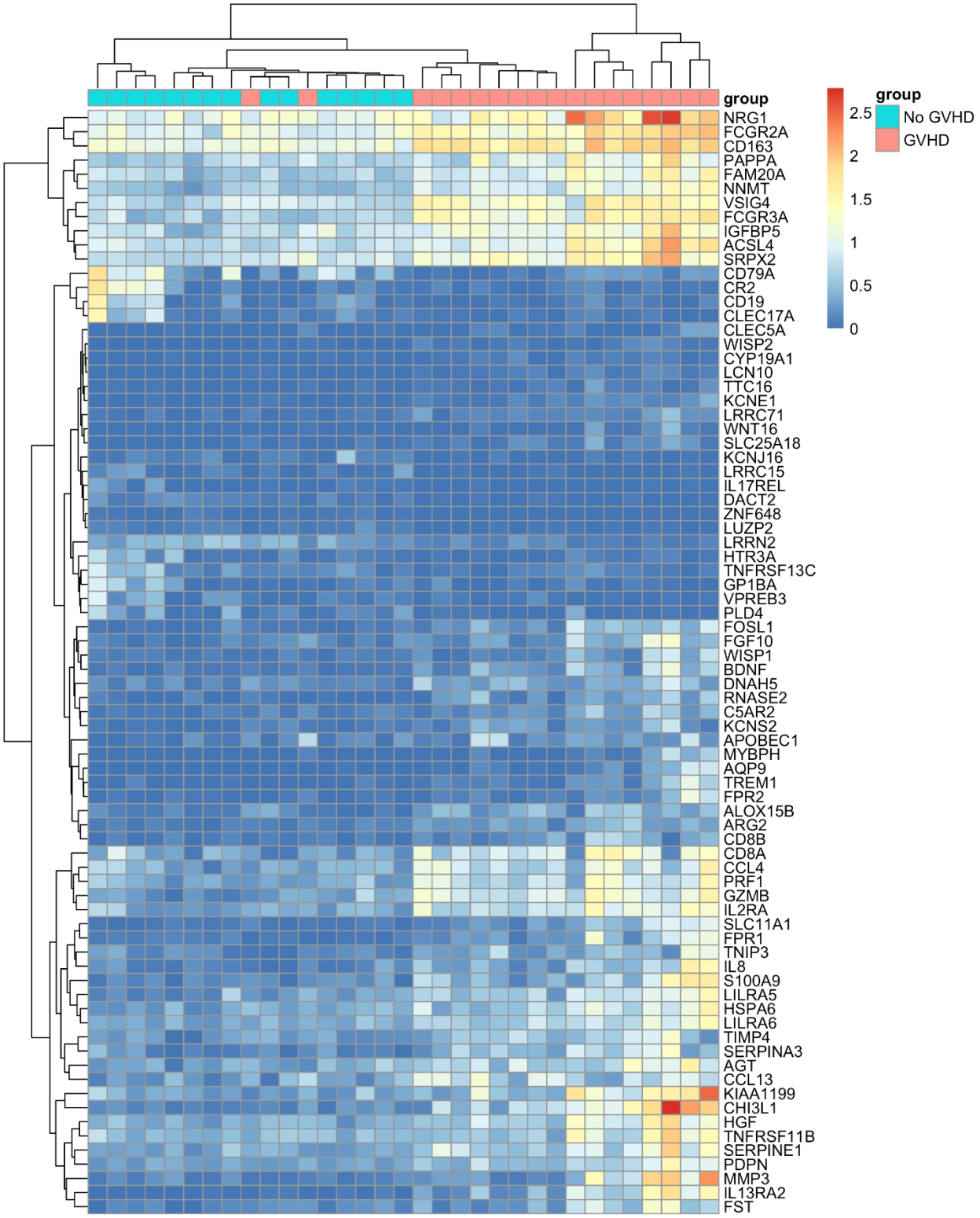
Variable	Adult GI GVHD (3 groups: paired samples GVHD at onset and steroid refractory GVHD and normal controls)	Pediatric GI GVHD freshly frozen biopsies (2 groups: Patients with and without GI GVHD)	Pediatric GI GVHD FFPE biopsies (2 groups: Patients with and without GI GVHD)
CHI3L1	Top differentially expressed gene in acute GVHD compared to normal biopsies (Fold change 3.39169 p= 0.00284)	Top differentially expressed gene in acute GVHD compared to no GVHD (Log2 fold change 4.5 p=0.001)	Log2fold change 3 P=2.10E-12
AQP8	Top decreased gene in acute GVHD compared to normal biopsies (Fold change –5.0; p< 0.001),	Not differentially expressed	Not seen
CCL18	Decreased at steroid refractory GVHD compared to onset of acute GVHD (Fold Change –1.55402 p=0.00007)	Not seen	Not seen
ITLN1	Decreased at steroid refractory GVHD compared to onset of acute GVHD (Fold change –1.58999 p=0.02574)	Not seen	Not seen
Metallothionein genes (MT1H, MT-TG, MT-TR)	Increased at steroid refractory GVHD onset compared to GVHD onset	Not seen	Not seen
IDO1	Increase at GVHD onset, decrease in steroid refractory GVHD	Differentially expressed in GVHD compared to no GVHD (Log2 fold change 3.3 p=0.003)	Not seen
PDL1	Increase at GVHD onset, decrease in steroid refractory GVHD	Not seen	Not seen
TIGIT	Increased at GVHD onset, decreased in steroid -refractory GVHD	Not seen	Log2fold change 1.3 P= 0.003
AhR	Increased at GVHD onset and in steroid refractory GVHD	Not seen	Not seen
AREG	Increased at GVHD onset and in steroid refractory GVHD	Not seen	Not seen
HMGCS2	Most significantly downregulated compared to normal samples on gene enrichment analyses	Second most decreased differentially expressed gene in acute GVHD compared to no GVHD (Log2 fold change -2.2 p=0.001)	Not seen
SETD7	Most highly upregulated in acute GVHD onset compared with	Not seen	Not seen

	normal samples on gene enrichment analyses		
GK *PGK1P2* *HIST1H1A* *COX7BP2*	Those who died early had higher expression of these 4 genes:	Not seen	Not seen
Smoothened	Associated with survival	Not seen	Not seen
Inflammatory response (GO:0006954)	Top upregulated biological process in acute GVHD *P* < 0.001	Sixth top upregulated biological process in acute GVHD p=2.67E-13	GO:0002673 regulation of inflammatory response p=0.007
Immune Deconvolution	Onset of acute GVHD:CD4+ activated memory T cells and M0 macrophages Steroid refractory GVHD: M2 macrophages and plasma cells	M1 macrophages Plasma cells Follicular helper T-cells	Not done

Supplement Table 7. Comparison of findings between adult GI GVHD transcriptome study and our study (freshly frozen GI Biopsies and paraffin embedded GI Biopsies).



Supplement Figure 1. Heat Map of all 164 genes in acute GI GVHD and no GVHD.



Supplement figure 2. Heat Map of 78 protein coding genes, log-transformed FPKM values with an adjusted p-value < 0.05, and a fold change of 3 from paraffin embedded GI biopsies

Supplementary Methods

Patient selection and exclusion for analyses

The endoscopist collected up to two additional rectosigmoid mucosal biopsies for research in addition to clinically indicated samples, when deemed safe. Patients were divided into 2 groups for analyses: patients with a diagnosis of clinical acute GI GVHD, supported by characteristic supportive features on pathological review of biopsies and patients without acute GI GVHD. Patients who had evidence of inflammatory colitis due to underlying disease (e.g., chronic granulomatous disease) were excluded from the analyses, despite our small sample size. These excluded patients were enrolled on the trial before results of GI biopsies were available, as we wanted to maximize opportunities for enrollment. These excluded patients had colitis due to their underlying disease, which would have confounded our transcriptome results of colitis due to acute GI GVHD, and therefore justified exclusion. In addition, patients were not evaluated if their research biopsy was obtained from a site other than recto-sigmoid location

Transcriptomics of freshly frozen GI biopsies:

Samples were stored in RNALater at -80◦C. Total DNA and RNA were isolated using the Qiagen AllPrep RNA/DNA mini kit. PolyA-RNA selection, fragmentation, cDNA synthesis, adaptor ligation and library preparation were performed using TrueSeq RNA Sample Preparation (Illumina). Single read 1x51 base pair, ~25M pass filter reads using the Illumina HiSeq 2000 was performed.

Following removal of primers and barcodes, reads (median coverage of 29.8M and (IQR 28.1M, 32.7M) were quantified by kallisto, using Gencode v24 as the reference genome

and Transcripts per Million (TPM) as an output. We included 14,229 protein-coding genes with TPM above 1 in 20% of samples. Differentially expressed genes between those who developed GVHD and those that did not with fold change differences (FC) >=1.5 and using false discovery rate correction (FDR<0.05) were determined using R package DESeq2 version 1.24.0 and importing and summarizing the kallisto output files to gene level with R package tximport version 1.12.3. Euclidean distance metric and Ward's linkage rule was used for unsupervised hierarchical clustering. Those gene sets were used for ToppGene and ToppCluster functional annotation enrichment analyses with visualization using Cytoscape.v3.0.2. Principal component analysis (PCA) distills large gene sets into component variables that are indicative of overarching biological themes among the original gene sets, and we focus on the first two component, which explained the greatest amount of variability.

Transcriptomic analyses for FFPE tissue

One slide of each sample was baked under 65℃ for 20-30 minutes. The baked slides were deparaffined with Xylene and hydrated with graded ethanol. The hydrated slides were treated with haematoxylin staining method and proceeded for pathological review to diagnose tumor content and select extraction area. RNA was isolated from FFPE biopsies using the Qiagen AllPrep DNA/RNA FFPE kit and PolyA-RNA selection, fragmentation, cDNA synthesis, adaptor ligation and library preparation were performed using TrueSeq RNA Sample Preparation (Illumina), 12Gb (40 million reads) raw data, Q30≥80%.

Weighted gene co-expression network analyses

Briefly, WGCNA framework identifies co-expressed gene clusters based on pairwise-correlations between gene expression profiles across all the samples (equation 1) with a signed weighted adjacency matrix determined using the gene co-expression similarities (equation 2).

$$\boldsymbol{S_{ij}} = \frac{\mathbf{1}+\boldsymbol{cor(i,j)}}{\mathbf{2}} \quad (1)$$

$$\boldsymbol{a_{ij}} = \boldsymbol{power}(\boldsymbol{s_{ij}}, \boldsymbol{\beta}) = |\boldsymbol{s_{ij}}|^{\boldsymbol{\beta}} \quad (2)$$

where cor(i,j) is the Pearson correlation coefficient between the expression profiles of a pair of genes i & j. The parameter β is selected based on the scale-free topology criterion. The adjacencies are used to compute topological overlaps between two genes and represent their interconnectedness in the gene co-expression network (equation 3).

$$\boldsymbol{TOM_{ij}}(\boldsymbol{A}) = \frac{\sum_{k \neq i,j} \boldsymbol{a_{ik}a_{kj}} + \boldsymbol{a_{ij}}}{\boldsymbol{min}\,(\sum_{k \neq i} \boldsymbol{a_{ik}}, \sum_{k \neq j} \boldsymbol{a_{jk}}) + \mathbf{1} - \boldsymbol{a_{ij}}} \quad (3)$$

Average linkage hierarchical clustering is performed on TPM-based dissimilarities to detect modules of highly correlated genes across the samples. Disease-related candidate gene modules are selected based on the strength and statistical significance (Fisher's asymptotic p-values) of the gene module correlations with the traits. We performed functional enrichment analysis of modules generated from the WGCNA to further interrogate pathogenesis of GVHD and search for potential druggable targets.

Immunohistochemistry for pERK

Samples were sectioned at 4.5um onto positive charged slides and baked at 60 degrees for 30 minutes. The slides were stained with p-ERK antibody (Cell Signaling,

4370S) at 1:400 using a HIER Citrate pretreatment for 40 minutes. The slides were stained using the BenchMark DISCOVERY platform.Slides were digitally scanned with an Aperio ScanScope scanning system (Aperio Technologies Inc.) and analyzed by using the Aperio ImageScope Viewer software. The positive pixel count algorithm was used to measure the intensity of pERK (brown signal). The entire slide with the exception of necrotic tissue was chosen for positive pixel counting. Analysis was carried out using the Positive Pixel Count v9 algorithm from the Aperio ImageScope software. Staining (% positive pixels) was scored according to the intensity and percentage of cells stained. Values of positive pixel counts between acute GI GVHD and no GVHD were compared using the Mann Whitney U test.

Plasma CD64

Plasma CD64 was measured by the human FCγR1A/CD64 sandwich ELISA kit (LSBio, WA, USA) according to the manufacturer's instructions. Statistical comparisons between cohorts were performed by the one-way ANOVA. Time points of measurements were at diagnosis of acute isolated skin or GI GVHD or a comparable time point after HSCT in patients without acute GVHD (~ day+35). We had plasma CD64 values available on 47 children with Crohn's disease at diagnosis and 42 non-IBD controls measured by the same ELISA method, for comparison.

Supplemental Dataset 1: Full list of differentially expressed genes by DESeq analyses in acute GI GVHD from freshly frozen and paraffin embedded GI biopsies along with the gene list of up and downregulated genes from patients with Ulcerative colitis. Upregulated and downregulated genes in acute GI GVHD along with details of analyses such as top

25 molecular functions, biological processes, cellular components and pathways are shown.

Supplemental Dataset 2: Full analyses of the weighted gene co-expression network analyses are shown with hubs and all modules with genes, adjusted p-values and functions and the M3, M51 and M30 module analyses presented separately.