SUPPLEMENTARY APPENDIX

Increased mitochondrial apoptotic priming of human regulatory T cells after allogeneic hematopoietic stem cell transplantation

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Supplementary Materials and Methods

Sample processing

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples by density gradient centrifugation (Ficoll-Paque; GE Healthcare) and cryopreserved in aliquots until needed for in vitro assays.

Antibodies, buffer and peptide concentration for BH3 profiling

Antibodies used were anti-CD4 Pacific blue (clone RPA-T4; BD Biosciences), anti-CD25 PE-Cy7 (clone M-A251; BD Biosciences), anti-CD127 APC eFluor780 (clone eBioRDR5; eBioscience) and anti-CD8 PE/Cy5 (clone RPA-T8; BioLegend). T-EB Buffer contains 300 mM trehalose, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 80 mM potassium chloride, 1 mM ethyleneglycoltetraacetic acid, 1 mM ethylenediaminetetraacetic acid, 0.1% BSA and 5 mM succinic acid in distilled water at pH 7.4. BH3 peptide concentrations: BIM 10 µM, BIM 0.03 µM, BID-Y 10 µM, BAD 10 µM, NOXA 10 µM, BAD 5 µM + NOXA 5 µM, PUMA 10 µM, BMF 10 µM, HRK 10 µM. Media for BH3 peptide incubation: T-EB Buffer containing 0.002% digitonin and 10 µg/ml oligomycin.

Flow cytometric analysis of Ki-67, BCL2 and CD95 expression

PBMC were first incubated with anti-CD4 Pacific blue, anti-CD25 PE-Cy7, anti-CD127 APC eFluor780 and anti-CD8a PE-Cy5 (clone RPA-T8; BioLegend). For CD95 staining, anti-CD95 PE (clone DX2; BD Biosciences) was added. For Ki-67 and BCL2 staining, cells were washed twice in PBS and suspended in 100 µL of Fixation/Permeabilization solution (BD Biosciences) for 20 minutes at 4°C. After fixation, cells were incubated with anti-Ki-67 PE (clone B56; BD Biosciences), anti-BCL2 PE (clone Bcl-2/100; BD Biosciences), or isotype-matched IgG-PE (clone MOPC-21; BD Biosciences) for 30 minutes at 4°C. Stained cells were analyzed on the BD FACSCanto II using FACS Diva software. CD95 median fluorescence intensity (MFI)

and Ki-67 positivity were determined for each T cell subset. Relative BCL2 expression was calculated by dividing the MFI for BCL2–PE by the MFI for the isotype matched IgG.

Flow cytometric analysis of BCLXL, BIM and MCL1 expression

PBMC were first incubated with anti-CD3 APC (clone SK7; eBioscience), anti-CD4 BV570 (clone RPA-T4; BioLegend), anti-CD8 BV421 (clone RPA-T8; BioLegend), anti-CD25 PE-Cy7 (clone M-A251; BD Biosciences), and anti-CD127 BV650 (clone A019D5; BioLegend). Surface stained cells were washed twice in PBS and suspended in 100 µL of Fixation/Permeabilization solution for 20 minutes at 4°C. Fixed cells were washed and incubated with rabbit anti–BCLXL Alexa Fluor 488 (clone 54H6), rabbit IgG Isotype Control Alexa Fluor 488, unconjugated rabbit mAb IgGXP Isotype Control, unconjugated anti-BIM (all from Cell Signaling Technology), or unconjugated rabbit anti–MCL1 (clone Y37; EPITOMICS) for 30 minutes at 4°C. Cells stained with unconjugated antibodies were subsequently incubated with anti-rabbit IgG Fab2 Alexa Fluor 488 (Cell Signaling Technology) and analyzed on the BD LSRFortessa. Relative BCLXL, BIM and MCL1 expression in each subset was calculated by dividing the MFI by the median value of the MFI for the isotype matched IgG.

Staurosporine (STS) and Fas (CD95) - induced apoptosis

T cell subsets were cultured separately with 0.5 µM STS[27] which induce intrinsic pathway of apoptosis or 5 µg/ml purified mouse anti-human CD95 antibody (clone EOS9.1; BD Biosciences)[14] or medium only in 96-well round-bottom plates at a concentration of 1×10^5 PBMC cells per well. Apoptosis induction was measured 6 hours after addition of anti-CD95 or STS. Cell death was assessed by annexin V/7-AAD co-staining and forward to side scatter profiles. Fas and STS-specific cell death were calculated as follows: (percent experimental cell death – percent spontaneous cell death)/(100% – percent spontaneous cell death) × 100.

Supplementary Table 1A

P-values from Multivariate Linear Model* (Supp. Information for Fig 2 C & D)

	P-value			
	CD95	Ki67	Apop STS	Apop CD95
CD8 vs Tcon	<0.0001	0.56	<0.0001	0.03
CD8 vs Treg	<0.0001	<0.0001	0.01	0.08
Tcon vs Treg	<0.0001	<0.0001	<0.0001	0.0002

* Modeled on T cell type (CD8, Treg, Tcon, i.e. comparing T cell type). Factors included in the model are age, cGVHD (Yes or No), grade 2-4 aGVHD (Y/N), prednisone use (Y/N), donor type, conditioning intensity, and time from HSCT to Sample date. The model was repeated excluding donor type and age and the results were consistent.

Supplementary Table 1B

P-values from Multivariable Linear Models* (Supp. Information for Fig 4 B & C)

		P-value			
		CD95	Ki67	Apop STS	Apop CD95
Treg	cGVHD vs no cGVHD	0.52	0.1	0.23	0.57
	Time from HSCT to Sample Date	0.02	0.052	0.97	0.27
Tcon	cGVHD vs no cGVHD	0.49	0.78	0.21	0.09
	Time from HSCT to Sample Date	0.11	0.25	0.57	0.4
CD8	cGVHD vs no cGVHD	0.84	0.93	0.3	0.11
	Time from HSCT to Sample Date	0.41	0.25	0.32	0.21

* Each model includes age, cGVHD (Yes or No), grade 2-4 aGVHD (Y/N), Steroid use (Y/N), donor type, conditioning intensity, time from HSCT to Sample date.

Supplementary Table 2

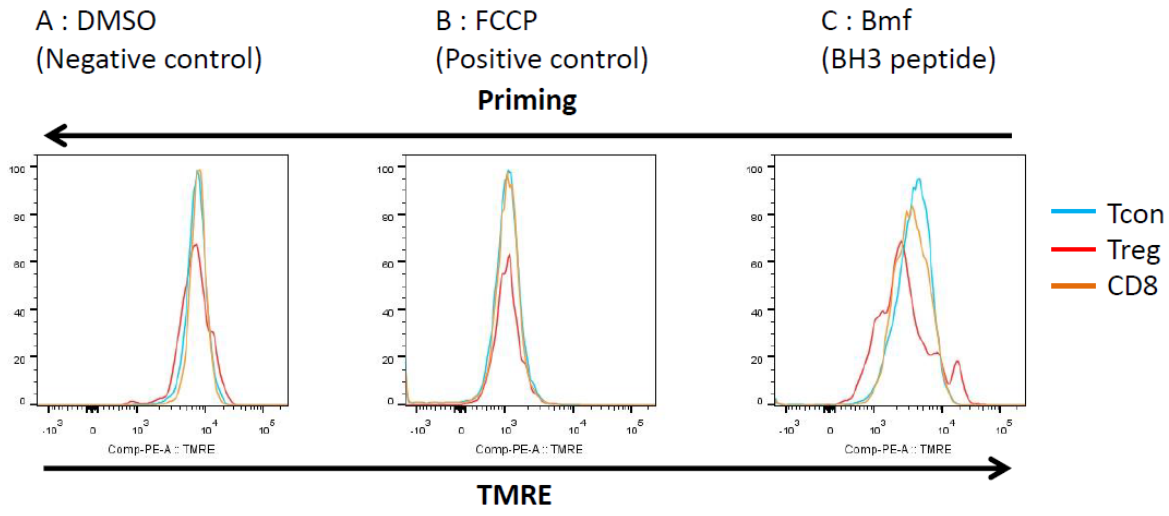
p-values from multivariable linear analysis for BH profile and CD95: cGVHD patients only (N=30)

model for		Bim	Bim0.03	Bid	Bad	NOXA	BAD+NOXA	PUMA	BMF	HRK	CD95	Ki67	Apop STS	Apop CD95	Blc2
Treg	age	0.07	0.46	0.66	0.84	0.44	0.37	0.18	0.75	1.00	0.94	0.44	0.74	0.69	0.78
	prednisone > 0	0.64	0.30	0.30	0.66	0.29	0.79	0.99	0.32	0.93	0.89	0.70	0.62	0.40	0.80
	gr 2-4 aGVHD Y/N	0.09	0.41	0.14	0.86	0.89	0.09	0.11	0.02	0.83	0.83	0.78	0.02	0.98	0.79
	severe cGVHD	0.88	0.01	0.36	0.02	0.21	0.0007	0.03	<.0001	0.08	0.41	0.16	0.55	0.12	0.001
	time from HSCT to sample	0.64	0.26	0.23	0.07	0.91	0.06	0.05	0.24	0.14	0.18	0.16	0.56	0.45	0.92
Tcon	age	0.16	0.79	0.30	0.48	0.95	0.46	0.35	0.75	0.72	0.76	0.72	0.58	0.64	0.66
	prednisone > 0	0.81	0.32	0.10	0.81	0.22	0.79	0.29	0.92	0.32	0.55	0.58	0.73	0.55	0.88
	gr 2-4 aGVHD Y/N	0.00	0.25	0.00	0.41	0.38	0.05	0.20	0.06	0.63	0.29	0.58	0.16	0.23	0.47
	severe cGVHD	0.61	0.11	0.12	0.02	0.17	0.02	0.06	0.007	0.51	0.85	0.78	0.76	0.49	0.037
	time from HSCT to sample	0.83	0.53	0.86	0.33	0.55	0.24	0.41	0.65	0.68	0.29	0.39	0.79	0.83	0.26
CD8	age	0.36	0.85	0.48	0.96	0.84	0.91	0.76	0.83	0.47	0.65	0.22	0.04	0.74	0.98
	prednisone > 0	0.69	0.98	0.10	0.72	0.40	0.95	0.47	0.62	0.74	0.77	0.86	0.06	0.78	0.54
	gr 2-4 aGVHD Y/N	0.01	0.76	0.01	0.16	0.54	0.02	0.13	0.11	0.95	0.37	0.11	0.83	0.29	0.03
	severe cGVHD	0.99	0.04	0.74	0.02	0.23	0.0045	0.04	0.002	0.70	0.33	0.23	0.51	0.45	0.029
	time from HSCT to sample	0.67	0.32	0.64	0.04	0.53	0.02	0.04	0.35	0.40	0.52	0.59	0.60	0.35	0.11

All P values indicate multivariable linear analysis for BH3 profile and CD95. Prednisone is included as Table 2 predinisone use or not.

Supplementary Figure 1

% Depolarization measurements used for the determination of depolarization in healthy donor T cell. (A) TMRE staining of cells incubated with DMSO is used for negative control (Tcon:blue, Treg:red, CD8:orange). (B) TMRE staining of cells incubated with FCCP is used for positive control. (C) TMRE staining of cells challenged with Bmf (representative BH3 peptide). Loss of TMRE staining indicates a high level of priming. The values in the table indicate the (Median Fluorescence Intensity) MFI for each condition in the figure above. The equation shown was used to calculate percent depolarization for each peptide using MFI values.



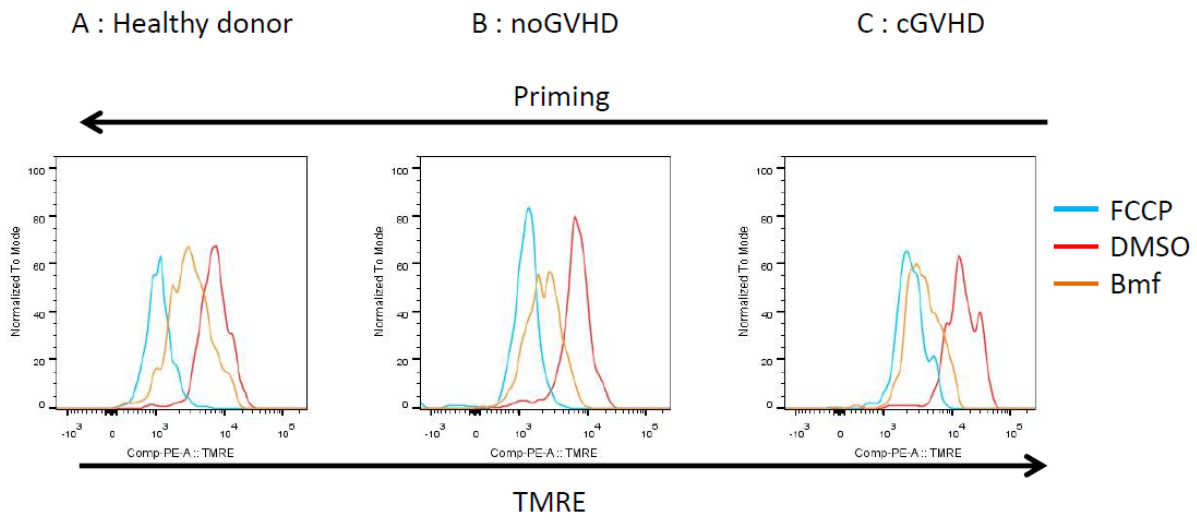
MFI	DMSO	FCCP	Bmf
Tcon	7596	1156	3855
Treg	7064	1213	2539
CD8	7992	1165	3455

$$\% \text{ Depolarization by peptide} = \frac{100 \times [(\text{Median PE of DMSO}) - (\text{Median PE of peptide})]}{[(\text{Median PE of DMSO}) - (\text{Median PE of FCCP})]}$$

Supplementary Figure 1.

Supplementary Figure 2

% Depolarization measurements used for the determination of depolarization in human T cells after HSCT. (A) Healthy donor TMRE staining after challenge with FCCP, DMSO and Bmf peptide (FCCP:blue, DMSO:red, Bmf:orange). (B) TMRE staining in a patients without GVHD. (C) TMRE staining in a patient with cGVHD. TMRE staining after challenge with Bmf was relatively lower in the patient with cGVHD than either healthy donor or patient without GVHD.



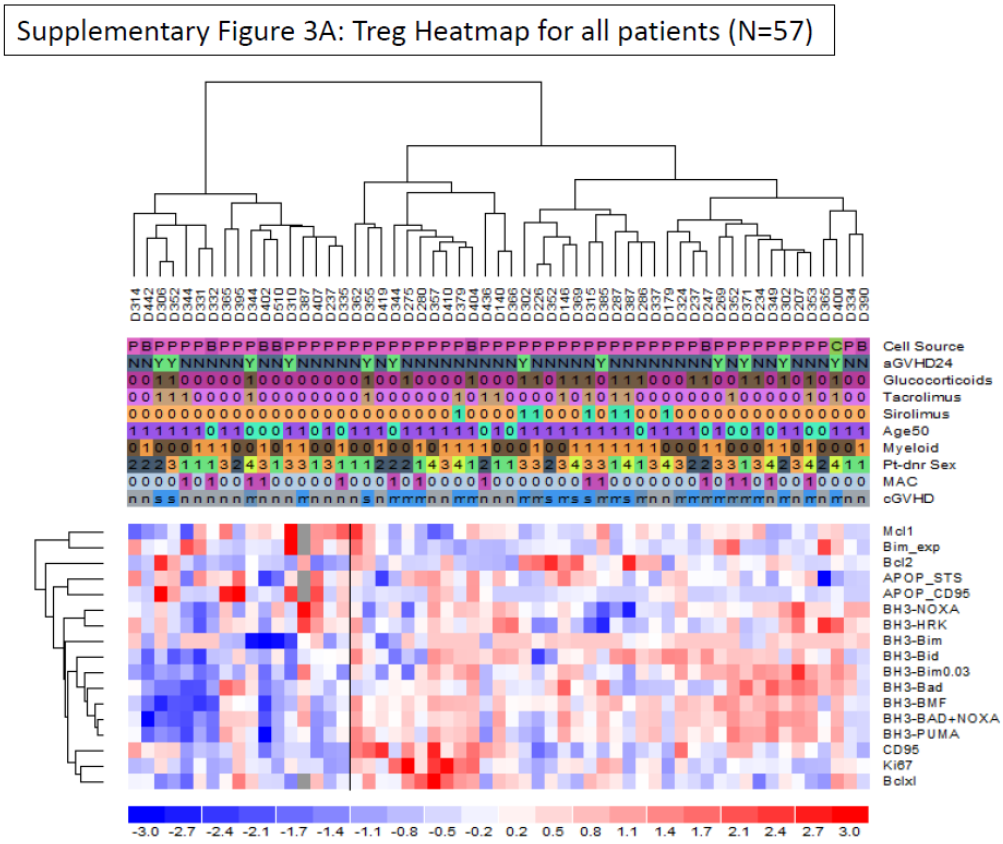
MFI	DMSO	FCCP	Bmf
HD	7064	1213	2539
noGVHD	7120	1327	2911
cGVHD	7142	1728	2500

Supplementary Figure 2.

Supplementary Figure 3

Unsupervised clustering of BH3 profile data. (A) – All HSCT Patients; B) Patients with cGVHD. Explanation of legend:

Cell Source: P - peripheral blood stem cell transplant, B - bone marrow stem cell transplant. aGVHD24: Y - grade II-IV acute GVHD, N - no grade II-IV aGVHD. Glucocorticoids=1 if any amount of prednisone, 0 if No. Tacrolimus: 1 - Yes, 0 - No. Sirolimus: 1 - Yes, 0 - No. Age50: 1 if age>=50: 0 if <50. MAC: 1 - myeloablative conditioning, 0 - reduced intensity conditioning. Myeloid: 1 - myeloid disease, 0 - other. Pt-dnr Sex: 1 - M->M, 2 - F->M, 3 - M-> F, 4 - F->F. cGVHD: 'n' for no cGVHD, 'm' in blue denotes 'mild' and 'm' in gray denotes 'moderate', 's' denotes severe.



Supplementary Figure 3B: Treg Heatmap for cGVHD only (N=30)