bjh research paper

CD52-negative T cells predict acute graft-versus-host disease after an alemtuzumab-based conditioning regimen

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Received 16 January 2020; accepted for publication 9 April 2020

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Summary

Allogeneic haematopoietic stem cell transplantation (HSCT) after a reduced-intensity conditioning (RIC) regimen with fludarabine, melphalan and alemtuzmab is an effective therapy for haematological malignancies. Alemtuzumab, a monoclonal antibody against CD52, a glycosylphosphatidylinositol-anchor-bound surface protein on lymphocytes, depletes T cells to prevent graft-versus-host disease (GVHD). Despite this, acute and chronic GVHD (a/cGVHD) remain life-threatening complications after HSCT. The aim of the present study was to identify parameters to predict GVHD. In 69 patients after HSCT, T-cell subsets were functionally analysed. Reconstitution of $CD52^{neg}$ T cells and $CD52^{neg}$ regulatory T cells (Tregs) correlated with onset, severity and clinical course of aGVHD. Patients with aGVHD showed significantly lower levels of $CD52^{pos}$ T cells compared to patients with cGVHD or without GVHD ($P < 0{\cdot}001$). Analysis of T-cell reconstitution revealed a percentage of <40% of $CD52^{pos}CD4^{pos}$ T cells or $CD52^{pos}$ Tregs at day +50 as a risk factor for the development of aGVHD. In contrast, $CD52^{neg}$ Tregs showed significant decreased levels of glycoprotein A repetitions predominant (GARP; $P < 0{\cdot}001$), glucocorticoid-induced TNFR-related protein (GITR; $P < 0{\cdot}001$), chemokine receptor (CXCR3; $P = 0{\cdot}023$), C-C chemokine receptor type 5 (CCR5; $P = 0{\cdot}004$), but increased levels of immunoglobulin-like transcript 3 (ILT3; $P = 0{\cdot}001$), as well as a reduced suppressive capacity. We conclude that reconstitution of $CD52^{neg}$ T cells and $CD52^{neg}$ Tregs is a risk factor for development of aGVHD.

Keywords: GVHD, stem cell transplantation, T-cell depletion, T cells.

A reduced-intensity conditioning regimen (RIC) followed by allogeneic haematopoietic stem cell transplantation (HSCT) is an effective therapy to cure haematological malignancies. Acute and chronic graft-versus-host disease (a/cGVHD) are common and life-threatening complications after HSCT. One of the most effective ways to prevent GVHD is T-cell depletion (TCD) using alemtuzumab, a humanised monoclonal antibody against CD52.[1] CD52 is a glycosylphosphatidylinositol (GPI)-anchor-bound protein on the surface of lymphocytes, monocytes, eosinophils and present on cells of the male reproductive tract.[2] In alemtuzumab-treated patients >95% of $CD3^{pos}CD4^{pos}$ T cells and >80% $CD3^{pos}CD8^{pos}$ T cells were depleted.[3]

In 2019, Finazzi *et al.*[4] showed that despite TCD with alemtuzumab the incidence of aGVHD Grades II–IV (Grades III–IV) was 34% (13%) and of cGVHD was 4%.

Many studies revealed that $CD3^{pos}CD4^{pos}$ and $CD3^{pos}CD8^{pos}$ T cells play a crucial role in the development of GVHD.[5] Regulatory $CD3^{pos}CD4^{pos}CD25^{pos}FoxP3^{pos}$ T cells (Tregs) are responsible for induction and maintenance of self-tolerance and are required for prevention of GVHD.[6–8] In 2010, we found that $CD52^{neg}$ T cells reconstitute after alemtuzumab-based TCD, donor $CD3^{pos}CD4^{pos}$ T cells convert mixed to full donor T-cell chimerism and replenish the $CD52^{pos}$ T-cell pool after alemtuzumab-based TCD.[9] In the present study, we focussed on immune reconstitution of $CD3^{pos}CD4^{pos}$, $CD3^{pos}CD8^{pos}$ T cells and Tregs after TCD with alemtuzumab in the context of GVHD and showed for the first time that reconstitution of $CD52^{neg}$ T cells is associated with development, severity and clinical course of aGVHD.

 British Journal of Haematology, 2020, **191,** 253–262
First published online 14 May 2020
doi: 10.1111/bjh.16706

Patients, materials, and methods

Patients and conditioning regimen

Five buffy coats, as healthy controls, and peripheral blood samples were collected from 69 patients who underwent HSCT at the University Medical Center of the Johannes Gutenberg University Mainz, Germany between 2010 and 2018. Patients were treated with RIC: fludarabine (30 mg/m^2 days −7 to −3), melphalan (140 mg/m^2 day 2) and alemtuzumab.[4,8,10] All patients in our cohort received viral/antifungal/anti-microbial prophylaxis with aciclovir, posaconazole, cotrimoxazole/trimethoprim and ciprofloxacin. GVHD prophylaxis contained cyclosporine A (day −1 to +50). Donor selection involved molecular typing for human leukocyte antigen (HLA)-A, -B, -C, -DRB1, and -DQB1 and were matched at least in eight of 10 HLA alleles.

The protocol was approved by the local ethics committees [837·185·00 (2551)] and national authorities. All patients gave written informed consent.

GVHD was staged by National Institute of Health criteria.[11,12] Patients' characteristics are summarised in Table I.

Chimerism analyses were performed by the Institute of Forensic Medicine in Mainz, Germany: DNA was extracted from bone marrow and analysed for chimerism by polymerase chain reaction (PCR) in a multiplex-PCR-System 'PowerPlex ESX17' (Promega, Walldorf, Germany). Detection limit for patient/donor cells ~5%.

Phenotypic analyses by flow cytometry

Peripheral blood mononuclear cells (PBMCs) were stained with the following antibodies: anti-CD3-PE-Cy7, anti-CD4-allophycocyanin (APC)-H7, anti-CD25-V450, anti-CD39-APC, anti-HLA-DR-APC, anti-IgG-phycoerthyrin (PE), anti-IgG-V450, anti-IgG-APC-H7, anti-IgG-PE-Cy7, anti-CXCR3-APC, anti-CD3-V450, anti-CD3-APC-H7, anti-CD3-PE-Cy7 (BD Biosciences, San Jose, CA, USA), anti-CD8-APC, anti-IgG-fluorescein isothiocyanate (FITC) and anti-IgG-APC, anti-CD3-FITC, anti-CD3-PE, anti-CD3-APC (Beckmann Coulter, Brea, CA, USA), anti-CD127-FITC, anti-GARP-APC (eBioscience, Vienna, Austria), anti-CD52-PE (Serotec, Raleigh, NC, USA), fluorescent-labelled aerolysin variant (FLAER)-FITC (Biozol, Eching, Germany), anti-CD44-APC, anti-GITR-APC, anti-CD62L-APC (BioLegend, San Diego, CA, USA), anti-CD45RA-APC (Miltenyi, Bergisch Gladbach, Germany) and anti-ILT3-APC (Invitrogen, Carlsbad, CA, USA). For intracellular staining of forkhead box P3 (Foxp3) and the transcription factor HELIOS, cells were prepared using Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the

Table I. Patient characteristics.

Characteristic	Total	Acute GVHD	Chronic GVHD	No GVHD
Total patients, *n* (%)	69	35 (50·7)	12 (17·3)	22 (31·8)
Age, years, mean (range)	58 (27–75)	58 (27–73)	53 (36–68)	59 (39–75)
Sex, M;F, *n*	44;25	20;15	8;4	16;6
Disease, *n* (%)				
AML	24 (34·7)	9 (25·7)	5 (41·6)	10 (45·5)
CML	2 (2·8)	2 (5·7)	0 (0)	0 (0)
MDS	12 (17·3)	9 (25·7)	1 (8·3)	2 (9·0)
Lymphoma	14 (20·2)	8 (22·8)	3 (25)	3 (13·6)
Myeloma	14 (20·2)	6 (17·1)	2 (16·6)	6 (27·2)
MPS	2 (2·8)	0 (0)	1 (8·3)	1 (4·5)
Others	1 (1·4)	1 (2·8)	0 (0)	0 (0)
Source of graft, *n* (%)				
MUD	59 (85·5)	32 (91·4)	11 (91·6)	16 (72·7)
Sibling	10 (14·5)	3 (8·6)	1 (8·3)	6 (27·2)
HLA matching, *n* (%)				
10;10	47 (68·1)	22 (62·8)	7 (58·3)	18 (81·8)
9;10	19 (27·5)	11 (31·4)	4 (33·3)	4 (18·1)
8;10	3 (4·3)	2 (5·7)	1 (8·3)	0 (0)
$CD3^+$ ($\times 10^8$)/kg in the graft, mean, median (range)	243, 205, (7–879)	280, 243, (7–879)	155, 149, (104–251)	215, 203, (121–325)
Full donor chimerism, *n* (%)	61 (88)	31 (88)	10 (83)	20 (90)
Virus reactivation, *n* (%)				
EBV	23 (33)	12 (34)	4 (33)	7 (31)
CMV	37 (53)	17 (48)	8 (66)	12 (54)
Day of analysis post-HSCT, mean, median (range)	243, 162, (40–2007)	154, 120, (40–441)	578, 352, (94–2007)	204, 179 (48–480)

Day of analysis post-HSCT provides the information on which day after HSCT blood draw for the comparison between the three GVHD groups was performed. M, male; F, female; HLA, human leucocyte antigen; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; MPS, myeloproliferative syndrome; MUD, matched unrelated donor; EBV, Epstein–Barr virus; CMV, cytomegalovirus.

manufacturer's protocol and stained with anti-Foxp3-FITC and anti-HELIOS-APC (eBioscience). For intracellular staining of cytotoxic T-lymphocyte antigen 4 (CTLA-4), Granzyme A and chemokine (C-C motif) receptor 5 (CCR5) a fixation/permeabilisation solution kit (BD Biosciences) was used according to the manufacturer's protocol and stained with anti-CTLA4-APC, anti-Granzyme A-APC and anti-CCR5-APC (BioLegend, USA). Analyses were performed on a BD Biosciences fluorescence-activated cell sorting (FACS) Canto II Flow Cytometer and analyzed with BD FACSDiva Software (BD Biosciences).

Tregs isolation

PBMCs were labelled with anti-CD4-MicroBeads (MACS, Miltenyi) and isolated according to the manufacturer's protocol followed by FACS with FACS Aria IIu and FACSDiva 6.13 software (BD Biosciences) of the $CD25^{hi}CD127^{int/low}$ cells and sorted into $CD52^{pos}$ and $CD52^{neg}$ Tregs.

CD52-based Tregs suppression assay and Treg-marker expression under stimulation

Third party PBMCs were incubated with $CD52^{pos}$ or $CD52^{neg}$ Tregs of the same patient and proliferation was measured after 6 days. The cell suspension was incubated in AIMV medium (Gibco, Life Technologies, Carlsbad, CA, USA) and 10% human serum albumin and stimulated with recombinant human interleukin 2 (rhIL-2, 100 iu/ml) (Novartis, Basel, Switzerland) and OKT3 (30 ng/ml) (eBioscience) at day 0. Proliferation was evaluated by measuring carboxyfluorescein succinimidyl ester (CFSE) concentration performed with CellTrace© CFSE Cell Proliferation Kit (ThermoFisher, Waltham, MA, USA) according to the manufacturer's protocol. PBMCs from six patients were incubated for 24 h with IL-2 and OKT3 and marker expression analysed by flow cytometry.

Statistics

Graph Pad Prism 5 software (Graphpad Software Inc., La Jolla, CA, USA) and the Statistical Package for the Social Sciences (SPSS®), version 23 (SPSS Inc., IBM Corp., Armonk, NY, USA) were used. For all analyses, a $P < 0{\cdot}05$ was considered to be statistically significant. Non-parametric methods were used for group comparisons (Mann–Whitney test or Kruskal–Wallis test). Related group comparison for two groups was performed by Wilcoxon signed-rank test or Kruskal–Wallis test for more than two related groups.

Results

Reconstitution of leucocytes after alemtuzumab-based RIC HSCT

Absolute leucocyte count and lymphocyte counts did not differ significantly between patients with aGVHD, cGVHD or without GVHD ($P = 0{\cdot}23$; $P = 0{\cdot}09$).

Analysing relative counts of T cells, we found that patients with aGVHD had significantly higher amounts of $CD3^{pos}CD4^{pos}$ T cells (mean = 29·8%, median = 25·60%) compared to patients with cGVHD (mean = 17·15%, median = 12%; $P = 0{\cdot}033$) or without GVHD (mean = 13·83%, median = 13·0%; $P = 0{\cdot}001$). While, patients with cGVHD had significantly higher amounts of $CD3^{pos}CD8^{pos}$ T cells (mean = 30·11%, median = 28·3%) compared to patients with aGVHD (mean = 15·3%, median = 8·55%; $P = 0{\cdot}004$).

$CD52^{neg}$/GPI^{neg} T cells reconstitute after alemtuzumab-based RIC HSCT

In healthy controls ($n = 5$) >98% of $CD3^{pos}CD4^{pos}$, $CD3^{pos}CD8^{pos}$ T cells or Tregs expressed CD52. Patient-derived T-cell subsets [median (range) time of analysis after HSCT was 162 (40–2007) days; Table I] had a significantly lower frequency of $CD52^{pos}$ T cells: $CD3^{pos}CD4^{pos}$ T cells had the lowest CD52 expression (mean: 37·28%, median: 27·40%) followed by Tregs (mean: 50·30%, median: 44·50%) and $CD3^{pos}CD8^{pos}$ T cells (mean: 63·62%, median: 80·00%). CD52 expression differed statistically significantly between $CD3^{pos}CD4^{pos}$ T cells and $CD3^{pos}CD8^{pos}$ T cells ($P < 0{\cdot}0001$), between $CD3^{pos}CD4^{pos}$ T cells and Tregs ($P < 0{\cdot}0001$) and between $CD3^{pos}CD8^{pos}$ T cells and Tregs ($P = 0{\cdot}046$).

$CD52^{neg}$ T cells lack GPI anchor consistently, indicating a loss of GPI-anchor expression on the lymphocyte surface, leading to missing surface expression of GPI-anchor bound molecules like CD52 (Fig 1).

Reconstitution of $CD52^{neg}$ T cells correlates with development of aGVHD

Reconstitution of $CD52^{neg}$ T cells differed significantly between patients with aGVHD, cGVHD or without GVHD: $CD3^{pos}CD4^{pos}$ T cells from patients with aGVHD expressed lower levels of CD52 (mean: 11·33%, median: 5·89%) compared to $CD3^{pos}CD4^{pos}$ T cells from patients with cGVHD (mean: 63·19%, median: 77·85%; $P < 0{\cdot}001$) and compared to $CD3^{pos}CD4^{pos}$ T cells of patients without GVHD (mean: 66·97%, median: 62·50%; $P < 0{\cdot}001$). There were no significant differences in CD52 expression of $CD3^{pos}CD4^{pos}$ T cells between patients with cGVHD and without GVHD ($P = 0{\cdot}65$). Similar results were found within Tregs and $CD3^{pos}CD8^{pos}$ T cells: Tregs from patients with aGVHD also expressed lower levels of CD52 (mean: 16·31%, median: 5·56%) compared to Tregs from patients with cGVHD (mean: 75·47%, median: 91·00%; $P < 0{\cdot}001$) and compared to Tregs from patients without GVHD (mean: 91·30%, median: 94·50%; $P < 0{\cdot}001$). There were no significant differences in CD52 expression of Tregs between patients with cGVHD and without GVHD ($P = 0{\cdot}40$). The $CD3^{pos}CD8^{pos}$ T cells of patients with aGVHD expressed the highest levels

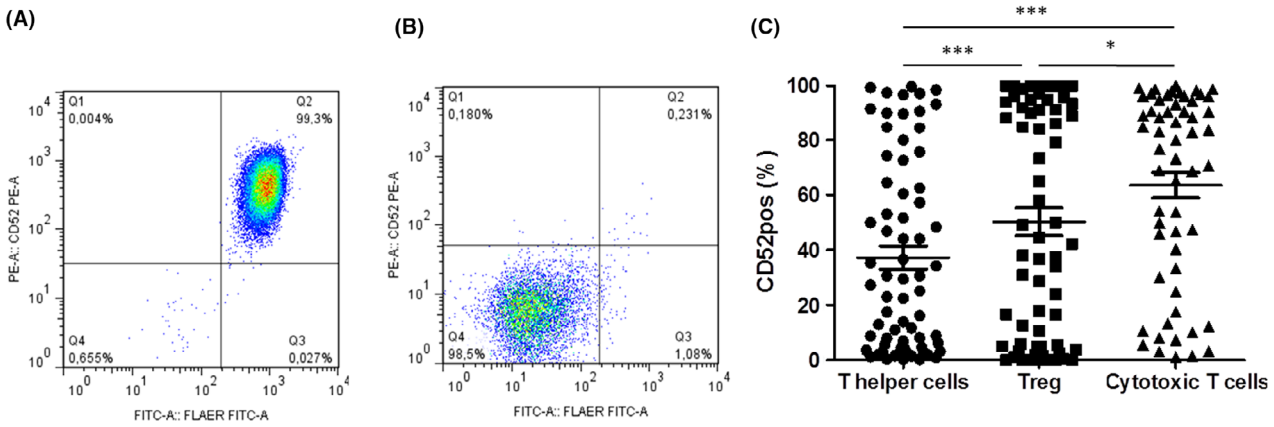

Fig 1. CD52/GPI-anchor expression in different T-cell subsets. Healthy individuals show >98% CD52/GPI-anchor expression within T-cell subsets, here an example is shown of $CD3^{pos}$ T cells in a healthy individual (A). In contrast to healthy individuals patients after alemtuzumab-based conditioning regimen had lower CD52/GPI-anchor expression within the T-cell subsets, here an example is shown of $CD3^{pos}$ T cells in a patient at day +60 after HSCT (B). $CD3^{pos}CD4^{pos}$ T-helper cells had the lowest CD52/GPI-anchor expression, followed by Tregs and $CD3^{pos}CD8^{pos}$ cytotoxic T cells in the post-HSCT cohort (C). Dot plots with mean ± SEM are shown. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$. [Colour figure can be viewed at wileyonlinelibrary.com]

of CD52 within the T-cell subsets of patients with aGVHD (mean: 43·82%, median: 45·80%), but CD52 expression of $CD3^{pos}CD8^{pos}$ T cells was again significantly lower in comparison to patients with cGVHD (mean: 76·10%, median: 92·75%; $P = 0{\cdot}004$) or without GVHD (mean: 88·60%, median: 90·65%; $P < 0{\cdot}001$). Comparison of CD52 expression of $CD3^{pos}CD8^{pos}$ T cells between patients with cGVHD or without GVHD showed no significant differences ($P = 0{\cdot}59$). These results indicate that patients with a combination of high numbers of $CD52^{pos}CD3^{pos}CD8^{pos}$ T cells and low numbers of $CD52^{pos}$ Tregs and $CD3^{pos}CD4^{pos}$ T cells are at risk of aGVHD (Fig 2).

Longitudinal development of CD52 expression in T-cell subsets after alemtuzumab-based TCD

We analysed CD52 expression of T-cell subsets at different time-points in 12 patients who developed aGVHD, three patients with cGVHD and seven patients who never developed any GVHD. In patients who never developed any GVHD, $CD52^{pos}$ T cells reconstituted early after HSCT: >80% Tregs and $CD3^{pos}CD8^{pos}$ T cells expressed CD52 at day +50, but reconstitution of $CD52^{pos}CD3^{pos}CD4^{pos}$ T cells was later in comparison to $CD52^{pos}$ Tregs and $CD52^{pos}CD3^{pos}CD8^{pos}$ T cells of patients without GVHD, but faster in comparison to patients with aGVHD. Patients with aGVHD showed delayed reconstitution of $CD52^{pos}$ T-cell subsets in comparison to patients with cGVHD or no GVHD: none of the patients with aGVHD displayed >60% $CD52^{pos}CD3^{pos}CD4^{pos}$ T cells at day +350. Reconstitution of $CD52^{pos}$ T-cell subsets over time correlated with better clinical outcome. Three patients died of severe aGVHD. These patients showed very low CD52 expression in $CD3^{pos}CD4^{pos}$ T cells (<40%) and Tregs (<20%), but a high CD52 expression within the $CD3^{pos}CD8^{pos}$ T cells (up to 82%).

Referring to patients with cGVHD reconstitution of $CD52^{pos}$ T cells showed broad differences. One patient in the cGVHD group died of severe cGVHD of the skin and never reached >40% CD52 expression in Tregs and $CD3^{pos}CD4^{pos}$ T cells, but had high levels within $CD3^{pos}CD8^{pos}$ T cells (up to 100%). In summary, the results indicate a rate of <40% $CD52^{pos}CD3^{pos}CD4^{pos}$ T cells and $CD52^{pos}$ Tregs at day +50 as a predictive marker for the development of aGVHD (Fig 3).

Influence of Treg counts and functional Treg markers

In recent literature a low Treg count,[13,14] altered Treg function[15–17] and changes of Treg-marker expression[7,18] are described as risk factors for a development of GVHD. Therefore, we analysed the Treg count and expression of functional markers within each GVHD group, their expression on $CD52^{pos}$ and $CD52^{neg}$ Tregs, and performed functional analysis of $CD52^{pos}$ and $CD52^{neg}$ Tregs derived from the same patient.

In our cohort, PBMCs of patients with aGVHD contained less Tregs (% Tregs of $CD3^{pos}CD4^{pos}$ T cells mean: 3·63%, median: 1·91%) in comparison to patients with cGVHD (mean: 3·98%, median: 3·56%) and those without GVHD (mean: 5·4%, median: 3·61%), but with no significant differences ($P = 0{\cdot}25$).

Cell surface receptor glycoprotein A repetitions predominant (GARP) is described as a marker of activated Tregs.[19] GARP expression was higher on $CD52^{pos}$ Tregs (mean: 7·83%, median: 3·51%) compared to $CD52^{neg}$ Tregs (mean: 1·5%, median: 0%; $P < 0{\cdot}001$). GARP expression on Tregs of patients with aGVHD (mean: 3·1%, median: 1·08%) was higher in comparison to patients with cGVHD (mean: 1·58%, median: 1·5%; $P = 0{\cdot}91$), but significantly lower in comparison to patients without GVHD (mean: 4·32, median:

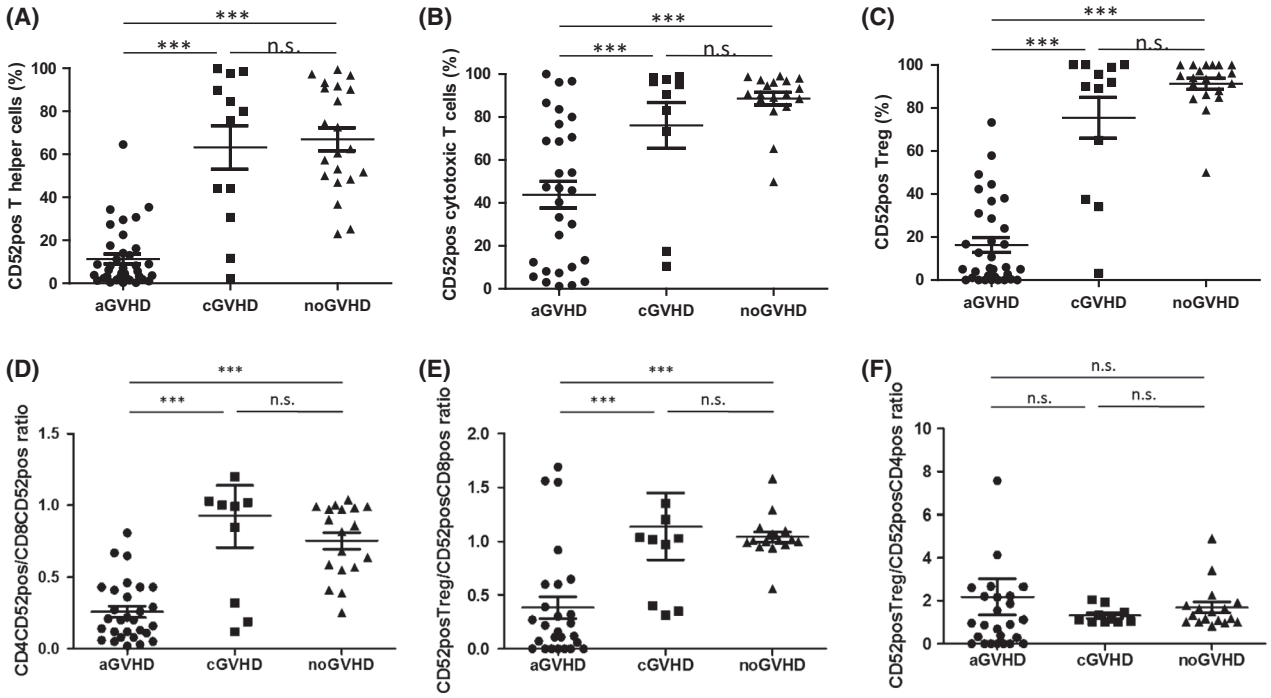

Fig 2. CD52/GPI-anchor expression in different T-cell subsets predicts aGVHD. $CD3^{pos}CD4^{pos}$ T cells (A), $CD3^{pos}CD8^{pos}$ T cells (B) and Tregs (C) show significantly lower CD52/GPI-anchor expression in patients with aGVHD in comparison to patients with cGVHD or without GVHD. No significant differences in CD52/GPI expression were seen between the T-cell subsets of patients with cGVHD or no GVHD. Significantly lower ratios of $CD52^{pos}CD4^{pos}/CD52^{pos}CD8^{pos}$ T cells (D) and $CD52^{pos}$ Treg/$CD52^{pos}CD8^{pos}$ T cells (E) were found in patients with aGVHD. $CD52^{pos}$ Treg/$CD52^{pos}CD4^{pos}$ T-cell ratio (F) was not altered between the three groups. Dot plots with mean ± SEM are shown $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$..

4·52%; $P = 0{\cdot}006$). Also significant differences were seen between patients with cGVHD and without GVHD ($P = 0{\cdot}029$).

Glucocorticoid-induced TNFR-related protein (GITR)[20] expression was significantly higher on $CD52^{pos}$ Tregs (mean: 11·91%, median: 4·81%) compared to $CD52^{neg}$ Tregs (median: 5·65%, median: 1·48%; $P < 0{\cdot}001$). In patients with aGVHD, GITR expression (mean: 3·85%, median: 2·01%) was significantly lower compared to patients without GVHD (mean: 14·6%, median: 8·85; $P = 0{\cdot}017$).

Chemokine receptor CXCR3[10] showed higher expression in the $CD52^{pos}$ Tregs group (mean: 11·16%, median: 7·2%) compared to $CD52^{neg}$ Tregs (mean: 5·81%, median: 3·44%; $P = 0{\cdot}023$). Between the GVHD groups there were no significant differences ($P = 0{\cdot}22$).

C-C chemokine receptor type 5 (CCR5)[21] had higher expression on $CD52^{pos}$ Tregs (mean: 4·75%, median: 2·71%) than on $CD52^{neg}$ Tregs (mean: 2·3%, median: 0·69%; $P = 0{\cdot}004$). Between the GVHD groups there were no significant differences ($P = 0{\cdot}21$).

Surface receptor ILT3, also known as leucocyte immunoglobulin-like receptor B4 (LILRB4),[22] was really only detectable on $CD52^{pos}$ Tregs (mean: 29·11%, median: 26·45%) compared to $CD52^{neg}$ Tregs (mean: 1·45%, median: 1·27%; $P = 0{\cdot}001$), but showed no significant differences between the GVHD groups ($P = 0{\cdot}93$).

For CD39, CD44, CD62L, CD45RA, HLA-DR, Granzyme A and transcription factor HELIOS there were no significant differences between $CD52^{pos}$ Tregs and $CD52^{neg}$ Tregs or between the GVHD groups. The expression of CTLA4 was significantly higher in the Tregs of aGVHD patients (mean: 92·58%, median: 94·3%) compared to the Tregs of patients without GVHD (mean: 79·7%, median: 83·95%; $P = 0{\cdot}036$) (Fig 4).

Functional analysis of $CD52^{neg}$ and $CD52^{pos}$ Tregs

To analyse potential differences of suppressive capacity between $CD52^{pos}$ or $CD52^{neg}$ Tregs, we isolated $CD52^{pos}$ and $CD52^{neg}$ Treg at one time-point from three different patients (patient 1: aGVHD, day 105 after HSCT; patient 29: aGVHD, day 126 after HSCT; patient 68: no GVHD, day 136 after HSCT) and compared their function in the above mentioned experiments. Analysing proliferation of $CD3^{pos}CD8^{pos}$ T cells after 6 days showed that $CD52^{pos}$ Tregs and $CD52^{neg}$ Tregs both were able to suppress effector T-cell proliferation, but with different efficiency: in all experiments $CD52^{pos}$ Tregs showed a stronger suppressive capacity than their $CD52^{neg}$ Treg counterparts. To gain more information about possible mechanisms of different suppressive capacities of $CD52^{pos/neg}$ Tregs we stimulated patients' PBMCs ($n = 6$): GARP expression on patient Tregs was increased in both groups but to a greater extent in $CD52^{pos}$ Tregs (Fig 5).

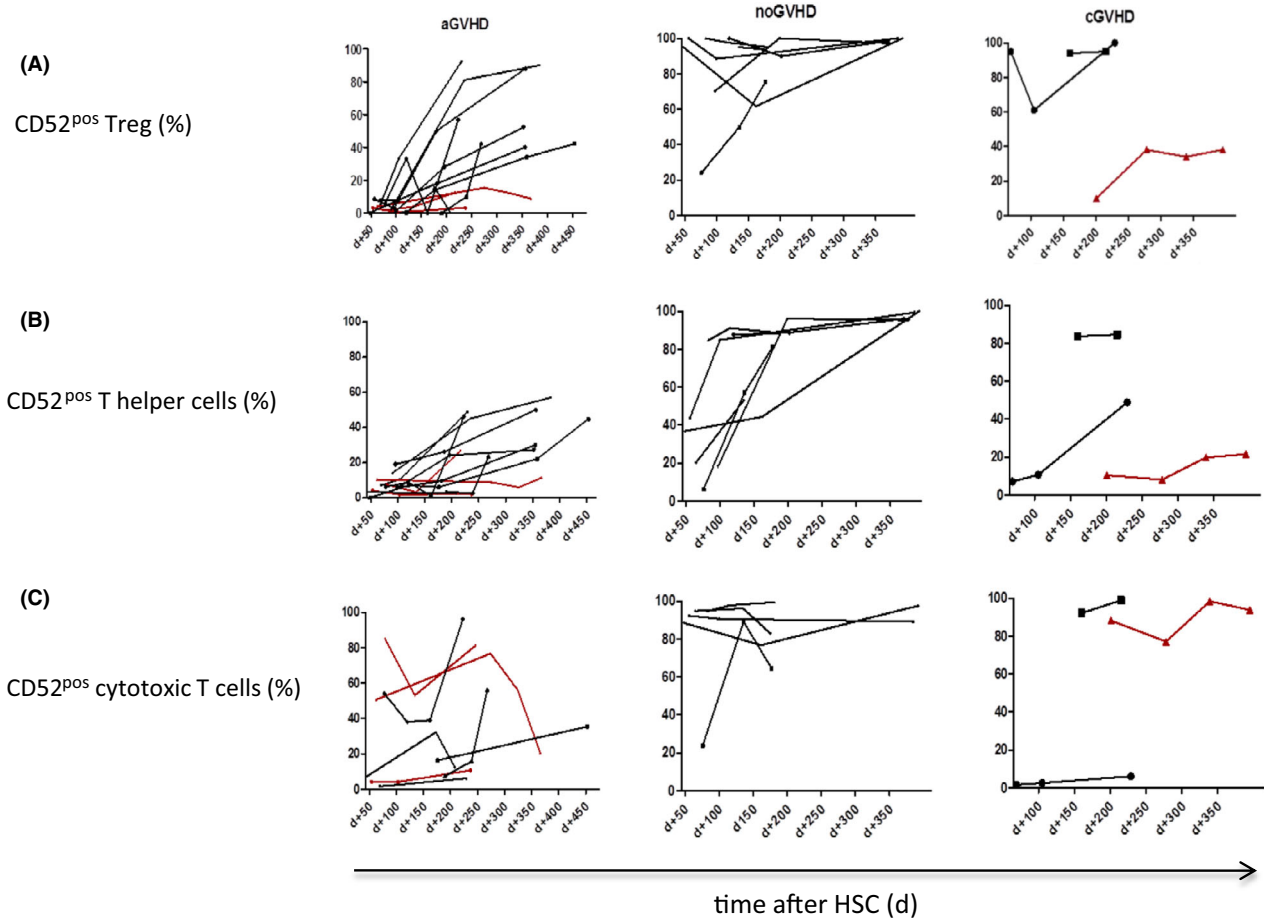

Fig 3. Longitudinal reconstitution of $CD52^{pos}/GPI^{pos}$ T-cell subsets. Longitudinal reconstitution of $CD52^{pos}/GPI^{pos}$ Tregs (A), $CD52^{pos}/GPI^{pos}CD3^{pos}CD4^{pos}$ T-helper cells (B) and $CD52^{pos}/GPI^{pos}CD3^{pos}CD8^{pos}$ cytotoxic T cells (C) in patients with aGVHD (n = 12), cGVHD (n = 3) or without GVHD (n = 7). Every point in a row represents one point of time when the relative CD52/GPI anchor expression analysis was performed. Red rows represent patients who died of aGVHD (n = 3) or cGVHD (n = 1). x-axis, time after transplantation (days) and y-axis: percentage of $CD52^{pos}/GPI^{pos}$ T cells (%). [Colour figure can be viewed at wileyonlinelibrary.com]

Discussion

Reduced-intensity conditioning HSCT protocols using alemtuzumab, are feasible to treat older and pretreated patients with low tumour-related mortality; however, despite TCD GVHD occurs to some extent.[4,23–26] Different schedules and levels of alemtuzumab can contribute to clinical effects and different T-cell reconstitution pattern.[27–29] Patients treated with 'proximal alemtuzumab' (close to the time of graft infusion) developed more mixed chimerism[29] and less aGVHD compared with patients treated with 'distal alemtuzumab" (more distant from the time of graft infusion) schedules, whereas 'intermediate alemtuzumab' (e.g. days −14 to −10) is described to reduce the incidence of mixed chimerism and is associated with a low incidence of aGVHD and decreases the need for additional haematopoietic cell products after HSCT.[28] In a larger cohort of 101 patients treated in our clinic with the same 'proximal alemtuzumab' conditioning regimen, as in our present cohort, 46% of the patients developed aGVHD. In patients receiving a conditioning regimen without alemtuzumab, $GPI^{neg}/CD52^{neg}$ T-cell populations are detected at the same low frequencies as in healthy individuals (<2%).[30] In 2003 it was reported that alemtuzumab is detectable in patients serum up to 56 days after HSCT[31] and that patients receiving alemtuzumab show a poor immune reconstitution, particularly with slow recovery of the $CD3^{pos}CD4^{pos}$ T-cell subset.[32] In our present cohort of 69 patients after alemtuzumab-based RIC, reconstituting T cells lacked CD52 up to 2006 days after HSCT. This is consistent with previous publications by our group[9] and others: Loeff *et al.*[33] showed that lack of CD52 expression following alemtuzumab-based TCD results from loss of GPI-anchor expression caused by a highly polyclonal mutation frequency of the phosphatidylinositol glycan anchor biosynthesis Class A (*PIGA*) gene in T cells. Loeff *et al.*[33] described the occurrence of very low frequencies of $CD52^{neg}$ T cells in the peripheral blood of healthy donors, suggesting that gaining mutations in the *PIGA* gene of T cells is a general

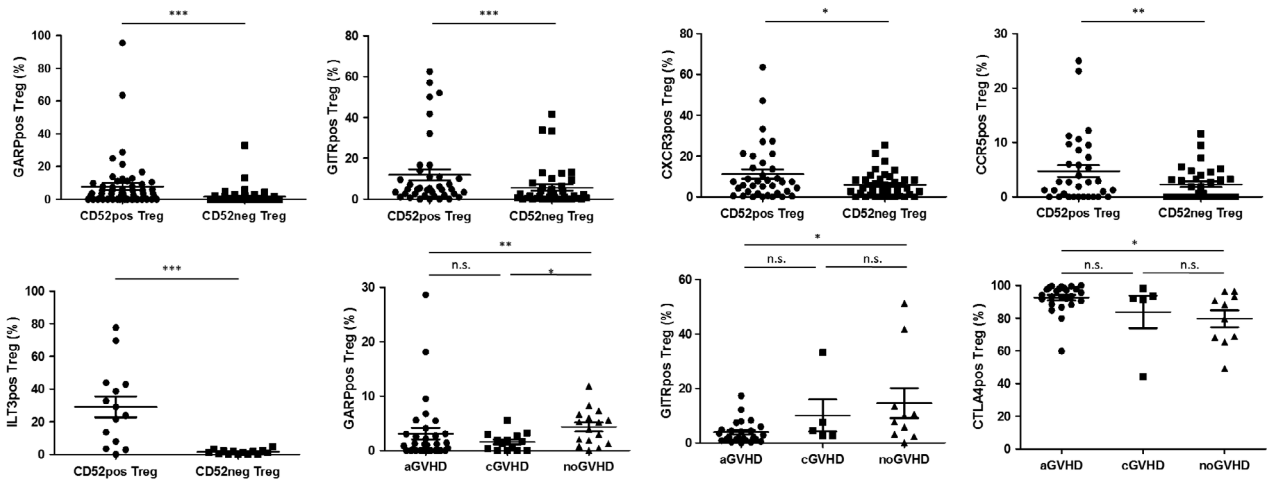

Fig 4. Treg marker expression. Significantly higher expression of GARP, GITR, CXCR3 and CCR5 were found in CD52^{pos}/GPI^{pos} Tregs. Converse results were observed relating to ILT3. CTLA4 was expressed significantly higher in the Tregs of patients with aGVHD. CD39, CD44, CD62L, CD45RA, HLA-DR, Granzyme A and transcription factor HELIOS showed no significant differences between the groups. Dot plots with mean ± SEM are shown $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

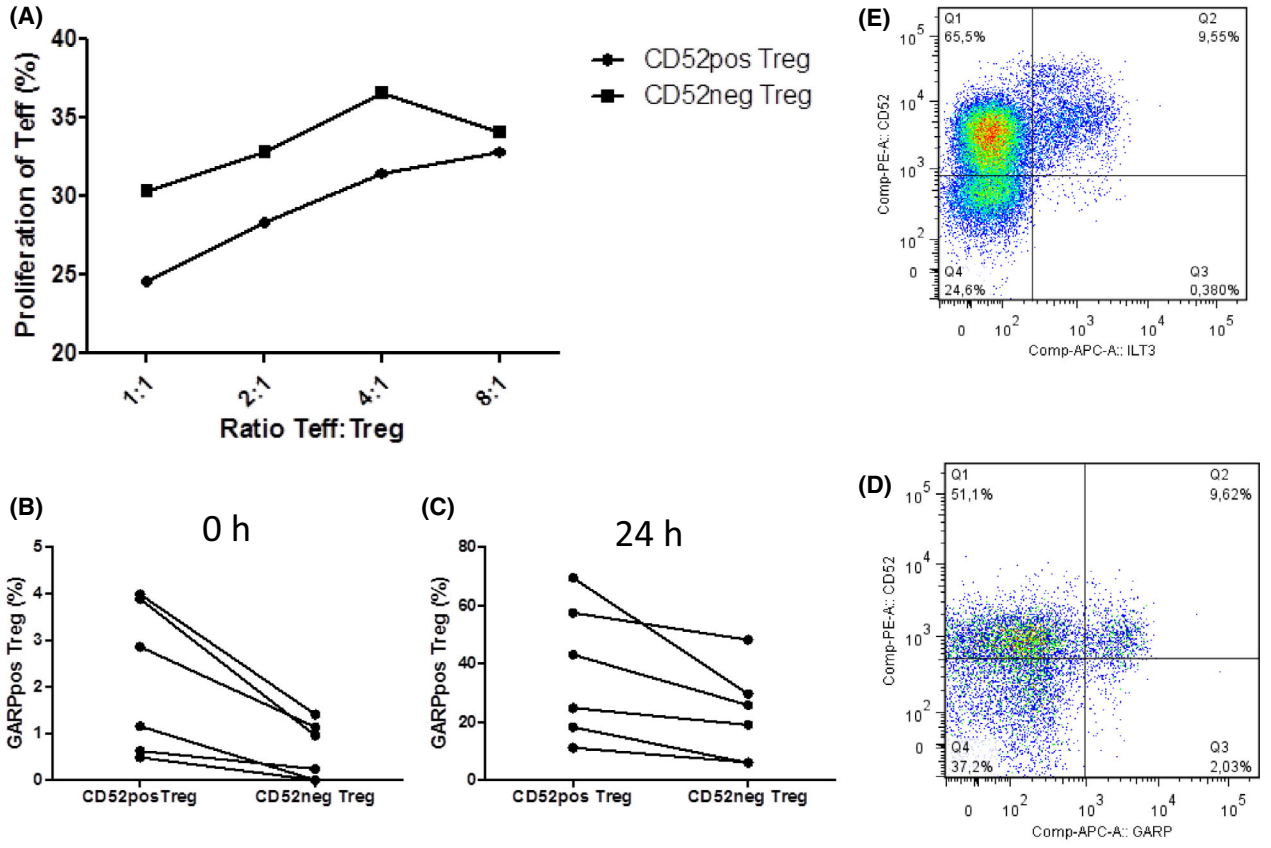

Fig 5. Functional differences between CD52^{pos} Tregs and CD52^{neg} Tregs. Both, CD52^{pos} Tregs and CD52^{neg} Tregs were able to suppress effector T-cell (Teff) proliferation in MLR, but Teff incubated with CD52^{pos} Tregs showed lower proliferation rates compared to Teff incubated with CD52^{neg} Tregs (n = 3) (A). Steady state GARP expression in CD52^{pos} Tregs and CD52^{neg} Tregs (B) and GARP expression after stimulation for 24 h (C) showed higher GARP expression of CD52^{pos} Tregs, an example is shown for one patient after stimulation (D). The expression of ILT3 strongly correlated with the expression of CD52/GPI anchor, here an example is shown in the CD3^{pos} T cells of a patient after alemtuzumab-based TCD at day +146 (E). [Colour figure can be viewed at wileyonlinelibrary.com]

phenomenon, but indicating that treatment with alemtuzumab allows the preferential outgrowth of $GPI^{neg}/CD52^{neg}$ T cells. Persistence of $GPI^{neg}/CD52^{neg}$ T cells for years after HSCT may be caused by the long lifespan of memory T cells derived from the graft[34] before they are replaced by new naïve $GPI^{pos}/CD52^{pos}$ T cells from engrafted stem cells.[30] We could show for the first time, that early and long-lasting reconstitution of $GPI^{neg}/CD52^{neg}$ T cells significantly correlates with the onset and clinical course of aGVHD. Reconstitution of $CD52^{neg}$ T cells also correlates with mixed donor chimerism, possibly caused by higher selective pressure of $CD52^{neg}$ T cells after alemtuzumab treatment.[9] Regarding function, some authors have reported that $GPI^{neg}/CD52^{neg}$ $CD3^{pos}CD4^{pos}$ and $CD3^{pos}CD8^{pos}$ T cells in mice and men are functional,[33,35,36] but these analyses solely focussed on effector T-cell functions, but exclude Tregs function.

Next to other modulators and triggers such as the microbiome, drug therapy or tissue damage,[37,38] $CD3^{pos}CD4^{pos}$ T cells and $CD3^{pos}CD8^{pos}$ T cells are described to be able to induce aGVHD.[39,40] In the present study, we investigated our hypothesis that $CD52^{pos}$ T cells are functional and that $CD52^{neg}$ T cells lack function. Our present results confirm this hypothesis, as patients with aGVHD had higher levels of functional $CD52^{pos}CD3^{pos}CD8^{pos}$ T cells compared to the relative lower levels of functional $CD52^{pos}CD3^{pos}CD4^{pos}$ T cells in the aGVHD group (Fig 2). Data of longitudinal $CD52^{pos}$ T-cell reconstitution indicate that patients with aGVHD start with low levels of $CD52^{pos}$ cells before day +100 compared to patients that never developed any GVHD (Fig 3), pointing out that dysfunctional $CD52^{neg}$ T cells are a risk for the development of aGVHD generally. Our present data presume a value of <40% $CD52^{pos}CD3^{pos}CD4^{pos}$ T cells and $CD52^{pos}$ Tregs at day +50 as a predictive marker for the development of aGVHD. Particularly, a combination of high levels of $CD52^{pos}CD3^{pos}CD8^{pos}$ T cells and low levels of $CD52^{pos}CD3^{pos}CD4^{pos}$ T cells or $CD52^{pos}$ Tregs seem to identify patients at risk of developing aGVHD (Figs 2 and 3). Those patients are supposed to have functional $CD52^{pos}CD3^{pos}CD8^{pos}$ T cells as key drivers for the development of aGVHD and dysfunctional $CD52^{neg}CD3^{pos}CD4^{pos}$ T cells and $CD52^{neg}$ Tregs being unable to maintain immune homeostasis in these patients.

To date, functional data on $GPI^{neg}/CD52^{neg}$ Tregs have been missing. Data from patients with multiple sclerosis treated with alemtuzumab (12 mg/day for 5 consecutive days, and again after 12 months for 3 days) also showed delayed $CD3^{pos}CD4^{pos}$ T-cell repopulation with expanding Tregs, but there was no monitoring of the CD52 expression.[41] The *in vitro* studies of Havari *et al.*[42] showed an increase in Treg frequency after alemtuzumab exposure of $CD3^{pos}CD4^{pos}$ T cells and adequate suppressive function dependent on both cell–cell contact and IL-2 consumption assuming an alemtuzumab-mediated effect promoting the long-term efficacy of alemtuzumab, but again without monitoring GPI-anchor or CD52 expression. CD52 is also described as a co-stimulatory molecule able to induce Tregs: in one study alemtuzumab treatment induced Tregs, which were able to suppress effector T cells in mixed lymphocyte reaction (MLR) but also without analysing CD52 expression.[43] In the present study, we found for the first time that patient-derived $GPI^{neg}/CD52^{neg}$ Tregs lack suppressive capacity in MLR and that they persist in high frequencies in patients with aGVHD. Testing functional Treg markers, $GPI^{pos}/CD52^{pos}$ Tregs exhibit higher levels of GARP,[44,45] GITR,[46–48] CXCR3,[10] and CCR5[21] confirming a more sufficient function and protection of aGVHD compared to their $CD52^{neg}$ counterparts. The role of ILT3 as a marker for reduced function in Tregs[22] remains controversial. Ulges *et al.*[22] described that $ILT3^{pos}$ Tregs negatively correlated with $CK2^{pos}$ Tregs, which enables Tregs to suppress T-helper cell type 2 responses *in vivo*, but not T-helper cell type 1 response. We found that ILT3 is almost only present on $GPI^{pos}/CD52^{pos}$ T cells, suggesting it to be a GPI-anchored molecule as well (Fig 5E).

The role of CD52 in itself is not completely understood: Bandala-Sanchez *et al.*[49]published data in which a suppressive population of $CD52^{high}CD4^{pos}$ T cells inhibit $CD52^{low}CD4^{pos}$ T-cell activation through release of 'soluble' CD52 (sCD52) released by phospholipase C cleavage. sCD52 interacts with Siglec-10 on responder $CD52^{low}CD4^{pos}$ T cells and inhibits activation of responder cells by blocking lymphocyte-specific protein tyrosine kinase (Lck) and zeta chain of T cell receptor associated protein kinase 70 (Zap70) phosphorylation. These $CD52^{high}CD4^{pos}$ T cells lack the molecular nomenclature of Tregs (CD25, Foxp3).[50] Toh et al.[51] also described immune modulation by $CD52^{pos}CD3^{pos}CD4^{pos}$ T cells by sCD52 or CD52-cross-linking as an option to suppress T-cell activity, suggesting CD52 as a suppressive molecule in $CD3^{pos}CD4^{pos}$ T cells.

Our present results give more insight into the complex interaction of T cells and drugs in the development and maintenance of GVHD, suggesting a prospective study on the clinical role of the reconstitution of $CD52^{neg}$ T-cell subsets with different schedules of alemtuzumab administration.

Acknowledgments

We thank Mainz Research School of Translational Biomedicine (TransMed) for the support and providing the grant.

Author contributions

Pascal Woelfinger planned the experiments, collected blood samples and performed the flow cytometry analysis and suppression assays, interpreted the data, and wrote the paper. Katharina Epp and Lukas Schaefer collected blood samples and performed the flow cytometry analysis and suppression assays. Diana Kriege collected blood samples and performed the flow cytometry analysis. Matthias Theobald carefully read the paper, and discussed the results. Tobias Bopp provided infrastructure for the suppression assays, read the paper, and

discussed the results. Eva-Maria Wagner-Drouet initiated and supervised the study, provided clinical data and wrote the paper. The manuscript contains parts of the doctoral thesis of Katharina Epp and Lukas Schaefer at the Department of Hematology, Oncology and Pneumology, University Cancer Center Mainz (UCT), University Medical Center Mainz.

Conflict of interest

The authors declare no conflicts of interest.

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