The immunological phenotype of rituximab-sensitive chronic graft-*versus*-host disease: a phase II study

Suzanne van Dorp,[1,2#] Henrike Resemann,[2#] Liane te Boome,[2] Floor Pietersma,[1] Debbie van Baarle,[1,3] Frits Gmelig-Meyling,[1] Roel de Weger,[4] Eefke Petersen,[2] Monique Minnema,[2] Henk Lokhorst,[2] Saskia Ebeling,[1] Scott J.P. Beijn,[5] Edward F. Knol,[5] Marijke van Dijk,[3] Ellen Meijer,[2*] and Jürgen Kuball[1,2]

[1]Dept of Immunology, [2]Hematology and [4]Pathology, [3]Internal Medicine and Infectious Diseases (DvB), and [5]Dermatology/Allergology UMC Utrecht, Utrecht, The Netherlands

#Both authors contributed equally to this work.

*Citation: van Dorp S, Resemann H, te Boome L, Pietersma F, van Baarle D, Gmelig-Meyling F, de Weger R, Petersen E, Minnema M, Lokhorst H, Ebeling S, Beijn SJP, Knol EF, van Dijk M, Meijer E, and Kuball J. The immunological phenotype of rituximab-sensitive chronic graft-*versus*-host disease: a phase II study. Haematologica 2011;96(9):1380-1384. doi:10.3324/haematol.2011.041814*

Online Supplementary Design and Methods

FACS staining

For phenotypic analysis, peripheral blood mononuclear cells (PBMC) from patients, No-GVHD controls (n=5) with a fully reconstituted lymphocyte repertoire at 12 months after allogeneic stem cell transplantation (allo-SCT) treated within the identical transplantation and post-transplantation regimen, as all included patients and healthy donor (n=5) controls (*Online Supplementary Tables S1 and S2*) were stained with antibodies with fluorescent labels as indicated against the following markers: CD3-PerCP, CD4-PerCP, CD80-R-PE, IFN-g-FITC, CD69-FITC, CD137-PE, CD5-PE and CD62L-PE-Cy5 (all from BD Pharmingen), CD8-PerCP, CD69-APC, CD19-APC, CD138-PerCP, IL-17-PacBlue (all from BioLegend), CD3-eFlour-450, CD4-PE-Cy7, CD4-Alexa Flour 750, CD8-APC, CD25-FITC, CD127-PE-Cy7, HLA-DR-Alexa Flour 750,CD38-PE-Cy7, CD86-PE-Cy5, CD27-eFour 780, FoxP3-APC, CD20-PacBlue, IL-4-PE-Cy7, and IL-10-PE (all from eBioscience).

For FACS analysis, 300,000 cells were analyzed. For evaluation of cytokine production capacities of lymphocytes, cells were stimulated with IL-2 (20 u/mL, Novartis Pharmaceuticals) and PHA-L (30 μg/mL, Sigma Aldrich). After 4 h of stimulation, cells were stained for extracellular and intracellular markers. FoxP3-staining was performed according to the manufacturer's instructions for intracellular staining (eBioscience) in unstimulated samples. Samples were analyzed with an LSR-II flow cytometer (BD Biosciences). The acquired data were analyzed using FACS Diva software (BD Biosciences).

Cytokine analysis

For cytokine analysis, plasma samples from patients, No-GVHD and healthy-donor controls were examined for their content of interleukin (IL)-2, IL-10, IL-12p70, interferon-gamma (IFN-g), tumor necrosis factor-alpha (TNF-a), IL-4, IL-13, IL-6, IL-17, and IL-21 using multiplex immunoassays as described earlier.[1]Transforming growth factor-beta (TGF-b), BAFF and platelet-derived growth factor-AA (PDGF-AA) were measured with ELISA according to manufacturer's instructions (BD Biosciences, Bender MedSystems (TGF-b and BAFF) and Antigenic America (PDGF-AA)). PDGF-AA was measured in plasma, while TGF-b and BAFF were measured in serum samples.

B-cell clonality, chimerism, and auto-antibodies

For clonality assessment of B cells, genomic DNA was isolated from patient PBMC samples using a nucleospin blood quick pure kit (Qiagen). B-cell receptor diversity was analyzed using BIOMED multiplex PCR assays as described earlier.[2] Chimerism analysis of T and B cells was performed by PCR-based amplification of short tandem repeats sequences as described earlier.[3] For analysis of auto-antibodies, an immunoblot for SSc-specific auto-antibodies was used according to the manufacturer's instructions (Euroimmun, Lübeck, Germany). Sera of patients and No-GVHD controls were analyzed for IgG antibodies against Scl-70, CENP A, CENP B, RP11, RP155, Fibrillarin, NOR90, Th/To, PM-Scl100, PM-Scl75, Ku, PDGFR and Ro-52.

Histological stainings

Skin biopsies were stored in 4% formalin and embedded in paraffin. Slides were stained with hematoxylin and eosin (H&E, Klinipath), and monoclonal antibodies against CD3 (A0452), CD8 (M7103), CD20 (M0755; all from Dako), CD4 (Monosan, monx10326), CD5 (Novocasta, NCL-CD5-4C7) and FoxP3 (eBioscience, 14-4776). Slides were stained for all markers, except FoxP3, using a BondmaX stainer (Leica). Slides were stained for FoxP3 manually. Epidermal involvement and dermal sclerosis was scored as described earlier.[4] Pathologists were clinically blinded during analysis. Nine skin biopsies of the leg, arm and trunk which were obtained from healthy donors after receiving their informed consent served as controls.

References

1. de JW, Bourcier K, Rijkers GT, Prakken BJ, Seyfert-Margolis V. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. BMC Immunol. 2009;10:52.
2. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003;17(12):2257-317.
3. Huisman C, de Weger RA, de VL, Tilanus MG, Verdonck LF. Chimerism analysis within 6 months of allogeneic stem cell transplantation predicts relapse in acute myeloid leukemia. Bone Marrow Transplant. 2007;39(5):285-91.
4. Canninga-van Dijk MR, van der Straaten HM, Fijnheer R, Sanders CJ, van den Tweel JG, Verdonck LF. Anti-CD20 monoclonal antibody treatment in 6 patients with therapy-refractory chronic graft-versus-host disease. Blood. 2004;104(8):2603-6.

Online Supplementary Table S1. Characteristics of No-GVHD control group used for flow-cytometry analyses.

No-GVHD controls (n=5)	
Median age (yrs; range)	50 (45-63)
Sex M/F (%)	60/40
Disease (n)	
• AML	3
• CML	1
• NHL	1
Related donor (n,%)	4 (80)
NMA conditioning (n,%)	5 (100)
ATG (n,%)	1 (20)
Acute GVHD (n)	0

AML: acute myeloid leukemia; ATG: antithymocyte globuline; CML: chronic myeloid leukemia; NHL: non-Hodgkin's lymphoma; NMA: non-myeloablative.

Online Supplementary Table S2. Characteristics of healthy controls used for flow-cytometry analyses.

Healthy controls (n=5)	
Median age (yrs; range)	30 (23-40)
Sex M/F (%)	40/60

Online Supplementary Table S3. Patient characteristics.

	Total population	Responding patients	Non-responding patients	p-value
N (%)	18 (100)	11 (67)	7 (33)	
Median age (years; range)	53 (39-66)	53 (39-64)	55 (44-66)	0.751
Sex male/female (%)	78/22	73/27	86/14	0.485
Median follow-up (months; range)	7 (1-13)	8 (4-13)	4 (1-4)	0.001
Number of pre-treatments	1	1	1	1.000
Median time after allo-SCT (months; range)	34 (10-61)	38 (9-77)	34 (11-62)	0.319
Months from onset of chronic GVHD until RTX-treatment (median; range)	12 (2-51)	15 (3-51)	8 (2-43)	0.441
Disease				0.450
• AML/MDS	2	0	2	
• CLL	2	0	2	
• CML	1	1	0	
• MM	8	5	3	
• Myelofibrosis	1	1	0	
• NHL	4	4	0	
Related donor (n; %)	14 (78)	9 (82)	5 (71)	0.515
NMA conditioning (n; %)	16 (89)	10 (91)	6 (86)	0.641
ATG (n; %)	4 (22)	2 (18)	2 (29)	0.515
Acute GVHD (n; %)	14 (78)	8 (73)	6 (86)	0.428

*Allo-SCT indicates allogeneic stem cell transplantation; AML: acute myeloid leukemia; ATG: antithymocyte globuline; CLL: chronic lymphocytic leukemia; CML: chronic myeloid leukemia; Disease, disease for which allo-SCT was given; GVHD: graft-versus-host disease; MDS: myelodysplastic syndrome; MM: multiple myeloma; NHL: non-Hodgkin's lymphoma; NMA: non-myeloablative. * P values: Mann-Whitney-U test for age, follow up, months after allo-SCT and chronic GVHD; Fisher's exact test for other factors.*