SUPPLEMENTARY MATERIAL

Abbreviations

Anti-VLA-4: anti-very late antigen-4

APC: allophycocyanin

APC-H7: allophycocyanin-hilite 7

FITC: flurescein isothiocyanate

mAbs: monoclonal antibodies

mDCs: myeloid dendritic cells

NK: natural killer

PB: peripheral blood

PBS: phosphate-buffered saline

pDCs: plasmacytoid dendritic cells

PE: phycoerythrin

PE-Cy7: phycoerythrin-cyanine 7

PerCP-Cy5.5: peridinin chlorophyll proteincyanine 5.5

PO: pacific orange

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Table EI. Desensitization protocol to alemtuzumab.

Step	Solution	Rate (mL/h)	Minutes	Volume (ml/step)	Dose (mg/step)	Cumulative dose (mg)
1	A	2	15	0.5	0.00059	0.00059
2	A	5	15	1.25	0.001475	0.002065
3	A	10	15	2.5	0.00295	0.005015
4	A	20	15	5	0.0059	0.010915
5	B	5	15	1.25	0.01475	0.025665
6	B	10	15	2.5	0.0295	0.055165
7	B	20	15	5	0.059	0.114165
8	B	40	15	10	0.118	0.232165
9	C	10	15	2.5	0.295	0.527165
10	C	20	15	5	0.59	1.117165
11	C	40	15	10	1.18	2.297165
12	C	80	61 min	82.22	9.7029	12

Total time: 225 minutes. Solutions were prepared from the most concentrated bag followed by 1:10 fold dilutions of each solution: Solution C (0.118 mg/ml): 12 mg of alemtuzumab (vial: 1,2 ml) in 100 ml saline; Solution B (0.0118 mg/ml): 10 ml solution C in 90 ml saline; Solution A (0.00118 mg/ml): 10 ml of solution B in 90 ml saline.

Methods (Flow Cytometry):

The quantification and phenotypic characterization of peripheral blood (PB) cells were performed by flow cytometry. Samples were collected 1 hour before and 2 hours after therapy, on each day of treatment for an acute effect evaluation, and after 3 months of the treatment. The PB was stained with the following monoclonal antibodies (mAbs): CD4 V450 (clone RPA-T4, BD Biosciences, San Jose, USA), CD52 phycoerythrin (PE, clone 4C8, BD Pharmingen, San Diego, USA), CD3 peridinin chlorophyll proteincyanine 5.5 (PerCP-Cy5.5, clone SK7, BD), TCRγδ phycoerythrin-cyanine 7 (PE-Cy7, clone 11F2, BD); CD8 allophycocyanin-hilite7 (APC-H7, clone SK1, BD), HLA-DR V450 (clone L243, BD), CD45

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V500-C (clone 2D1, BD), CD11c PerCP-Cy5.5 (clone B-L46, BD), CD16 PE-Cy7 (clone 3G8, BD Pharmingen), CD123 APC (clone 9F5, BD), CD14 APC-H7 (clone MΦP9, BD), CD20 V450 (clone L27, BD), IgG flurescein isothiocyanate (FITC, clone G18-145, BD Pharmingen), IgA PE (clone IS11-8E10, Miltenyi Biotec, Cologne, Germany), CD27 PerCP-Cy5.5 (clone L128, BD), CD19 PE-Cy7 (clone J3-119, Beckman Coulter, Miami, USA), IgM APC (clone G20-127, BD Pharmingen), CD38 APC-H7 (clone HB7, BD). After an incubation period of 10 minutes, in the dark, at room temperature, 2 ml of FACSLysing solution (BD) was added, followed by another 10 minutes incubation period. Then, samples were washed in phosphate-buffered saline (PBS, Gibco, Life Technologies, Paisley, UK) and, finally, resuspended in 500 µl of PBS.

Samples were immediately acquired in a FACSCanto™II (BD) flow cytometer equipped with FACSDiva software (BD; version 8.0.1). The results were analyzed using Infinicyt (version 1.8) software (Cytognos S.L., Salamanca, Spain). The mAbs panel used enabled the identification of T cells (namely $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^+CD8^+$, $CD4^-CD8^-$ and γδ T cells), B cells (including immature, naive, plasmablasts and memory B cells' subsets), natural killer (NK) cells, monocytes (classical, intermediate and non-classical monocyte subpopulations), basophils, myeloid dendritic cells (mDCs), plasmacytoid dendritic cells (pDCs) and neutrophils. It also allowed to evaluate the expression of CD52 (measured as mean fluorescence intensity, MFI) in T cells, B cells, NK cells, monocytes, neutrophils, basophils, mDCs and pDCs.

Characterization of changes in immune cell populations (Table E2 and E3)

On the first day of the course, alemtuzumab induced a marked depletion in numbers of T-cells (349.18 cells/µl to 0.96 cells/µl) and B-cells (293.49 cells/µl to 9.02 cells/µl). In the following days of treatment, the numbers remained stable at low levels, with minimal additional decreases in the next two days of treatment. At the end of the course, total lymphocytes numbers decreased to a near-complete lymphopenia (826.98 cells/µl at baseline to 2.56 cells/µl).

Regarding T-cell subsets, the proportions of CD4+ T-cells did not suffer notorious changes, while the proportions of CD8+ T-cells transiently decreased after each treatment, recovering before next infusion. The proportions of γδ+ T-cells and CD4-CD8-γδ- T-cells increased after infusion, in each day of treatment. Concerning the B-cells subsets, immature B-cells and memory B-cells decreased to a complete depletion

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on the first day of treatment and remained undetectable during the complete course, while naïve B-cells maintained the same proportion of baseline during all courses.

Despite the alemtuzumab-induced lymphopenia, total numbers of leukocytes were, in general, increased more than 2-fold compared to baseline, during all days of treatment. The leukocytosis was mainly due to an increase in neutrophils, both in proportion and absolute number (2475 cells/µl in baseline; peak of 12048 cells/µl after day 2; 6307 cells/µl at the end of course). Conversely, NK cells, basophils, mDCs and pDCs were reduced after the first alemtuzumab infusion, despite the lower surface expression of CD52 in these cells compared with lymphocytes. In the same line, a strong reduction of monocytes is observed after alemtuzumab infusion. Interestingly, a marked decrease of the proportion of intermediate and non-classical monocytes is observed, which is consistent with the higher expression of CD52 in these subsets (MFI of 2662 and 4278, respectively, at baseline), compared to classical monocytes (MFI of 1277, at baseline).

After 3 months, total lymphocytes reach 38% of baseline levels. The repopulation of T and B-cells was slower compared to natural killer cells, basophils and monocytes.

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Table E2. Quantification of circulating peripheral blood cells, at baseline, 1 hour before (Pre-DZ) and 2 hours after (Post-DZ) each treatment, and 3 months after the treatment completion.

Cell population		Baseline	Day 1 Post-DZ	Day 2 Pré-DZ	Day 2 Post-DZ	Day 3 Pré-DZ	Day 3 Post-DZ	Month 3
WBC counts	(cells/µl)	4420	9600	11560	12170	11310	6570	4200
Lymphocytes	(% of total WBC)	18.71	0.133	0.094	0.042	0.024	0.039	7.44
	(cells/µl)	826.982	12.768	10.866	5.111	2.714	2.562	312.48
T-cells	(% of total WBC)	7.9	0.01	0.007	0.002	0.004	0.002	1.52
	(cells/µl)	349.180	0.960	0.809	0.243	0.452	0.131	63.840
CD4+ T-cells	(% of T-cells)	35	33	37	44	54	54	62
CD8+ T-cells	(% of T-cells)	61	38	51	22	18	38	35
γδ+ T-cells	(% of T-cells)	0.32	4.61	2.65	11	0	8	1.21
CD4-CD8-γδ-T-cells	(% of T-cells)	2.95	24	7.29	22	29	0	0.45
B-cells	(% of total WBC)	6.64	0.094	0.04	0.015	0.01	0.002	1.44
	(cells/µl)	293.488	9.024	4.624	1.826	1.131	0.131	60.480
Immature B-cells	(% of B-cells)	2.37	0	0	3	0	0	30
Naïve B-cells	(% of B-cells)	90	100	100	91	91	77	67
Memory B-cells	(% of B-cells)	7.46	0	0	6	9	23	1.75
Plasmablasts	(% of B-cells)	0	0	0	0	0	0	0.17
NK-cells	(% of total WBC)	4.17	0.029	0.047	0.025	0.01	0.035	4.01
	(cells/µl)	184.314	2.784	5.433	3.043	1.131	2.300	168.420
Neutrophils	(% of total WBC)	56	99	97	99	98	96	77
	(cells/µl)	2475.2	9504	11213.2	12048.3	11083.8	6307.2	3234
Basophils	(% of total WBC)	1.18	0.004	0.0027	0.001	0.007	0.001	0.87
	(cells/µl)	52.156	0.384	0.312	0.122	0.792	0.066	36.540
Monocytes	(% of total WBC)	8.74	0.14	0.3	0.317	0.82	0.061	5.04
	(/µl)	386.308	13.440	34.680	38.579	92.742	4.008	211.680
Classical Monocytes	(% of monocytes)	87	100	95	99	91	97	75
Intermediate Monocytes	(% of monocytes)	6.27	0	5	1	8	2	17
Non-classic Monocytes	(% of monocytes)	6.19	0	0	0	0	1	6.94
mDCs	(% of total WBC)	0.16	0	0.0002	0	0.014	0.003	0.07
	(cells/µl)	7.072	0.000	0.023	0.000	1.583	0.197	2.940
pDCs	(% of total WBC)	0.08	0	0.0001	0	0	0	0.06
	(cells/µl)	3.536	0.000	0.012	0.000	0.000	0.000	2.520

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Results expressed as absolute cell counts per µl of peripheral blood (/µL) and frequency (%); DZ: desensitization; mDCs: myeloid dendritic cells; pDCs: plasmacytoid dendritic cells; WBC: white blood cells.

	Baseline	Day 1 Post-DZ	Day 2 Pré-DZ	Day 2 Post-DZ	Day 3 Pré-DZ	Day 3 Post-DZ	Month 3
B Cells	5564	1272	1100	1134	1037	444	12912
T Cells	2372	371	228	0	1079	119	8796
NK cells	466	633	922	678	417	56	1967
Neutrophils	165	131	1945	227	326	190	826
Basophils	61	49	57	0	68	0	148
Monocytes	1641	218	577	329	267	415	3785
mDCs	771	307	n/a	n/a	256	324	1939
pDCs	528	281	n/a	n/a	n/a	n/a	1440

Table E3. Expression of CD52, measured as mean fluorescence intensity (MFI) in peripheral blood cells, at baseline, 1 hour before (Pre-DZ) and 2 hours after (Post-DZ) each treatment, and 3 months after the treatment completion.

DZ: Desensitization; mDCs: myeloid dendritic cells; pDCs: plasmacytoid dendritic cells; n/a: not available.

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