Focusing of the regulatory T-cell repertoire after allogeneic stem cell transplantation indicates protection from graft-*versus*-host disease

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Supplementary data

Supplementary methods

RNA isolation, cDNA amplification and Next-generation sequencing

RNA was isolated from the sorted cells using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA was directly reverse transcribed into cDNA using SMARTer RACE cDNA Amplification Kit (Clontech) with our own custom protocol. Briefly, 4 µl of the purified total RNA was combined with 1 µl of 12 µM oligo-dT primer in a PCR tube. Contents were mixed and incubated at 72°C in a thermal cycler for 3 minutes, then cooled down to 42°C for 2 minutes. The tubes were immediately removed from the cycler and 1x First-Strand Buffer (Clontech); 2.5 mM DTT (Clontech); 1 mM dNTP mix (Clontech); 0.6 µM SMARTer IIA Oligonucleotide; 10 units RNase Inhibitor (Clontech) and 50 units SMARTScribe Reverse Transcriptase (Clontech) were added to the tube. The PCR reaction was placed back into the 42°C cycler and the program continued at 42°C for 1 hour and 70°C for 10 minutes to terminate reverse transcription. Transcribed cDNA was used for a RACE-PCR reaction to amplify the VDJ (CDR3) region using a reverse primer specific for the TCRβ locus (TRB). PCR was done using the Advantage 2 Polymerase Mix (Clontech) with custom primers including adapter overhangs (**Table S1**)**.** We used custom Universal Primer Mix that included an overhang at the 5'-end of the short primer **(Table S1)**. The reverse primer was complementary to the C-region of the TRB (TRBC) and also included an adapter overhang **(Table S1)**. The entire volume of the cDNA (10 µl) was used as template for the 50 µl RACE-PCR. To sequence our amplicons generated via RACE, we used paired-end Illumina sequencing as described elsewhere[1]. Raw data extraction was performed with MIXCR (v2.1.11). Alignment of clonotypes based on IMGT database (22.05.2018) and assembling of clonotypes was done to correct PCR-errors. Default options were used for alignment and assembling respectively. Prior to downstream analysis, all samples were normalized to the

same number of productive reads to avoid bias. All bioinformatics and statistical analysis were performed on a Linux workstation running Ubuntu 14.04 LTS as operating system. Several bash and R-scripts were written as part of streamline data analysis method to form a standard, reproducible analysis pipeline. The pipeline included decompression, conversion into the FASTQ-file format and subsequent formatting. Analysis of TCR repertoire was done using VDJtools, tcR, or in-house scripts. All BASH and R scripts are uploaded to: https://github.com/MHHIMMUNOLOGY/MHHTCR_ANALYSIS.

T cell expansion ex vivo and in vitro suppression assay

Following re-isolation on day 14 or new freshly sorted (control), Tregs were expanded for a total of 14 days using Dynabeads™ Mouse T-Activator CD3/CD28 (ThermoFisher) according to manufacturer's instructions. Tregs were expanded for a total of 14 days in culture medium (RPMI 1640, Life Technologies) supplemented with 10% FCS, 1% penicillin/streptomycin, 5g/l glucose, 300U IL-2 (Sigma) and 50µM 2-mercapethanol (Sigma) with cells splitting according to need and IL-2 replenishing every second day. Treg suppression assay was performed as described elsewhere[2]. Briefly, responder conventional $CD4^+$ T cells were isolated using MojoSort™ Mouse CD4 Naïve T Cell Isolation Kit (Biolegend) according to manufacturer's instructions and stained with eFluor670 (Invitrogen). Tregs were co-cultured with responder cells in indicated ratios for 72h.

BMT in mouse model of GVHD

6-12 week old Balb/c (recipients, H-$2K^d$) female mice were irradiated with 800cGy from a Cs source. Within 24 hours, mice were transplanted with a mixture of $5x10^6$ of T cell-depleted BM and $5x10^5$ of pan-T cells from C57BL/6 mice (donors, H-$2K^b$) and $5x10^5$ Tregs from B6.Cg-Foxp3tm1Mal/J mice (donors, H-$2K^b$). Pan-T cells used in the experiment were isolated using Pan T cell Isolation Kit (Miltenyi) according to manufacturer's instructions.

Mice were sacrificed at 7 or 14 days after BMT. No animals showed signs of clinical GVHD by day 14.

Antibodies, flow cytometry and cell sorting

Antibodies were purchased from Miltenyi Biotec, eBiosciences, Biolegend and BD Biosciences. FACS data was acquired on a BD Pharmingen LSRII (BD Biosciences) and FlowJo software (Treestar) was used for data analysis. FACS sorting was carried out at the Cell Sorting Core Facility of Hannover Medical School on the FACS-Aria Fusion (BD Biosciences) or FACS-Aria IIu machine (BD Biosciences).

Supplementary Tables

Table S1: Patient and transplant characteristics.

Patient no.	Donor age	Disease	Patient Sex	Patient Age at Tx	Graft	Conditioning regimen	GVHD prophylaxis	Clinical GVHD
16	37	MDS	M	68	MMUD	BFM	ATG/CsA/ MMF	°IV
31	28	MPN	F	69	MUD	BFM	ATG/CsA/ MMF	°IV
44	49	MDS	M	55	MMUD	Flamsa-Bu	ATG/CsA/ MMF	°IV
49	52	MDS	M	64	MUD	BFM	ATG/CsA/ MMF	°I
65	28	MM	M	40	MUD	Flu/Melph	ATG/CsA/ MMF	°I
73	52	MPN	M	55	MUD	Flamsa-Bu	ATG/CsA/ MMF	°I
39	35	MDS	F	68	MMUD	Flu/Treo	ATG/CsA/ MMF	N/A
51	24	MPN	F	60	MUD	Flamsa-Bu	ATG/CsA/ MMF	N/A
55	39	AML	M	49	MUD	Flamsa-Bu	ATG/CsA/ MMF	N/A
61	32	AML	F	59	MUD	BFM	ATG/CsA/ MMF	N/A
78	50	MPN	F	57	MRD	Flamsa-Bu	ATG/CsA/ MMF	N/A
80	36	AML	M	62	MUD	BFM	ATG/CsA/ MMF	N/A

MDS=Myelodysplastic syndrome; MPN=Myeloproliferative Neoplasm; MM=Multiple Myeloma; AML=Acute Myeloid Leukemia; MUD=Matched Unrelated Donor; MRD=Matched Related Donor; MMUD=Mismatched Unrelated Donor; BFM= Berlin-Frankfurt-Munster therapy; ATG= Anti Thymocyte Globulin; Flamsa-Bu= Fludurabine + Cytarabine + Granulocyte colony-Stimulating Factor + Busulfan; Flu= Fludurabine; Melph= Melphalan; Treo= Treosulfan; MMF=Mycophenolate mofetil; CsA=Cyclosporine A

Table S2: Absolute numbers of conventional and regulatory $CD4^+$ T cells in peripheral blood.

Patient no.	$CD4^+$conv/Tregs	$CD4^+$conv (cell/µL)	Treg (cell/µL)	Clinical GVHD
16	12.86	2.7	0.21	°IV
31	7.10	19.8	2.79	°IV
44	2.65	0.9	0.34	°IV
49	69.13	31.8	0.46	°I
65	8.08	12.36	1.53	°I
73	7.83	7.52	0.96	°I
39	*	*	*	-
51	4.60	3.96	0.86	-
55	79.57	12.81	0.161	-
61	14.80	1.48	0.1	-
78	22.22	4	0.18	-
80	29.22	4.47	0.153	-

*Patient 39 had <100 lymphocytes on the day 30 as assessed by the hematology cell analyzer. Because of such small numbers, calculation of the exact number of $CD4^+$ cells in the blood for that patient was not possible.

Table S3: Primers with Illumina overhangs for RACE-PCR.

Primer	**Sequence (5'-3')**
(Forward) RACE-long universal primer	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
(Forward) RACE-short universal primer	[Illumina Overhang]-CTAATACGACTCACTATAGGGC
(Reverse) TRB (human)-primer	[Illumina Overhang]-GCACACCAGTGTGGCCTTTTGGG
(Reverse) TRB (mouse)-primer	[Illumina Overhang]-TGGCTCAAACAAGGAGACCT
(Forward) Illumina Overhang	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[Short universal primer]
(Reverse) Illumina Overhang	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[TRB]

Supplementary Figures

Figure S1

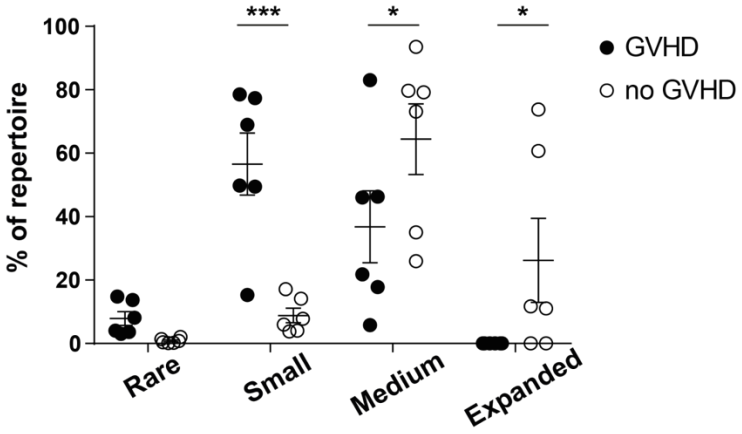

Figure S1: Clonotype proportions of the entire Treg TCR repertoire. Statistical analysis was performed two-way Anova. ***$p \leq 0.0005$; *$p \leq 0.05$.

Figure S2

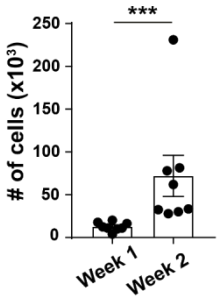

Figure S2: Treg recovery at week one and week two after BMT. Total number of Thy1.1^{+}CD4^{+}Foxp3^{+} cells recovered from secondary lymphoid organs of pooled recipient mice at week one and week two after BMT.

Figure S3

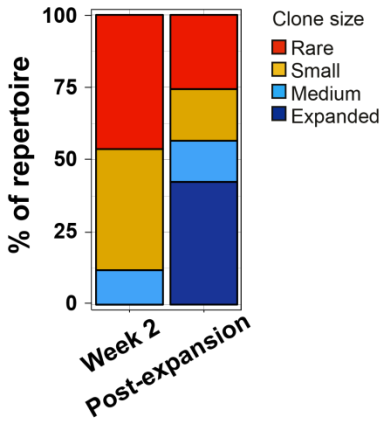

Figure S3: *Ex vivo* expansion does not diversify TCR repertoire. $CD4^{+}Foxp^{+}$ Tregs were sorted from B6.Cg-Foxp3tm1Mal/J mice. Cells were collected on week 2 after re-isolation as in Figure 3A (Pre-expansion) and after 2 weeks in vitro expansion protocol (Post-expansion). mRNA was collected and CDR3 region sequenced (**Supplementary methods**). Visualization of clone proportions: 0-0.1% (Rare clones), 0.1-1% (Small clones), 1-10% (Medium clones) and 10-100% (Expanded clones) of the total repertoire. One representative experiment out of two is shown.

Figure S4

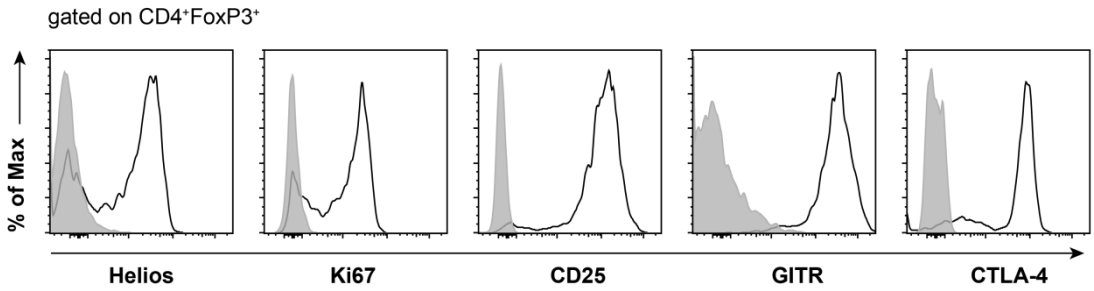

Figure S4: Expression of selected transcription factors and surface molecules in Tregs after two weeks expansion. Filled gray histograms represent respective isotype controls.

References

1. Ravens S, Schultze-Florey C, Raha S, et al. Human γδ T cells are quickly reconstituted after stem-cell transplantation and show adaptive clonal expansion in response to viral infection. *Nat. Immunol.* 2017;18(4):393–401.
2. Zhang P, Lee JS, Gartlan KH, et al. Eomesodermin promotes the development of type 1 regulatory T (T R 1) cells. *Sci. Immunol.* 2017;2(April):1–14.