Supplemental material

Supplemental methods

MSC production process

The MSC production process was identical to the process conducted for the clinical trial by Le Blanc et al in 2008[2]: Bone-marrow mononuclear cells were separated by density gradient centrifugation as previously described[3,4]. Washed cells were resuspended in Dulbecco's modified Eagle's medium–low glucose (Life Technologies, Gaithersburg, MD, USA, or Paisley, UK) supplemented with 10% fetal bovine serum (National Veterinary Institute, Uppsala, Sweden, or HyClone, Logan, UT, USA) and plated at a density of 160 000 cells per cm^2. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2 in 175 cm^2 flasks (Falcon, Franklin Lakes, New Jersey, USA, or Greiner Bio-One, Frickenhausen, Germany). When the cultures were near confluence (>80%), the cells were detached by treatment with trypsin and EDTA (Invitrogen, Grand Island, NY, USA, or Lonza Verviers, Verviers, Belgium) and replated at a density of 4000 cells per cm^2. When 2×10^6 cells or more were obtained, they were harvested and either cryopreserved in 10% dimethyl sulphoxide (Research Industries, Salt Lake City, UT, USA, or Leiden University Medical Centre Pharmacy, Netherlands) or washed repeatedly and resuspended to a final concentration of 2×10^6 cells per mL in saline solution according to local guidelines.

Criteria for release of mesenchymal stem cells for clinical use included absence of visible clumps, spindle-shape morphology, absence of contamination by pathogens (as documented by aerobic and anaerobic cultures before release), viability greater than 95%, and immune phenotyping proving expression of CD73, CD90, and CD105 surface molecules (>90%) and absence of CD34, CD45, CD14, and CD3[5].

Peripheral blood mononuclear cell and plasma separation

For plasma separation, the blood was collected in ethylene-diamine-tetraacetic acid (EDTA) tubes and kept on ice before centrifugation. The plasma was then frozen at -80ºC until analysis. Blood was collected in heparinized tubes for peripheral blood mononuclear cell (PBMC) separation. PBMCs were isolated by centrifugation using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, GE Healthcare, Uppsala, Sweden) and stored in 10% EDTA diluted in human ab plasma in aliquots in liquid nitrogen. Absolute lymphocyte counts were obtained from the Department of Clinical Chemistry, Karolinska University Hospital, Stockholm and absolute cell numbers calculated by multiplying absolute lymphocyte counts with the percentages obtained from flow cytometry analysis.

Supplemental table 1: Mesenchymal stromal cell donor and graft characteristics

Donor characteristics	
Number of donors	18
Donor sex (*male/female)*	10/8
Donor age (*median, range)*	29.5 (3-44)[#]
Number of doses per donor *(median, range)*	2.5 (1-12)
Number of donors per patient (*median, range)*	3 (1-6)
Graft characteristics	
Total number of grafts	71
MSC cell dose per graft *(mean, range)*	2.03 (1.3-3) x10^6/kg
Unrelated donor *(n, %)**	58 (82)
First degree relative *(n, %)**	10 (14)
Other relatives *(n, %)**	3 (4)
Culture passage at MSC harvest	
Passage 2 *(n, %)*	8 (11)
Passage 3 *(n, %)*	63 (89)

* No HLA matching was performed.

[#] Three children were among the MSC donors. All of them donated bone marrow to an HLA-identical sibling undergoing allogeneic haematopoietic stem cell transplantation. With their parents' permission, a minor part of that donated bone marrow was used for MSC production for this clinical trial.

Flow Cytometry Analysis
PBMCs were gently thawed and washed twice with a complete media RPMI containing 10% FCS. Cells were stained for various surface markers for 20 minutes at 4° C with antibodies described in **Suppl. Table 2**. Aqua fluorescent reactive dye for viability analysis was purchased from Invitrogen (Carlsbad, CA, USA).

Soluble Biomarker Analysis
The following cytokines and chemokines were analysed using a custom Bio-Plex® x-Plex™ (Bio-Rad Laboratories AB, Solna, Sweden): Interleukin (IL)-1b, -2, -6, -7, -8, -10, -17a, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, C-X-C motif ligand (CXCL)-2, -9, -10, chemokine C-C motif ligand 2, basic fibroblast growth factor (b-FGF). B-cell activating factor (BAFF) was analysed using ELISA as per the manufacturer´s instructions.

Micro RNA (miRNA) Analysis
Circulating plasma miRNA were analysed in seven patients (patients 1, 2, 5-9) before and at two time-points (1-3 hrs and 24 hrs) after the first MSC infusion. Total RNA isolation and analysis were conducted at Exiqon Services (Vedbaek, Denmark). Briefly, total RNA for miRNA analysis was extracted from 200µl plasma using the miRCURYTM RNA isolation kit (Exiqon). RNA spike-ins were added to the samples before isolation in order to monitor RNA extraction efficiency. RNA was reverse transcribed using the mercury LNATM Universal RT miRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon) and analyzed with in a LightCycler® 480 Real-Time PCR System (Roche Diagnostics Scandinavia AB, Bromma, Sweden) in 384 well plates using the protocol for mercury LNATM Universal RT miRNA PCR. All data were normalized to the average of assays detected in all samples (average assay Cq).

Linear mixed effects models
These models were applied for analysing absolute peripheral blood mononuclear cell subset counts. For analysing the differences between responders (R) and non-responders (NR), we used the following model: *Cell count = A x Responder (0 or 1) + B x Number of previous infusions*. Long and short term changes were evaluated for R and NR separately. For analysing long term changes, we used the model *Cell count = A x Number of previous infusions*, and for short term changes, we used *Cell count = A x Days since last infusion + B x number of previous infusions*. Subject was set as the random effect for all models. P-values were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question. Analysis was performed using R statistical software[6], with the *lme4* package[7].

Supplemental Table 2: Flow cytometry antibody panels used in the study.

		Antibody (concentration)										
Panel number	**Used for infusion(s)**	**PE**	**PE-Cy5**	**PE-Cy7**	**AF488**	**BV421**	**BV605**	**PerCP-Cy5.5**	**APC**	**APC-Cy7**	**AF700**	**AmCyan**
1	1, 3, 6, 9	CD14 (1/400)	CD3 (1/100)	CD21 (1/100)	IgD (1/100)	CD5 (1/100)	CD27 (1/100)	CD19 (1/100)	CD38 (1/100)	BAFF-R (1/100)		Aqua live dead (1/1000)
2	3	CD19 (1/100)	CD22 (1/100)	IgM (1/100)	IgD (1/100)	CD5 (1/100)	CD27 (1/100)	Ki67 (1/100)	CD38 (1/100)			Aqua live dead (1/1000)
3	3	CXCR3 (1/100)	CD4 (1/100)	CD127 (1/100)	CD8 (1/100)	CD3 (1/100)	CD56 (1/100)	CD19 (1/100)	CD27 (1/100)	CD45RA (1/100)		Aqua live dead (1/1000)
4	6	CXCR3 (1/100)	CD4 (1/100)	CD127 (1/100)	CD8 (1/100)	CD3 (1/100)	CD56 (1/100)	PD1 (1/100)	CD27 (1/100)	CD45RA (1/100)		Aqua live dead (1/1000)
5	1, 9	CXCR3 (1/100)	CD4 (1/100)	CD62L (1/100)	CD8 (1/100)	CD3 (1/100)	CD56 (1/100)	PD1 (1/100)	CD27 (1/100)	CD45RA (1/100)		Aqua live dead (1/1000)
6	6	FoxP3 (1/50)	CD4 (1/100)		CD8 (1/100)	CD3 (1/100)			IL17 (1/100)			Aqua live dead (1/1000)
7	1, 9	FoxP3 (1/50)	CD4 (1/100)	CD127 (1/100)		CD3 (1/100)			CCR7 (1/100)			Aqua live dead (1/1000)
8	3	FoxP3 (1/50)	CD4 (1/100)	CD62L (1/100)	CD31 (1/100)	CD3 (1/100)	CD56 (1/100)	Ki67 (1/100)	CCR7 (1/100)	CD45RA (1/100)	CD27 (1/100)	Aqua live dead (1/1000)

PB: Pacific Blue, PE: Phycoerythrin, Cy: Cyanine dye, FITC: Fluorescein Isothiocyanate, BV: Brilliant Violet, PerCP: Peridinin-chlorophyll-protein complex, APC: Allophycocyanin, AF: Alexa Fluor, CD: Cluster of Differentiation, PD: programmed death, FoxP3: Forkhead Box P3, CCR: C-C chemokine Receptor, CXCR3: C-X-C motif chemokine Receptor 3

Supplementary Table 3: Adverse events recorded before final evaluation

Adverse event*	**N**	**Comments**
Grade 3 infection	5	2 pneumonias (1 Metapneumovirus, 1 with unknown microbiology), 1 bacterial keratitis, 1 soft tissue infection, 1 adenovirus colitis.
Skin dysplasia	1	Melanocytic
Cervix dysplasia	1	
Recurrence of M-protein	1	After 7 infusions. The patient discontinued the study.
Increasing recipient chimerism	1	Patient with CLL. CD19 recipient chimerism increased after 3 infusions. The patient discontinued the study.
Death	1	Due to progressive cGvHD after 1 MSC infusion.

*Adverse events recorded until final evaluation at 12 months after discontinuing MSC treatment. N: Number of events, CLL: Chronic Lymphocytic Leukaemia, cGvHD: chronic Graft versus Host Disease, MSC: Mesenchymal Stromal Cell

Supplemental table 4: Histopathological evaluations of skin biopsies

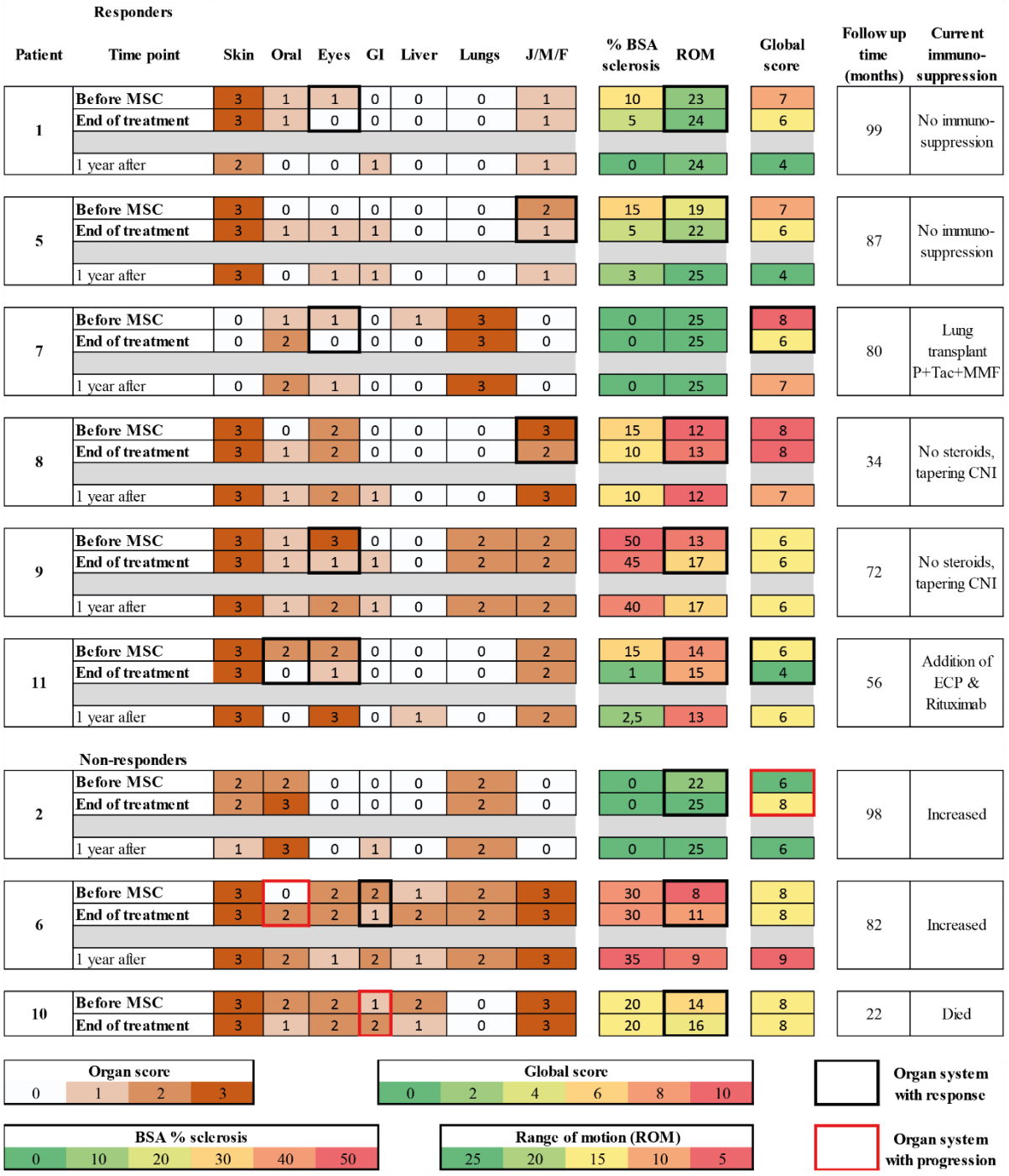
	Histopathology before treatment		Histopathology after treatment		
Responders					
Patient	**Location**	**Reveiw**	**Location**	**Review**	**Change**
5	Abdomen	Lamellar str. corneum, normal thickness, basal KC with vacuolization, basal membrane thickening, sparse dermal lymphocytic infiltrate, also at the dermal/subcutaneous border, sclerosis. **Diagnosis:** Sclerotic GvHD with discrete acute changes in the epidermis	Abdomen	Epidermis normal, sparse dermal lymphocytic infiltrate, sclerosis unchanged. **Diagnosis:** Sclerotic GvHD	Epidermal changes improved
7	Left part of back	Epidermis 3-4 layers, vacuolated melanocytes, melanophages. **Diagnosis:** Acute changes in the epidermis grade 1-2	Right part of back	Epidermis 6 layers. **Diagnosis:** Normal skin	Epidermal changes completely regressed
8	Right groin	Lamellar str.corneum, acanthosis (10 layers), dilated vessels, sclerosis superficial and deep, sparse infiltrate. **Diagnosis:** Sclerotic GvHD with discrete lichenoid aspects within the epidermis	Location unknown	Normal epidermis, sclerosis only deep, less than in 1 (could be location dependent). **Diagnosis:** Sclerotic GvHD	Epidermal changes improved
9	Right groin	Epidermis normal, vessels number increased, sclerosis, no infiltrate. **Diagnosis:** Sclerotic GvHD	Right groin	Vacuolized melanocytes, sclerosis slightly increased. **Diagnosis:** Sclerotic GvHD with some acute changes in the epidermis	No improvement, now some acute changes in the epidermis
11	Dorsal side of right thigh	Slight edema in papillary dermis, otherwise normal. **Diagnosis:** Dermal edema	Back of right thigh	Slight edema in papillary dermis, otherwise normal. **Diagnosis:** Dermal edema	No diagnostic changes
Non-responders					
10	Right breast	Epidermis 7 layers, vacuolated KC within basal cell layer, single cell dyskeratosis basal and suprabasal, attached lymphocytes, dermal edema, sparse lymphocytes, sclerosis deep dermis (superficial changes could be drug-induced-multiforme-like pattern. **Diagnosis:** Sclerotic GvHD with multiforme –like changes in the epidermis, drug-induced or GvHD	Left side of back	Epidermis normal, sclerosis superficial and deep (progression), dilated vessels. **Diagnosis:** Progression of Sclerotic GvHD	Epidermal changes improved Sclerosis progressed

GvHD: Graft versus Host Disease, KC: Keratinocytes

Supplemental table 5: Summary of immune phenotyping results.

	Long term values			1 day after infusion		7 days after infusion	
	R vs NR	N pre inf R	N pre inf NR	Days after inf R	Days after inf NR	Days after inf R	Days after inf NR
Subset	(A)	(A)	(A)	(A)	(A)	(A)	(A)
Lymphocytes	+ 0.37	- 0.75	- 0.29	+ **0.0014**	+ 0.16	+ **0.0005**	+ 0.53
CD3+ CD56-	+ 0.13	+ 0.96	- 0.51	+ **0.013**	+ 0.21	+ **0.0012**	+ 0.54
CD4+ CD8-	+ 0.26	- 0.63	- 0.25	+ **0.039**	+ 0.28	+ **0.0025**	+ 0.89
CD4+ CD27+ CD45RA+	+ **0.0062**	- 0.26	+ 0.75	+ **0.011**	+ 0.53	+ **0.0021**	- 0.26
CD4+ CD27+ CD45RA-	+ 0.47	- 0.24	- 0.28	+ **0.041**	+ 0.23	+ **0.0012**	+ 0.82
CD4+ CD27- CD45RA+	- 0.65	+ 0.36	- 0.13	+ 0.72	+ 0.66	- 0.93	+ 0.94
CD4+ CD27- CD45RA-	+ 0.63	+ 0.51	- 0.26	+ 0.65	+ 0.43	+ 0.89	+ 0.63
CD4+ FoxP3+	+ 0.29	- 0.46	+ 0.17	+ **0.0042**	+ 0.19	+ **0.0057**	- 0.83
CD4- CD8+	+ 0.13	+ 0.72	+ 0.28	+ 0.062	+ 0.43	+ **0.048**	+ 0.22
CD8+ CD27+ CD45RA+	+ 0.17	+ 0.92	+ **0.03**	+ 0.1	+ 0.86	+ **0.0097**	- 0.87
CD8+ CD27+ CD45RA-	- 0.67	+ 0.29	+ 0.47	+ 0.13	+ 0.3	+ 0.052	+ 0.64
CD8+ CD27- CD45RA+	+ 0.16	+ 0.82	+ 0.49	+ 0.14	+ 0.45	+ 0.26	+ 0.094
CD8+ CD27- CD45RA-	+ 0.17	+ 0.44	+ 0.87	+ **0.044**	+ 0.45	+ 0.26	+ 0.34
CD3- CD56+	- 0.32	+ 0.43	- **0.037**	+ 0.25	+ 0.93	+ 0.46	- 0.99
CD56bright	- 0.27	+ 0.26	+ 0.19	+ 0.13	- 0.7	+ 0.092	- 0.45
CD56dim	- 0.32	+ 0.45	- **0.033**	+ 0.28	+ 0.9	+ 0.5	+ 0.95
CD3+ CD56+	- 0.39	+ 0.84	- 0.22	+ 0.084	+ 0.6	+ 0.4	+ 0.46
CD3+ CD56+ CD8+ CD4-	+ 0.37	+ 0.77	+ 0.56	+ 0.097	+ 0.62	+ 0.24	+ 0.13
CD3+ CD56+ CD8- CD4+	- 0.26	- 0.38	- 0.13	+ 0.21	+ 0.84	+ 0.77	+ 0.9
CD3- CD19+	+ 0.17	- 0.31	+ 0.1	+ 0.14	- 0.13	+ **0.012**	- 0.34
CD19+ CD27+ IgD-	+ 0.57	- 0.37	+ 0.096	+ 0.2	- 0.15	+ 0.064	- 0.37
CD19+ CD21hi CD27- IgD+	+ **0.048**	- 0.32	- 0.73	+ 0.17	+ 0.66	+ **0.011**	+ 0.5
CD19+ CD5+	- 0.72	+ 0.75	+ 0.13	+ 0.23	- 0.32	+ 0.066	- 0.37
CD19+ IgD+ CD38low	+ 0.12	- 0.45	+ 0.098	+ 0.13	- 0.12	+ **0.0093**	- 0.36

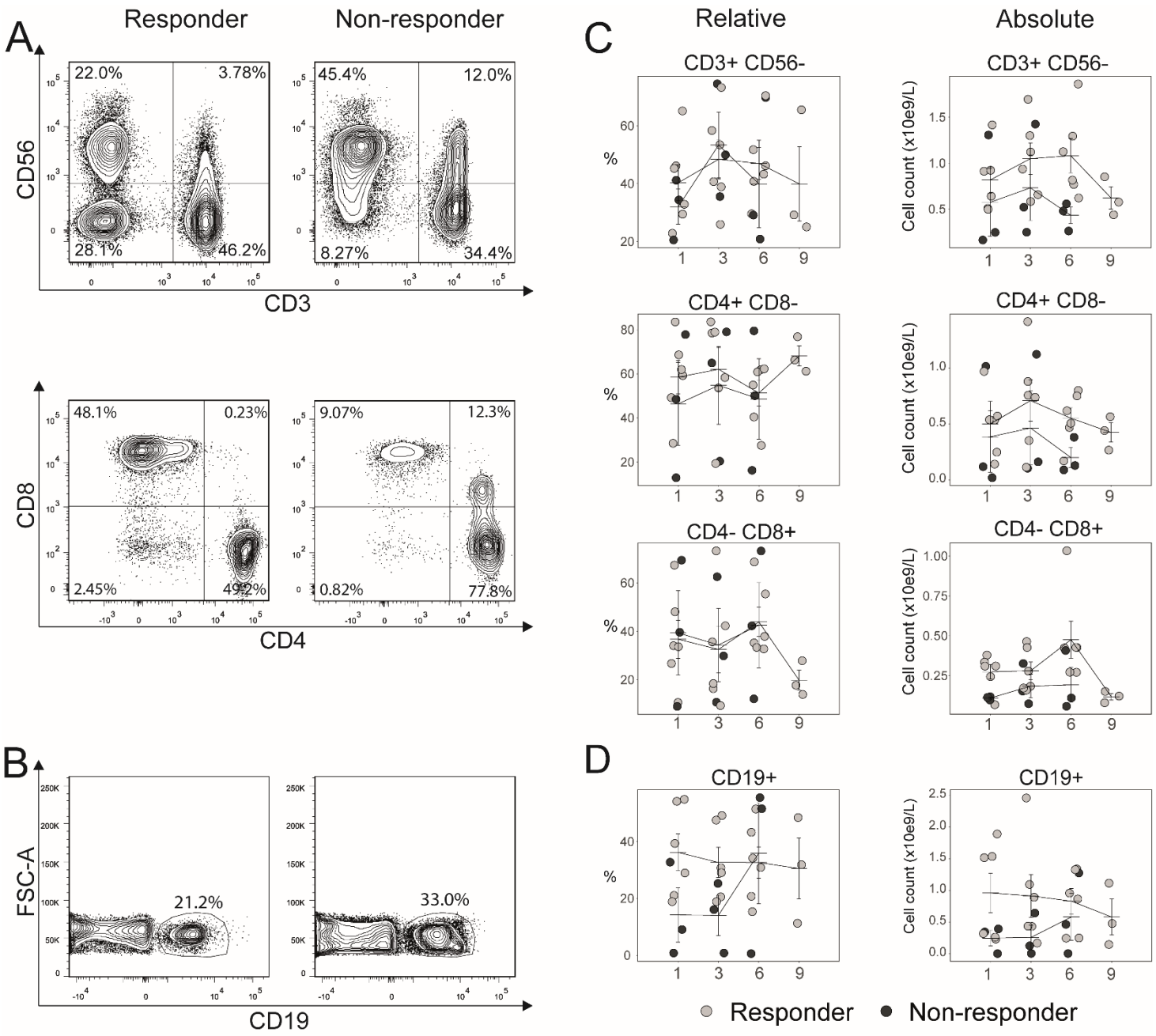
Values displayed are p-values for the coefficients in the linear mixed effects models: *Cell count = A x Responder (0 or 1) + B x Number of previous infusions* for long term changes and differences between responders and non-responders. *Cell count = A x Days since last infusion + B x number of previous infusions* for evaluating short term changes. The random effect was set to patient identifier. Different shades of green correspond to different significance levels. +/- indicates a positive or negative coefficient. In R vs NR + indicates higher levels in responders. In Num pre inf + indicates a trend to increasing levels long term during the study. In Days after inf + indicates increasing levels short term since last infusion. R: Responder. NR: Non-responder. pre: previous. inf: infusion.



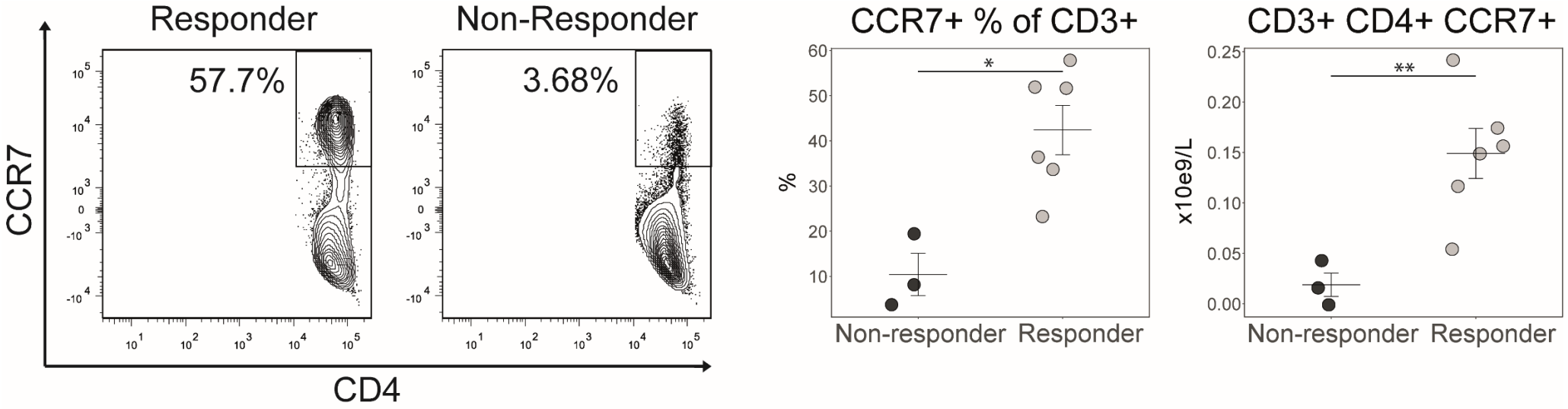
Supplemental figure 1: Response heatmap with final evaluation included. NIH organ score, NIH global score, range of motion (ROM) and body surface area (BSA) percentage involved with sclerosis. Time points are at study enrolment, end of MSC treatment and final evaluation, one year after end of treatment. Black boxes denote organs with response and red boxes denote organs with progression during MSC treatment. J: Joints, M: Muscles, F: Fascia, P: Prednisolone, Tac: Tacrolimus, MMF: Mycophenolate Mofetil, CNI: Calcineurin Inhibitors, ECP: Extracorporeal Photopheresis, MSC: Mesenchymal Stromal Cells

Supplemental figure 2: Timeline of clinical events during and after the study. The top row of each patient lists cGvHD treatments before and after the study. At the end of each timeline, current immunosuppressive therapy is listed in italic letters. The bottom row of each patient lists cGvHD status and clinical events. The heatmap coloured cells shows the global cGvHD grade at each study evaluation. Downwards pointing arrows depict cGvHD progression, new cGvHD treatments or death or relapse after inclusion.

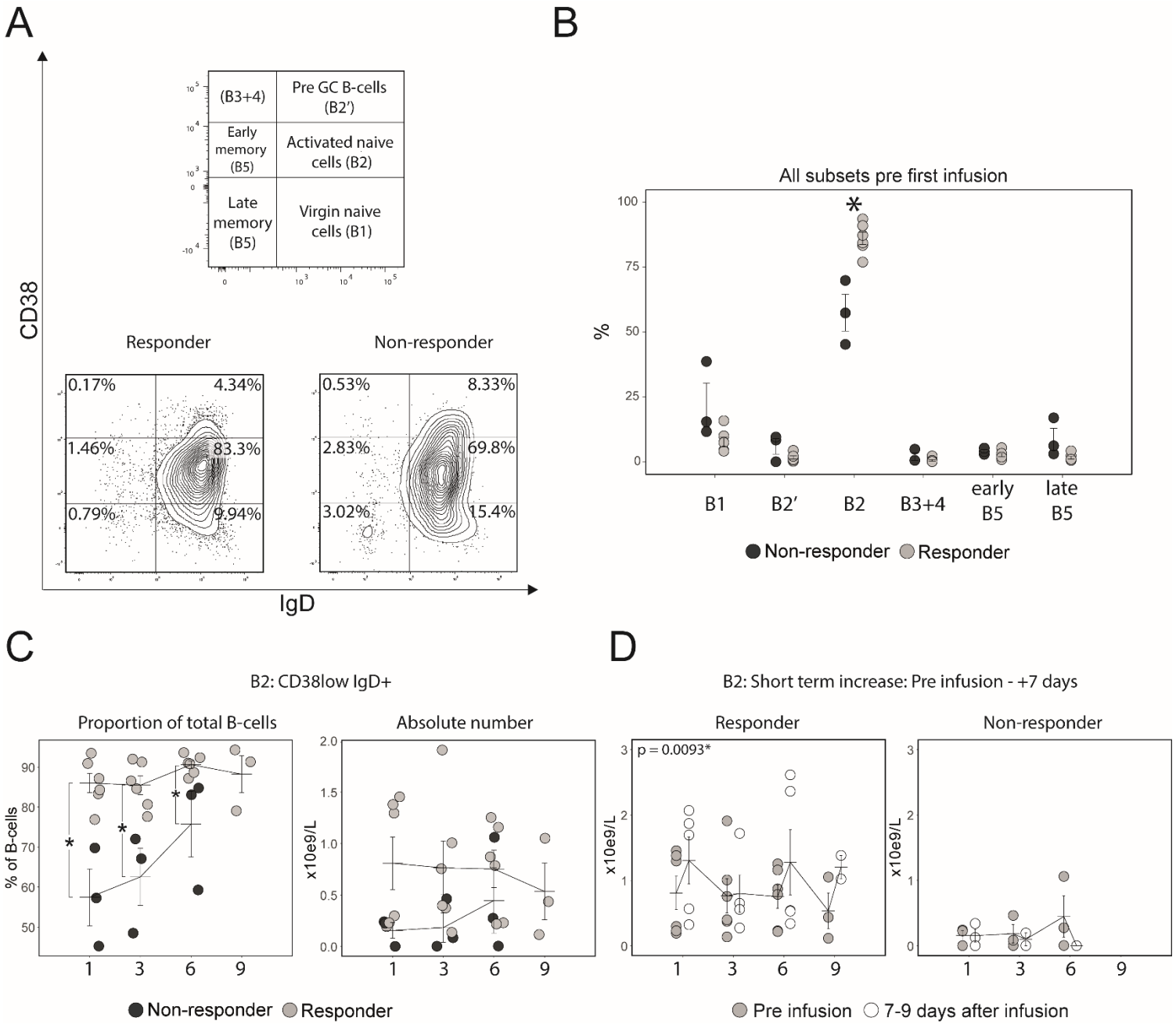
* Exact date of cGvHD onset unknown – patient is from another part of Sweden. ** Likely cause of death was sudden cardiac event. *** Cause of death was progressive cGvHD. cGvHD: chronic Graft versus Host Disease, CyA: Cyclosporine A, P: Prednisolone, FMC: Fetal Membrane Cells, Tac: Tacrolimus, Sir: Sirolimus, Eve: Everolimus, Thal: Thalidomide, Ruxo: Ruxolitinib, Infl: Infliximab, MMF: Mycophenolate Mofetil, MTX: Methotrexate, Ima: Imatinib, ChK: Chlorokine, R: Rituximab, C: Cyclophosphamide.



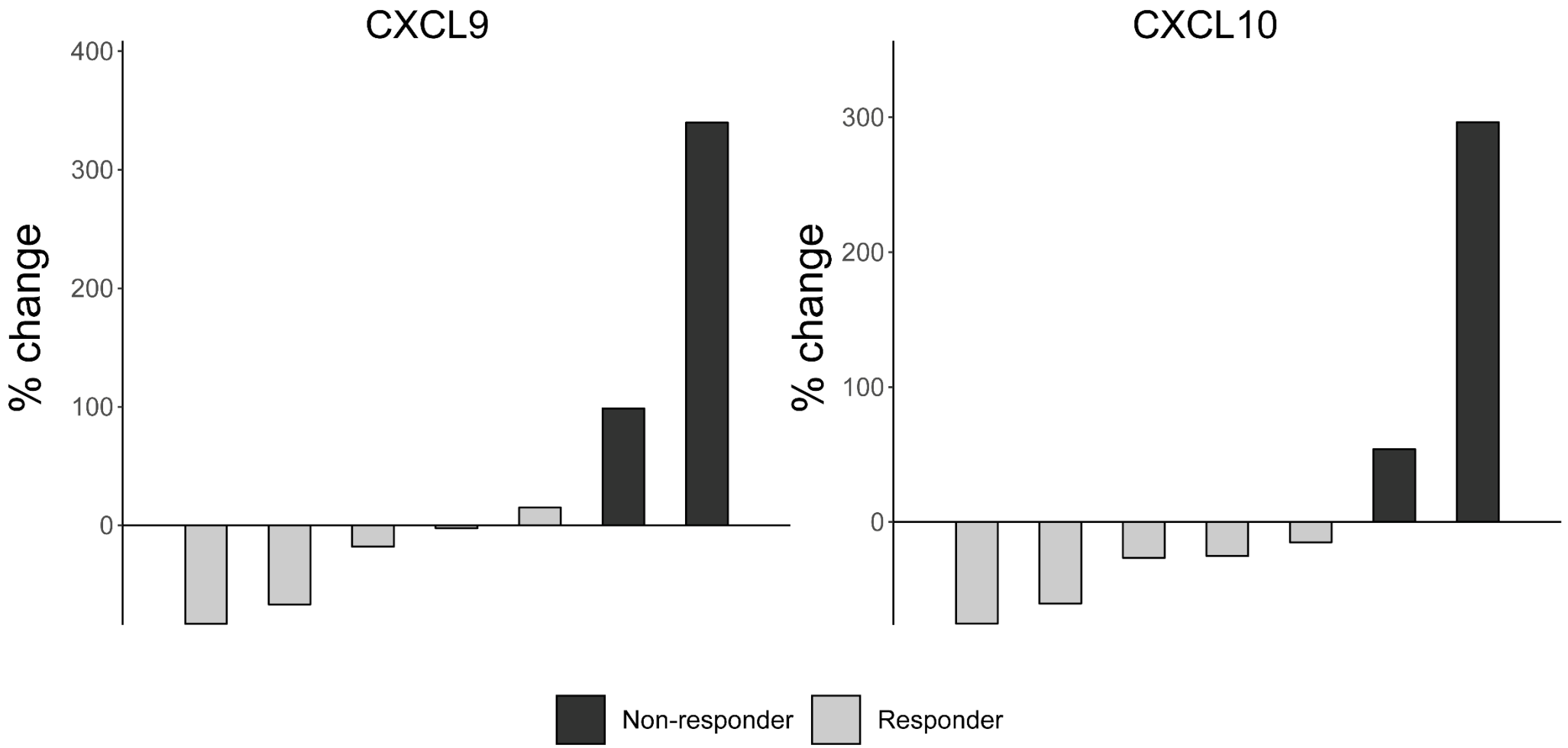
Supplemental figure 3: Basic B- and T-cell subsets. A and B: Representative plots of the flow cytometry analysis. **C and D:** Relative and absolute numbers of T-cells and B-cells were comparable between R and NR throughout the study. Error bars show mean +/- SEM.



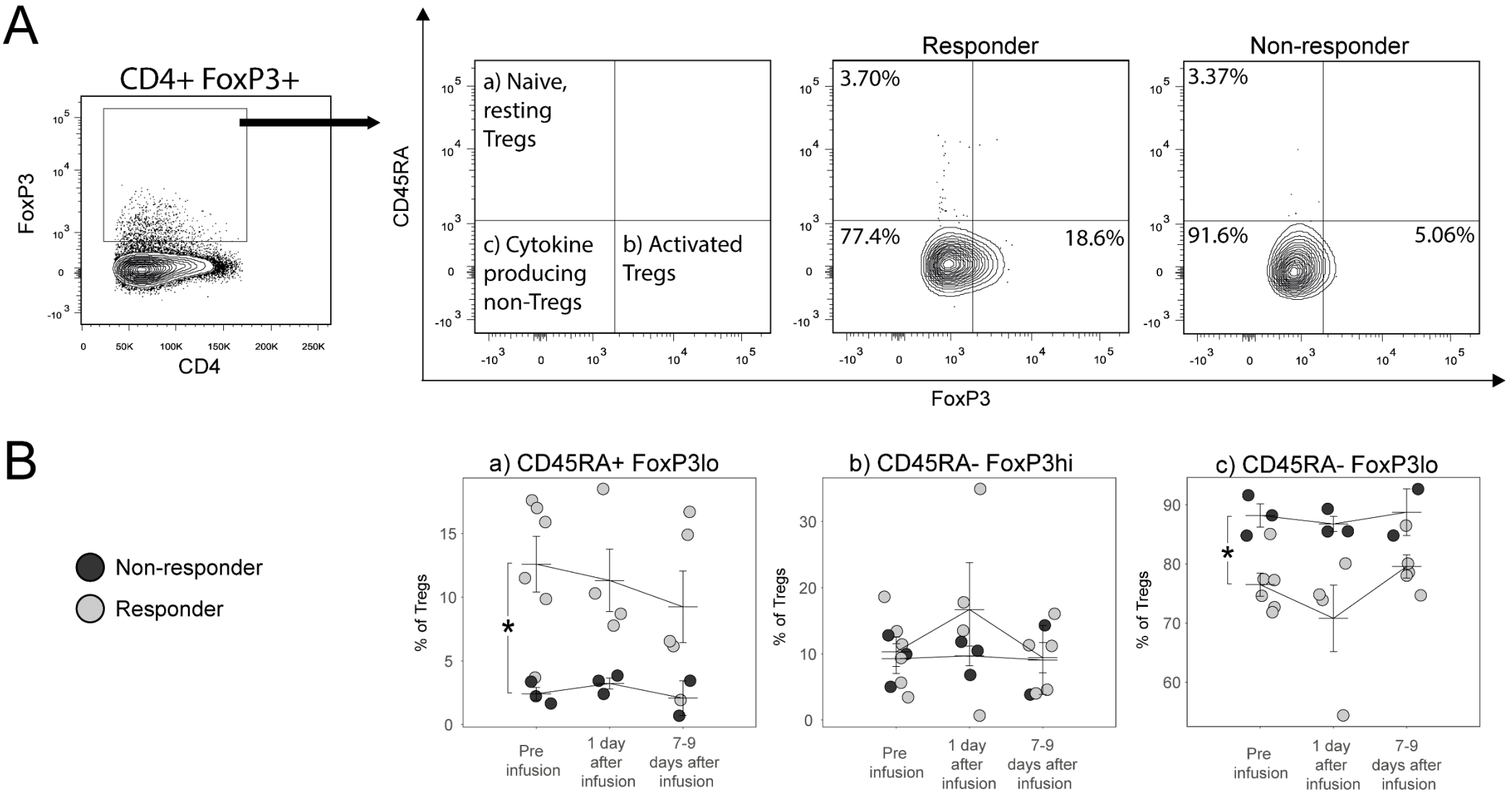
Supplemental figure 4: Responders had higher proportion of CD4+ CCR7+ T-cells compared to non-responders. The absolute numbers of CD4+ CCR7+ T-cells were higher in R compared to NR. P-value for absolute numbers with t-test and for relative numbers with Wilcoxon's rank sum test. * = $p < 0.05$, ** = $p < 0.005$



Supplemental figure 5: Activated naïve B-cells. A: Analysis was performed using IgD and CD38 to differentiate B-cells at different stages of development according to[1]. Representative plots and legend is shown. **B:** Relative numbers of B-cells at different development stages before first infusion. **C:** Relative and absolute numbers of activated naïve B-cells (B2) are higher in responders throughout the study. **D:** Absolute numbers of activated naïve B-cells increase 7 days after each MSC infusion. P-values for relative numbers are calculated using Wilcoxon's rank sum test. P-values in D are derived from the mixed effects model and represent the significance of the factor *Days since last infusion*. The P-values displayed are the P-values for coefficient A. * = $p < 0.05$, ** = $p < 0.005$. Error bars show mean +/- SEM. MSC: Mesenchymal Stromal Cell, CG: Germinal Center



Supplemental figure 6: Proposed biomarkers of response to MSC therapy in cGvHD: Relative changes of CXCL9 and CXCL10 concentrations from before the first infusion to before the 6:th infusion (5 months after treatment start) are shown. The relative change in CXCL10 was completely differential between responders and non-responders, increasing in non-responders and decreasing in responders. MSC: Mesenchymal Stromal Cell, cGvHD: chronic Graft-versus-Host Disease, CXCL: Chemokine (C-X-C motif) ligand



Supplemental figure 7: Treg subpopulation analysis revealed functional differences in Tregs between R and NR: A: representative plots of the flow cytometry analysis. Tregs were subdivided according to Sakaguchi et al[8] using CD45RA and FoxP3. **B:** R had a higher proportion of naïve Tregs (CD45RA+ FoxP3lo) while NR had a higher proportion of non-Tregs (CD45RA- FoxP3lo) among total Tregs. Data from infusion 3. P-values obtained by Wilcoxon rank-sum test. * = $p < 0.05$. Error bars show mean +/- SEM. MSC: Mesenchymal Stromal Cell, Treg: regulatory T-cell, Breg: regulatory B-cell

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