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

The major complication after allogeneic stem cell transplantation or bone marrow transplantation (BMT) remains acute graft-*versus*-host disease (GvHD). Therefore, the development of specific therapies aiming at the prevention of GvHD whilst retaining immune competence against infections and malignancies are of utmost interest. $CD4^+Foxp3^+$ regulatory T cells (Tregs) are crucial for maintaining immune homeostasis and prevention of autoimmune and inflammatory disorders, such as GvHD.[1] Due to these properties, Tregs are prime candidates for the suppression of GvHD. However, the exact mechanism of Treg-mediated suppression is still unclear. Much attention has been drawn to the role of T-cell receptor (TCR) specificity in Treg functionality. The importance of TCR diversity and at the same time Treg antigen specificity in maintaining tolerance was highlighted both in experimental models and patients.[2] Interestingly, a superior immunosuppressive capacity of allo-antigen-specific Tregs in the context of clinical trans-

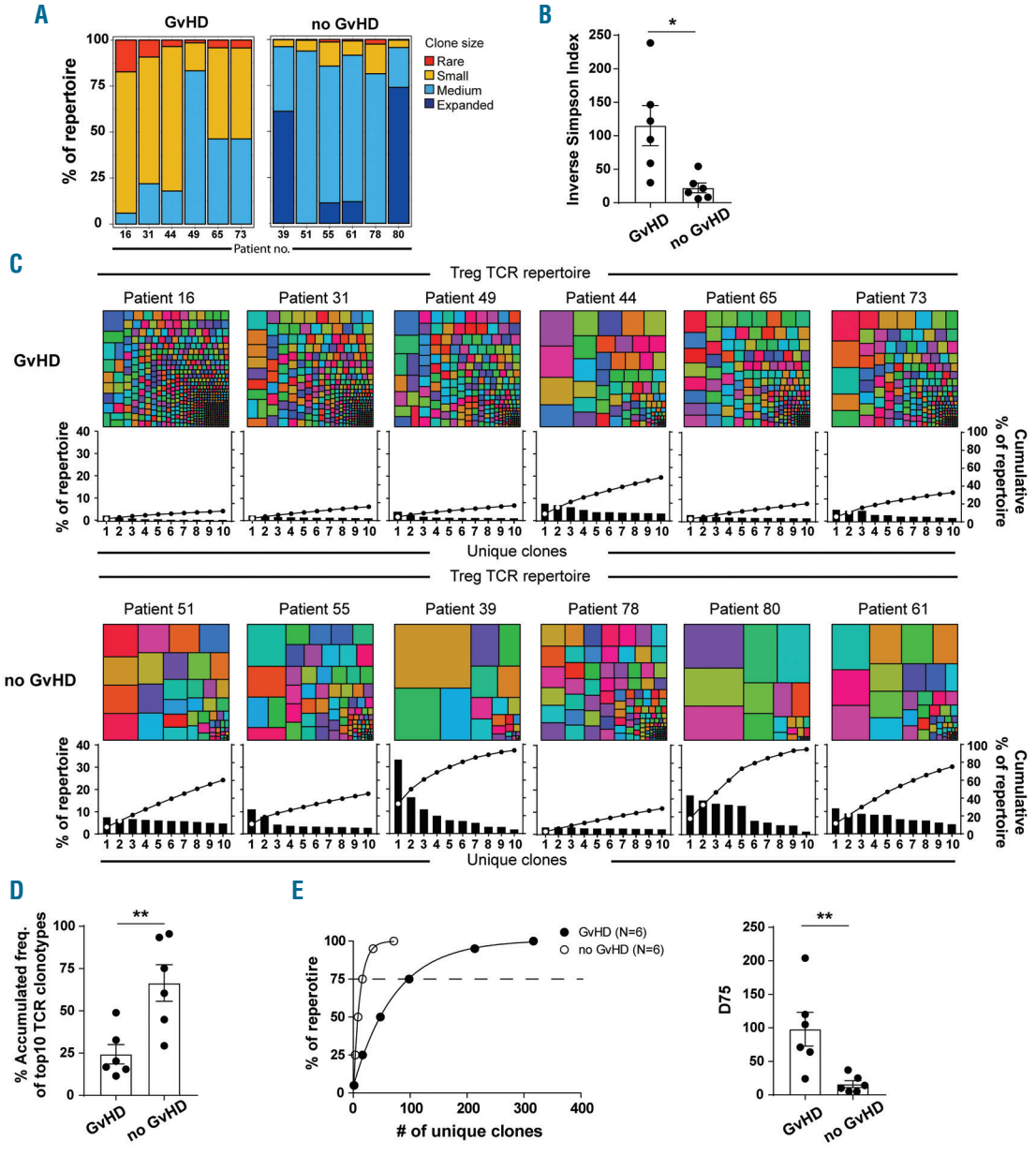

Figure 1. Treg TCR repertoire recovery and diversity after allogeneic hematopoietic stem cell transplantation. (A) Visualization of clone proportions: 0-0.1% (rare clones), 0.1-1% (small clones), 1-10% (medium clones) and 10-100% (expanded clones) of the total Treg TCR repertoire. (B) Inverse Simpson Index of Treg TCR repertoires. (C) Tree maps show each patient's Treg TCR repertoire, with each clone and its proportion to the repertoire represented by a differently colored square (each color is assigned randomly and does not match between plots). Plotted below is the clonotype frequency (bars) of each of the top 10 expanded clones (left y axis) and accumulated frequency (dots) of the top 10 expanded clones (right y axis) for each sample. (D) Percentage of overall Treg TCR repertoire taken up by the top 10 clones. (E) Left graph shows the number of unique clones in proportion to the overall TCR repertoire. Right graph displays a quantified comparison of the number of clones required to occupy 75% of the total TCR repertoire (D75). *$P \leq 0.05$ **$P \leq 0.01$.

plant tolerance has been demonstrated recently.[3] We hypothesize that optimal GvHD prevention is largely dependent on the recognition of allo-antigens by Tregs and subsequent expansion of specific Treg clones with a potent immunosuppressive capacity conferring protection from GvHD.

In this study, we suggest that Treg TCR specificity and repertoire diversity is crucial for the optimal function of Tregs, and therefore prevents GvHD early after transplantation. Patients which did not develop GvHD up to 100 days after stem cell transplantation (SCT) had a focused TCR repertoire with expanded Treg clonotypes, unlike patients suffering from acute GvHD, which show a more diverse Treg TCR repertoire. This observation was complemented in a well-established murine model[4] of acute GvHD, where we also observed a focused donor Treg TCR repertoire early after BMT suggesting a clonal expansion. These findings might provide a rationale for further development of TCR specific Treg cell therapies with a significant gain in efficiency.

We included 12 patients in this study, which was performed in accordance with the declaration of Helsinki and approved by the institutional review board at the Hannover Medical School (#2032-2013). All patients gave written informed consent. C57BL/6, B6.Cg-Foxp3tm1Mal/J (donors, H-2Kb) and BALB/c (recipients, H-2Kd) mice were bred at the Hannover Medical School. GvHD experiments[5] were performed as described in the *Online Supplementary Methods*. All animal experiments were carried out in accordance with institutional and governmental directives and approved under the permit number 33.12-42502-04-15/1856. RNA was isolated from the sorted cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using the SMARTer RACE cDNA Amplification kit (Clontech) followed by amplification of the *TCRβ CDR3* region. Amplicons were then subject to Illumina paired-end sequencing.[6] Analysis of the TCR repertoire was done using MiXCR, VDJtools and tcR (R-package) software.[7,8] Statistical analysis was done using Prism 7 (GraphPad) and the significance was calculated using the two-way Student's *t*-test, Mann-Whitney U test, or one-way ANOVA with Tukey post hoc testing. Mean values are shown ± standard error of mean (SEM). *P* values ≤0.05 were considered statistically significant. Details on the experiments are also provided in the *Online Supplementary Methods*.

To examine the Treg TCR repertoire, we sorted $CD4^+CD25^+CD127^-$ Tregs by fluorescence-activated cell sorting (FACS) from patients' peripheral blood mononuclear cells 30 days after allogeneic hematopoietic stem cell transplantation (*Online Supplementary Table 1 and 2*). In patients without GVHD the Treg TCR repertoire was less diverse than in GVHD patients (Figure 1A), as quantified according to the inverse Simpson Index (Figure 1B). Further analysis revealed a significantly greater number of medium and expanded clones in the patients without GvHD (*Online Supplementary Figure 1*). Of note, there was no significant correlation as assessed by the Pearson correlation analyses of sorted cell numbers *versus* the inverse Simpson Index (*data not shown*). This focused TCR repertoire in one, but not in the other patient group might point to an expansion of presumably allo-specific Tregs, which could be protective against GvHD. Accordingly, we observed a clonal Treg expansion as shown by tree maps (Figure 1C). However, we found no Treg clones shared by all patients (*data not shown*). Analysis of the highly expanded clones revealed that the top 10 expanded clones made up a significantly higher proportion of the overall Treg TCR repertoire in non-GvHD patients

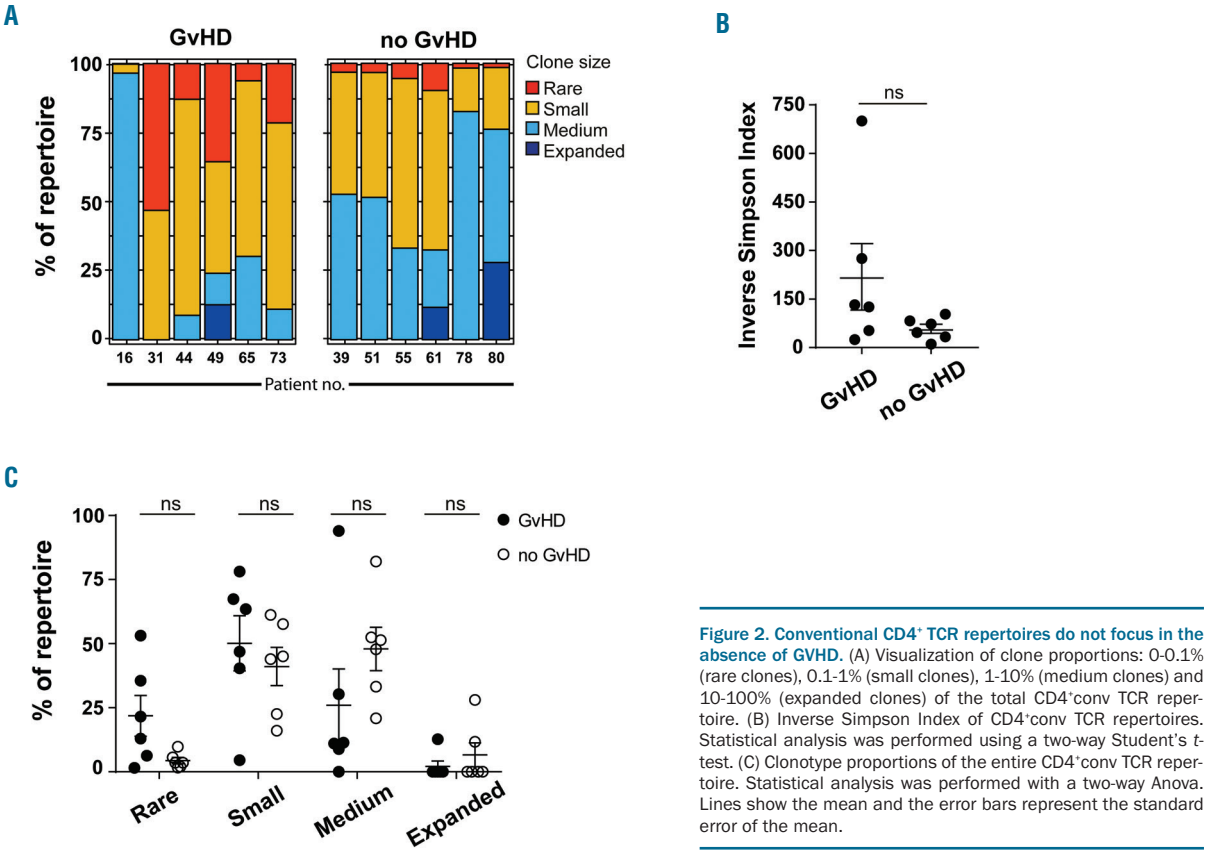

Figure 2. Conventional $CD4^+$ TCR repertoires do not focus in the absence of GVHD. (A) Visualization of clone proportions: 0-0.1% (rare clones), 0.1-1% (small clones), 1-10% (medium clones) and 10-100% (expanded clones) of the total $CD4^+$conv TCR repertoire. (B) Inverse Simpson Index of $CD4^+$conv TCR repertoires. Statistical analysis was performed using a two-way Student's *t*-test. (C) Clonotype proportions of the entire $CD4^+$conv TCR repertoire. Statistical analysis was performed with a two-way Anova. Lines show the mean and the error bars represent the standard error of the mean.

than in those with GvHD (Figure 1D). Further analysis of these TCR repertoires showed that in non-GvHD-patients less Treg clonotypes were needed to comprise 75% of the whole repertoire compared to those with GvHD (Figure 1E). These observations lead to the assumption that such expanded Treg clones are particularly effective in the prevention of GvHD. It is likely that specific T-cell clones evolve from a broad TCR repertoire after activation and proliferation, which then represent the protective focused repertoire. Of note, analyzing the TCR repertoire of conventional CD4$^+$ cells (CD4$^+$CD25$^{+/-}$CD127$^+$), we did not observe a significant change of diversity between patient cohorts (Figure 2). To test the hypothesis whether allo-activated Treg clones are more potent in preventing GvHD than polyclonal Tregs, we employed a murine model to examine the Treg TCR repertoire after BMT in more detail. To create BMT-recipients that are protected from GvHD, we co-injected Foxp3eGFP$^+$ Tregs with conventional T cells in a 1:1 ratio.[4,5] At one and two weeks after BMT, mice were sacrificed and donor Tregs from the spleen and lymph nodes were FACS-sorted based on the eGFP expression (Figure 3A). The number of recovered donor-derived Tregs was significantly greater on week two compared to week one post BMT (*Online Supplementary Figure 2*). The diversity of the Treg TCR repertoire on both time points was lower (Figure 3B) compared to the input Treg population as shown by the inverse Simpson Index (Figure 3C). By analyzing the Treg repertoire in more detail, we did not only find evidence of a focused TCR repertoire, but also of a substantial increase in the frequency of expanded clones (Figure 3D), similar to the observation in our patient cohort. Interestingly, 14 out of 20 samples collected two weeks after BMT (70%) comprised two expanded public CDR3 sequences (PS) CASSLGGESQNTLYF (PS1) and CASSPGSQNTLYF (PS2). Using Basic Local Alignment Search we compared the observed sequences with the NCBI database. Variant clonotypes with up to 94% similarity with PS1 and PS2 have been shown to recognize ribonucleoproteins (RNPs) and are affiliated with autoimmunity.[9] However, even though it is tempting to speculate about a possible connection of RNP-specific TCR with autoimmunity, more studies examining the antigen specificity of the TCR, along with paired

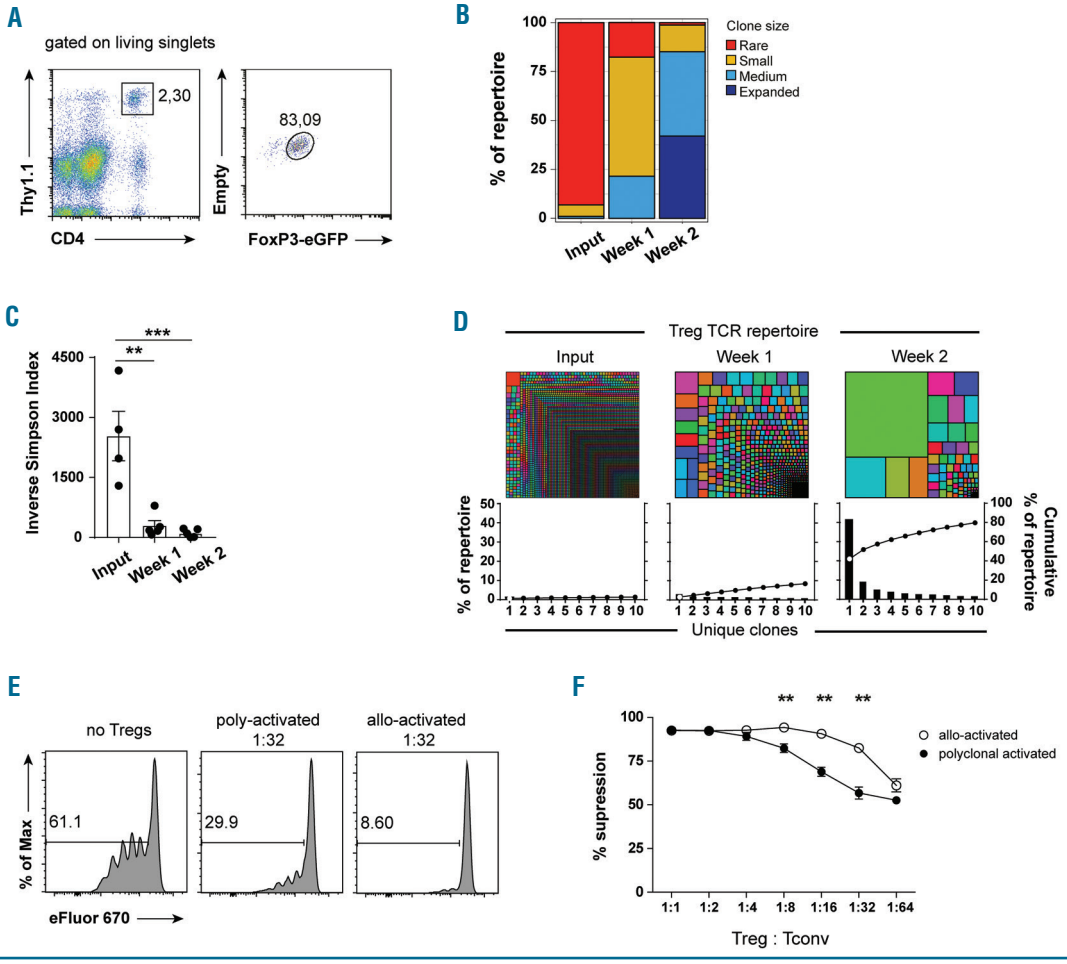

Figure 3. Allo-activated Tregs exhibit superior suppressive capacity. (A) Representative gating for re-isolation of donor FoxP3$^+$ Treg cells from recipient mice. (B) Visualization of clone proportions: 0-0.1% (rare clones), 0.1-1% (small clones), 1-10% (medium clones) and 10-100% (expanded clones) of the total repertoire. One representative experiment out of four is shown. (C) Inverse Simpson Indices of the initial input (n=4), week one (n=4), and week two (n=4) Treg TCR repertoire. (D) Tree maps showing Treg TCR repertoire and clonotype frequencies on different time points following bone marrow transplantation (BMT). Input Tregs were sorted by FACS according to the eGFP expression from a pool of spleenocytes and lymph node cells. One representative experiment out of five is shown. (E) Analysis of the *in vitro* Treg suppression assay. One representative experiment is shown. (F) Suppression of proliferation of eFluor670-labeled responder T cells *in vitro* by allo-activated *versus* polyclonal activated Tregs in indicated Treg : Tconv ratios. Data are combined from two independent experiments. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

TCRα- and TCRβ-chain sequencing are required.

To assess the suppressive capacity of allo-activated Treg clones, these were re-sorted 14 days post BMT, expanded *in vitro* to yield sufficient Treg cell numbers (*Online Supplementary Methods*), and tested in an *in vitro* suppression assay. Importantly, the TCR repertoire was still polyclonal, although the overall diversity dropped due to polyclonal expansion during *in vitro* culture (*Online Supplementary Figure 3*), similarly to findings published by Theil *et al*.[10] Detailed analysis of *in vitro* clonally expanded Tregs using intra- and extracellular markers showed that the cells retained Helios CD25, GITR, Ki67 and CTLA-4 expression (*Online Supplementary Figure 4*), indicating a stable Treg phenotype.[11,12] Next, the suppressive potential of such Tregs was assessed using an *in vitro* suppression assay. Compared to control polyclonal, activated Tregs, clonally expanded donor Tregs exhibited a superior suppressive capacity (Figure 3E). In repetitive experiments we could demonstrate that 4-fold diluted allo-activated Tregs were equally suppressive as activated polyclonal Tregs (Figure 3F).

Taken together, our results emphasize the importance of specific Treg clones for the prevention of GvHD. Similar findings have been suggested by Jin *et al*. using spectratyping and flow cytometry.[13] However, given the small patient sample size of the current study, these findings need to be validated in a larger patient cohort. Translation into clinical application is challenged by the complex process of identifying, isolating and expanding specific highly suppressive Tregs. Nonetheless, recent studies pave the way towards the discovery of neoantigens based on the single-cell *TCR*αβ sequencing, and engineering of specific Tregs using Chimeric Antigen Receptor technology.[14] This combined approach may facilitate the identification and engineering of allo-specific Tregs with a superior suppressive capacity. In conclusion, we and others[15] have shown that *TCR* sequencing of different T-cell subpopulations may emerge to predict the clinical outcome after HSCT.

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