Supplementary Data

1. eMethods

Separation and preparation of human PBMCs

Whole blood was collected by venepuncture in 3-4 EDTA blood tubes. Plasma was separated from heparinized blood and the remaining cell component was diluted with an equal amount of Dulbecco's Phosphate-Buffered Saline (DPBS, Thermo Fisher Scientific). PBMCs from samples collected between 06/2017 and 03/2020 were isolated by stratifying approximately 30 ml of diluted blood on 15 ml of lymphoprepTM (Density gradient medium, Stemcell Technologies), followed by centrifugation at 2000 rpm for 40 minutes at room temperature (RT). PBMCs were cryopreserved in a 10% dimethyl sulfoxide freezing solution (Cell Signalling Technology) and foetal bovine serum (Sigma Aldrich Co Ltd) for 24 hours, before transferring to liquid nitrogen tanks for storage.

CyTOF staining protocols and antibodies

The staining panel was designed using an Optimized Multicolour Immunofluorescence Panel with 34 markers (supplementary Table S4).

PBMCs (3-4 x 10^6 cells per tube) were incubated for 5 minutes with viability reagent Cisplatin. After washing the cell suspensions twice with MaxPar® cell staining buffer for 5 minutes, diluted Trustain (5µl/sample + 45µl cell staining buffer per tube) was added for 10 minutes. Directly conjugated antibodies (supplementary Table S4) for extracellular staining were incubated for 30 minutes in the dark at RT.

For intracellular staining, cells were washed twice in Nuclear Fix/Perm buffer prior to resuspending cells in 1 ml Nuclear Fix/Perm buffer (Thermo Fisher, 00-5523-00), and then incubated in the dark at 4°C for 30 minutes. Cells were washed twice (5 minutes at 600g) with permeabilization buffer. FoxP3 (259D/D7)-162Dy was added for 30 minutes incubation in the dark at RT. Cells were washed twice again and incubated overnight in the dark at 4°C with 1ml of fix/perm buffer and intercalator (diluted 1:4000) to identify single-cell events.

Flow cytometry (FC) staining protocol and antibodies

PBMCs (1-2 x 10^6 cells per tube) were stained with 100 µl of diluted viability dye zombie aqua (Biolegend®, 1:100 in DPBS) for 15 minutes. 50 µl diluted Fc Block (Human BD Fc BlockTM) was added to block potential non-specific antibody staining caused by IgG receptors. Subsequently, cell suspensions were washed with FC staining buffer (eBioscience) at 400g for 5 minutes) and unconjugated primary antibodies for the T cell panel

(CD127 (HIL-7R-M21)-BV510, CD3 (Hit3a)-BV605, CD8 (RPA-T8)-BV650, CD25 (2A3 and M-A251)-BV786, CD45RO (UCHL1)-PE-CF594, CD4 (L200)-PerCP-Cy5.5;), B cell panel (CD24 (ML5)-BV605, CD27 (M-T271)-BV650, CD19 (HIB19)-PE-CF594, IgD (IA6-2)-PerCP-Cy5.5;) or senescence markers (CD4 (RPA-T4)-PE-CF594, CD8 (SK1)-PerCP, CD27 (0323)-BV421, CD45RA (HI100)-BV605, CCR7 (G0343H7)-PeCy7, CD28 (CD28.2)-BV785, KLRG1 (REA261)-PE were added.

For T cell intracellular staining, the cell suspensions were washed, fixed (200 µl intracellular fixation buffer, eBioscience, 1:1 diluted with flow cytometry buffer) and permeabilized (1 ml of Nuclear fix / perm buffer, eBioscience 00-5523-00). Finally, cells were stained with FoxP3 (259D/C7 and PCH1010)-PE. To improve Tregs detection, two clones were used for CD25 and FoxP3 as previously reported.

2. eTables

eTable 1. List of flow cytometry markers used for the detection of T cell, B cell and senescent T cells.

MARKERS	SUBSETS	ABBREVIATION
T CELL MARKERS		
CD3+ (% lymphocytes)	All T cells	T
CD4+ (% T)	T helper cells	Th
CD8+ (% T)	CD8+ T cells	Tc
CD4+CD45RO+ (% CD4)	Memory T helper cells	M-Th
CD4+CD45RO- (% CD4)	Naïve T helper cells	N-Th
CD8+CD45RO+ (% CD8)	Memory CD8+ T cells	M-Tc
CD8+CD45RO- (% CD8)	Naïve CD8+ T cells	N-Tc
CD4+CD25high+CD127low (% CD4)	T regulatory cells	Tregs
CD4+CD25high+CD127low+FoxP3+ (% CD4)	FoxP3+ T regulatory cells	FoxP3+ Tregs
CD25high+CD127low+FoxP3+45RO+ (% CD4)	Memory T regulatory cells	M-Tregs
CD25high+CD127low+FoxP3+45RO- (% CD4)	Naïve T regulatory cells	N-Tregs
B CELL MARKERS		
CD19+ (% lymphocytes)	All B cells	B
CD19+CD27- (% B)	Naïve B cells	N-B
CD19+CD27+ (% B)	Memory B cells	M-B
CD19+CD27-CD24+IgD- (% CD24+ N-B)	Immature B cells	I-B
CD19+CD27-CD24+IgD+ (% CD24+ N-B)	Follicular B cells	F-B
CD19+CD27-CD24+IgDlow (% CD24+ N-B)	Transitional B cells	T-B
CD19+CD27+IgD- (% B)	Switched B cells	S-B
CD19+CD27+IgD+ (% B)	Unswitched B cells	US-B
CD19+CD27-IgD- (% B)	Late memory B cells	LM-B
T CELL SENESCENCE MARKERS		
CD8+ (% lymphocytes)	Cytotoxic T cells	Tc
CD8+CD45RA+CCR7-CD28+CD27+ (% CD8)	Early senescent cytotoxic T cells	ES-Tc
CD8+CD45RA+CCR7-CD28-CD27+ (% CD8)	Intermediate senescent cytotoxic T cells 1	IS1-Tc
CD8+CD45RA+CCR7-CD28-CD27- (% CD8)	Late senescent cytotoxic T cells	LS-Tc
CD8+CD45RA+CCR7-CD28+CD27- (% CD8)	Intermediate senescent cytotoxic T cells 2	IS2-Tc
CD8+CD27-CD45RA+KLRG1- (% CD8)	Non-viral associated senescent cytotoxic T cells	Non-viral-S Tc
CD8+CD27-CD45RA+KLRG1+(% CD8)	Viral associated senescent cytotoxic T cells	Viral-S Tc
CD4+ (% lymphocytes)	T helper cells	Th
CD4+CD45RA+CCR7-CD28+CD27+ (%CD4)	Early senescent T helper cells	ES-Th
CD4+CD45RA+CCR7-CD28+CD27- (%CD4)	Intermediate senescent T helper cells 1	IS1-Th

CD4+CD45RA+CCR7-CD28-CD27- (%CD4)	Late senescent T helper cells	LS-Th
CD4+CD45RA+CCR7-CD28-CD27+ (%CD4)	Intermediate senescent T helper cells 2	IS2-Th
CD4+CD27-CD45RA+KLRG1- (%CD4)	Non-viral associated senescent T helper cells	Non-viral-S Th
CD4+CD27-CD45RA+KLRG1+ (%CD4)	Viral associated senescent T helper cells	Viral-S Th

eTable 2. Assignment of meta-clusters from Phenograph identified clusters above the 1% frequency threshold with respective phenotypes and frequencies.

Cell type	Phenotype	Frequency (%)
CD4 T naïve (N)	CD3+ CD4+ CD27+ CD45RA+	7.4
CD4 T central memory (CM)	CD3+ CD4+ CD27+ CD45RA-	10.9
CD4 T effector (EFF)	CD3+ CD4+ CD27-	3.5
CD4 T senescent (SEN)	CD3+ CD4+ CD27- CD57+	1.6
CD8 T N	CD3+ CD8+ CD27+ CD45RA+	1.6
CD8 T CM	CD3+ CD8+ CD27+ CD45RA-	6.7
CD8 T EFF	CD3+ CD8+ CD27-	3.7
CD8 T SEN	CD3+ CD8+ CD27- CD57+	4.9
Regulatory T (Treg)	CD3+ CD4+ CD25+ FoxP3+	1.7
γδ T	CD3+ CD4- CD8- Vδ2+	1.3
Naïve B	CD3- CD19+ CD27-	8.1
Memory B	CD3- CD19+ CD27+	4.2
NK	CD3- CD19- CD14- CD56+	4.6
CD57+ NK	CD3- CD19- CD14- CD56+ CD57+	4.6
Classical Monocytes	CD3- CD19- HLADR+ CD11c+ CD14+ CD16-	18.6
Non-Classical Monocytes	CD3- CD19- HLADR+ CD11c+ CD14+ CD16+	4.2
CD57+ Monocytes	CD3- CD19- HLADR+ CD11c+ CD14+ CD16- CD57+	4.2

eTable 3. List of mass cytometry antibody panel and reagents.

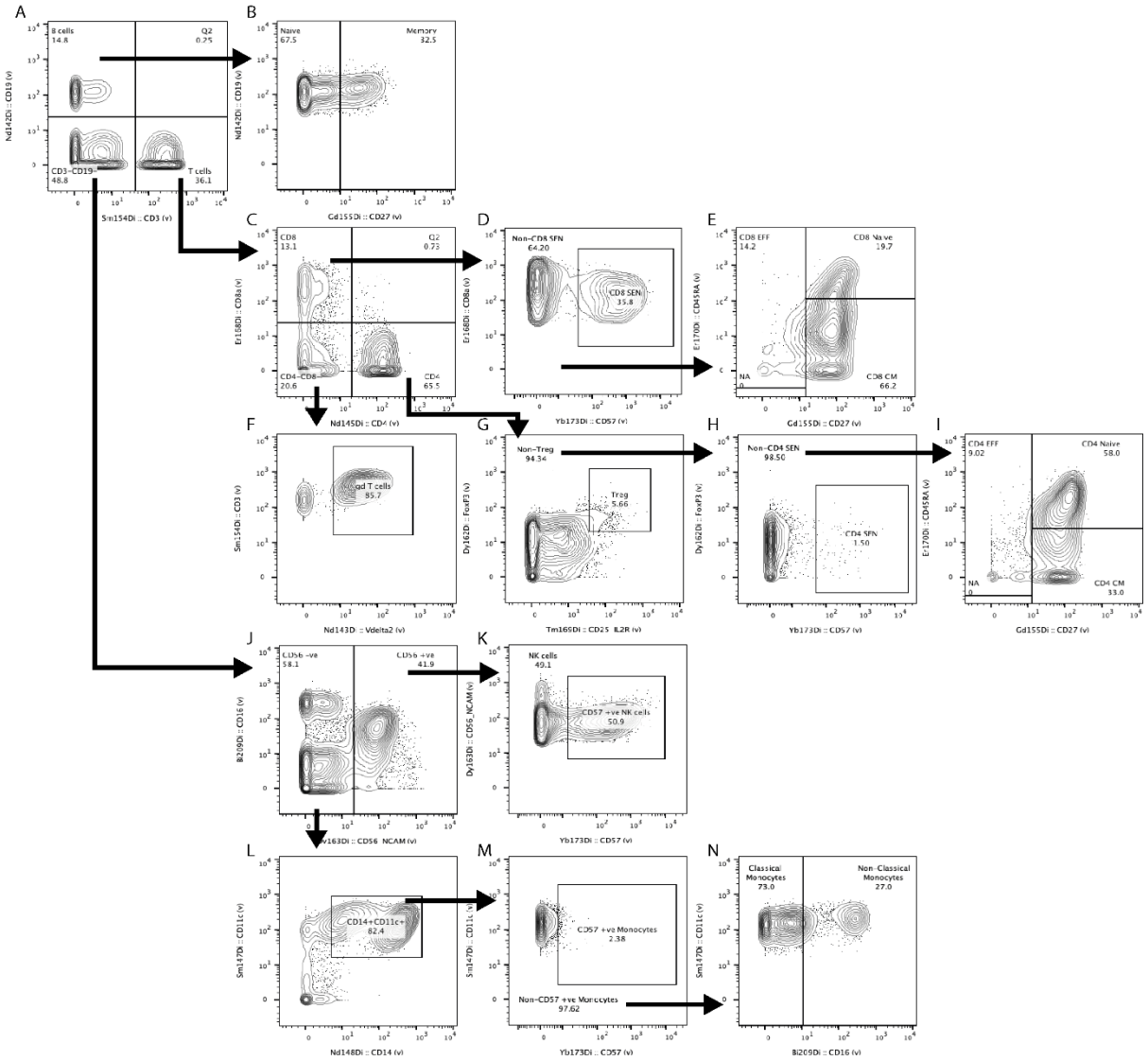
Marker	Clone	Protein	Target	Metal
CD45	HI30	Protein tyrosine phosphatase	All haematopoietic cells	89Y
CD3	UCHT1	CD3 antigen	T cells	154Sm
CD4	RPA-T4	CD4 T cell surface glycoprotein	T helper cells	145Nd
CD8a	SK1	CD8 T cell surface glycoprotein	Cytotoxic T cells	168Er
CD45RA	HI100	Isoform of CD45	Naïve / memory T cells	170Er
Vδ2	B6	T-cell receptors (TCRs)	Gamma-delta (γδ) T cells	143Nd
FoxP3	259D/C7	Forkhead box P3	T regulatory cells	162Dy
CD25-IL2R	2A3	Interleukin-2 receptor-α	T regulatory cells Mature B cells	169Tm
CD127-IL7Ra	A019D5	Interleukin-7 receptor-α	T cells, NK cells	176Yb
CD28	CD28.2	CD28 antigen	T cells	160Gd
CD57	HCD57	Beta-1, 3-glucuronyltransferase 1	T cell memory subset	173Yb
CD279-PD1	EH12.2H7	Programmed cell death protein 1	Exhausted T cells	175Lu
CD194-CCR4	205410	C-C chemokine receptor type 4	Th2 cells	149Sm
CD183-CXCR3	G025H7	Chemokine receptor / G protein-coupled receptor 9	Effector T cells	156Gd
CD197-CCR7	G043H7	C-C chemokine receptor type 7	Effector T cells	159Tb
CD38	HIT2	Cyclic ADP-ribose hydrolase	Activated cells	167Er
CD86	IT2.2	Type I membrane immunoglobulin	Activated cells	150Nd
CD19	HIB19	B cell antigen	B cells	142Nd
CD24	ML5	Signal transducer CD24	B cells, granulocytes	166Er
CD27	L128	Tumour necrosis factor receptor Family member CD27	Activated T cells, naïve / memory B cells	155Gd
IgD	IA6-2	Immunoglobulin D	Activated B cells	146Nd
CD14	RMO52	Monocyte differentiation antigen	Monocytes / macrophages	148Nd
CD16	3G8	Lymphocyte Fc gamma type III Low-affinity receptor	Monocytes / macrophages, NK cells	209Bi
HLA-DR	L243	HLA class II histocompatibility Antigen DR	Monocytes / macrophages, dendritic cells, Activated T cells, B cells, NK cells	174Yb
CD56-NCAM	NCAM16.2	Neural cell adhesion molecule 1	NK cells	163Dy
CD11c	Bu15	Integrin alpha-X	Dendritic cells, Monocytes / macrophages	147Sm
CD196-CCR6	11A9	C-C chemokine receptor type 6	dendritic cells, NK cells, Neutrophils	141Pr
CD123-IL3R	6H6	Interleukin-3 receptor-α	Dendritic cells, basophils	151Eu
CD40	5C3	Tumour necrosis factor receptor Superfamily member 5	M1 macrophage marker, dendritic cells, B cells	171Yb
CD192-CCR2	K036C2	C-C chemokine receptor type 2	Monocytes / macrophages	153Eu
CD169	7-239	Sialoadhesin	Macrophages	158Gd
CD61-ITGB3	VI-PL2	Platelet glycoprotein IIIa, Integrin-beta3	Platelets and megakaryocytes	165Ho
CD95-Fas	DX2	TNF receptor family	Apoptosis marker	152Sm

eTable 4. Cryopreservation time and frequencies of lymphocyte subsets.

Pairwise correlation analysis	ALS Cryopreservation time (days)	HC Cryopreservation time (days)
CyToff Median marker intensity		
FoxP3	*R*= -0.1286 *p*= 0.5786	*R*= -0.2188 *p*= 0.5414
CD4	*R*= 0.3857 *p*= 0.0842	*R*=0.009956 *p:* 0.9782
CD8	*R*= 0.1247 *p*= 0.5903	*R*= -0.1520 *p*= 0.6734
CD19	*R*= -0.07026 p= 0.7622	*R*= -0.2877 p= 0.4202
CD57	*R*= 0.3446 p= 0.1260	*R*= 0.5382 p= 0.1122
FC Lymphocyte subsets frequencies (%)		
B cells (CD19)	R=0.2249 p= 0.1630	*R*=-0.1277 *p*=0.7247
M-B (CD19+CD27+)	R=-0.2113 p= 0.2618	R= 0.1707 p= 0.4717
LM-B	R= 0.07260 p= 0.6562	R=-0.4116 p= 0.0714

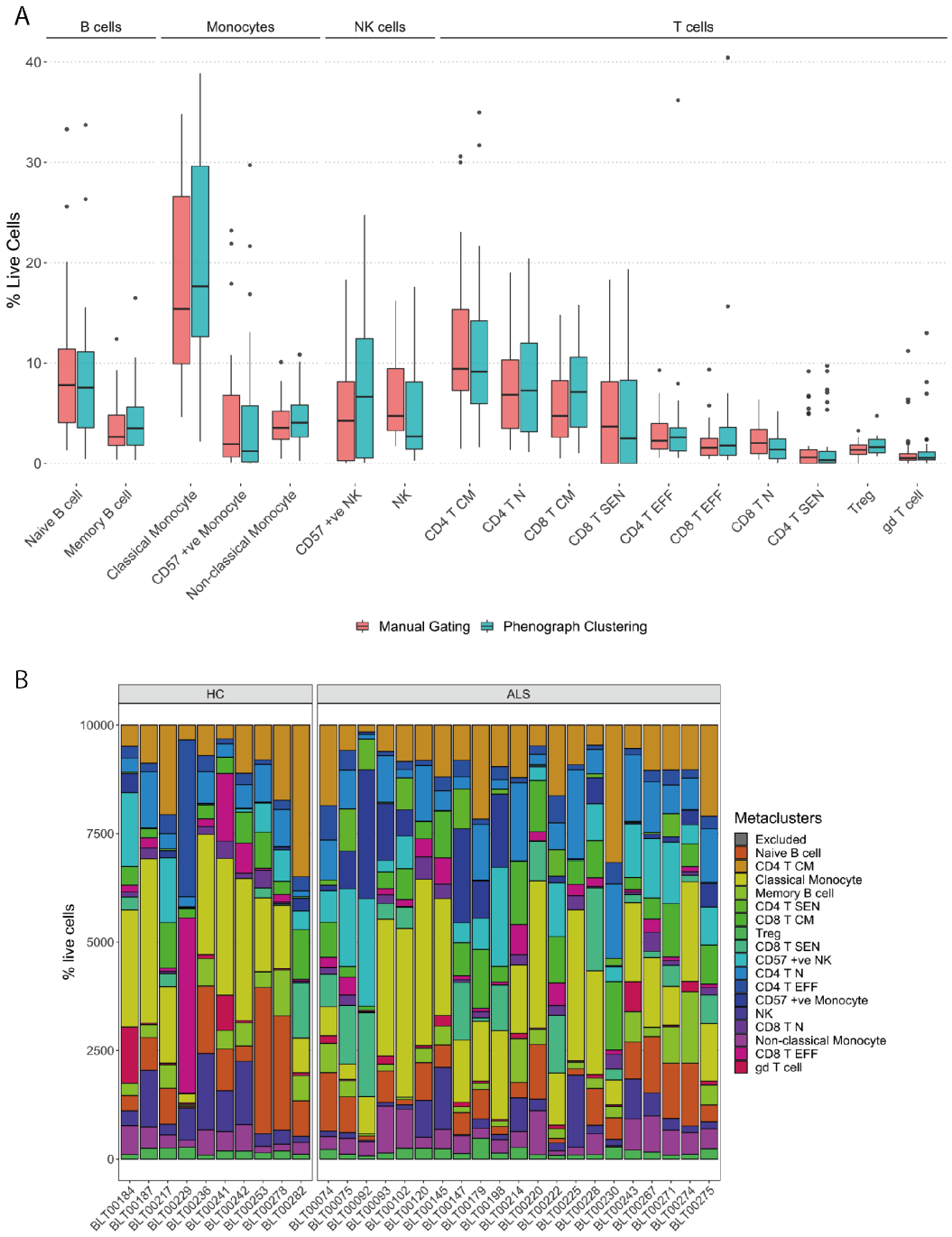
CD19+CD27-IgD-		
T cells (CD3)	R= 0.1621 p= 0.3176	R=-0.2717 p=3461
CD4 T cells	R= -0.1753 p= 0.2792	R= 0.1817 p= 0.4432
CD8 T cells	R= 0.1779 p= 0.2722	R= 0.1790 p= 0.4502
M-Tc (CD8+CD45RO+)	R= -0.2418 p= 0.1327	R= -0.02409 p= 0.9197
FoxP3+ Tregs (CD4+CD25high+CD127low +FoxP3+)	R=-0.1969 p=0.2234	R= -0.01805 p= 0.9398
M-Tregs (CD25high+CD127low+Fox P3+45RO+)	R=-0.1922 p= 0.2349	R=0.04328 p= 0.8562
CD4+CD27-CD45RA+KLRG1+	R=-0.06860 p= 0.7139	R=-0.1976 p= 0.4037
CD8+CD45RO+	R=--0.1364 p= 0.4013	R=--0.01805 p= 0.9398

3. eFigures

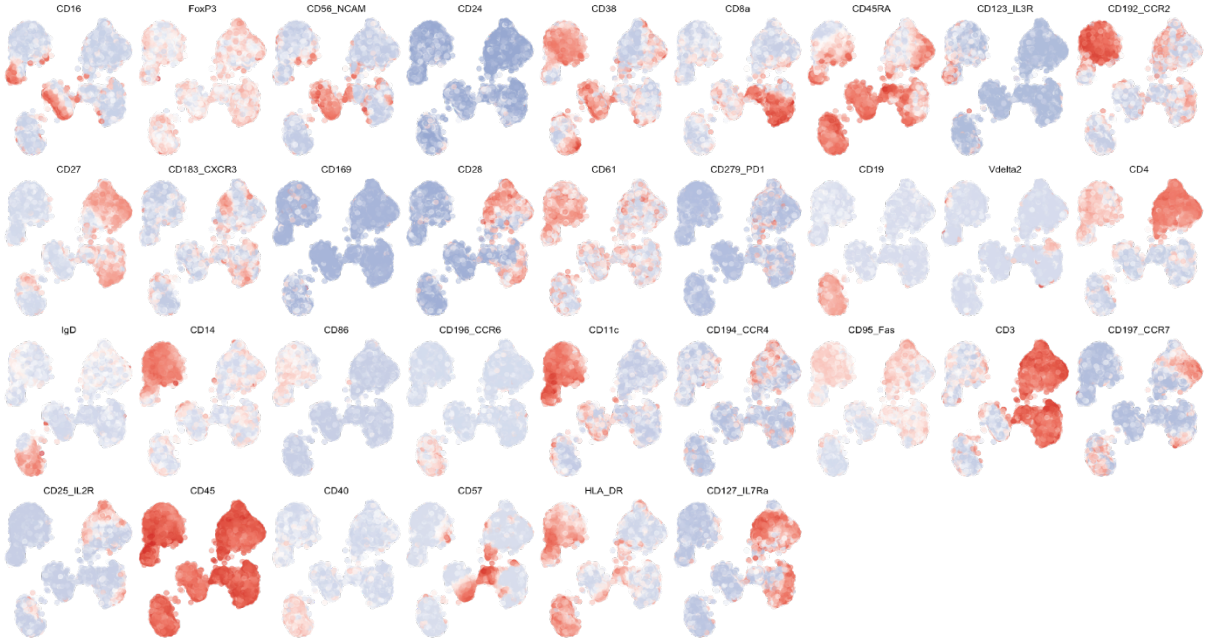


eFigure 1: CyTOF data manual gating strategy.

(A) T and B cells identified by CD3 and CD19 expression respectively from live single cells. **(B)** Naïve and memory B cells were determined by CD27 expression. **(C)** $CD3^+$ cells were subdivided into CD4 and CD8 T cells. **(D)** CD57 was used to identify senescent CD8 T cells (CD8 SEN). **(E)** Non-CD8 SEN cells were further subdivided into Naïve, central memory (CM) and effector (EFF) CD8 T cells via CD27 and CD45RA. **(F)** $CD4^-$ $CD8^-$ cells were gated on Vδ2 to identify γδ T cells. **(G)** Treg cells were identified from CD4 T cells via CD25 and FoxP3 expression. **(H)** Non-Treg cells were gated on CD57 expression to identify senescent CD4 T cells (CD4 SEN). **(I)** Non-CD4 SEN cells were further subdivided into Naïve, central memory (CM) and effector (EFF) CD4 T cells via CD27 and CD45RA. **(J)** $CD3^-CD19^-$ cells were subdivided into $CD56^-$ and $CD56^+$ cell populations. **(K)** $CD56^+$ were gated on CD57 to identify NK and $CD57^+$ NK cells. **(L)** $CD56^-$ cells were gated on CD14 and CD11c to identify $CD14^+CD11c^+$ cells. **(M)** $CD57^+$ monocytes were identified from the $CD14^+CD11c^+$ population via CD57 expression. **(N)** Non-$CD57^+$ monocytes were further subdivided into classical and non-classical monocytes based on CD16 expression.

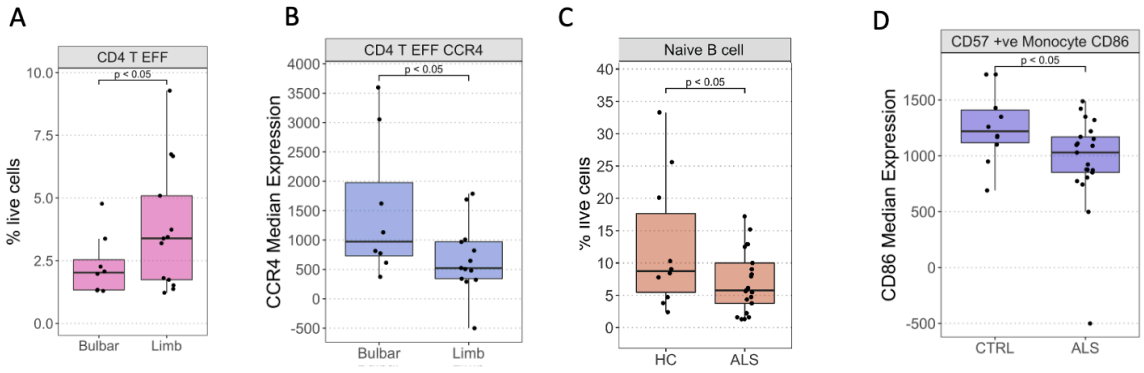


eFigure 2: Comparison between manual gating and unsupervised phenograph clustering of CyTOF data. **(A)** Frequency of cell populations as a percentage of live cells, identified via manual gating (red) and unsupervised phenograph clustering (blue). **(B)** Sample-wise cell type composition of individual samples in control and ALS samples.



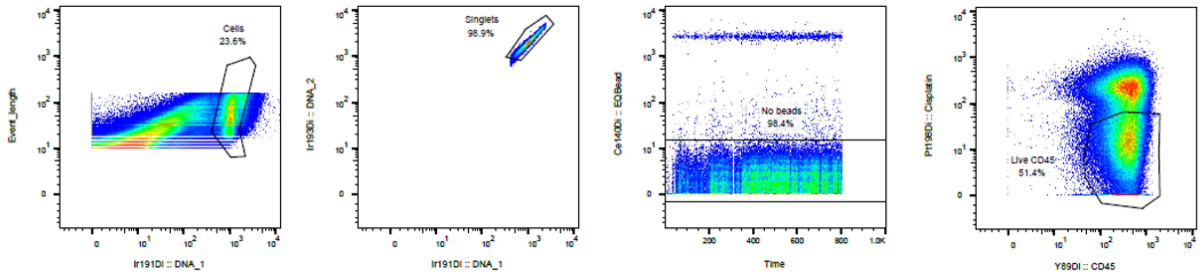
eFigure 3: t-Distributed Stochastic Neighbour Embedding (tSNE) with marker expression.

tSNE plots of 100,000 down sampled cells (50,000 in HC and ALS groups) overlayed with marker-wise expression. High (red) and low (blue) expression is shown relative to the marker specific mean.



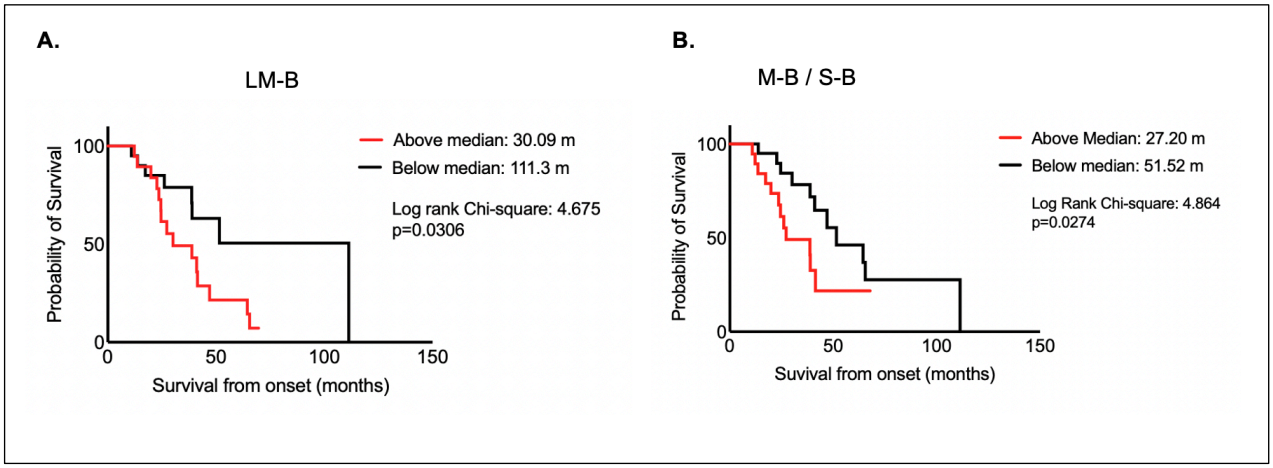
eFigure 4: High-dimensional mass cytometry (CyTOF) analysis: different abundance of manually gated lymphocyte and monocyte cell subsets in ALS compared to healthy controls and in phenotypic variants of the disease.

(A) Effector CD4 T cells were higher in blood from ALS patients with limb onset (A-L) compared to those with bulbar onset ALS (A-B). **(B)** Effector CD4 T cells CCR4 median expression is lower in blood from limb ALS compared to bulbar ALS. (**C**) Naïve B cells are less abundant in blood from ALS patients compared to HC ($p < 0.05$). **(D)** CD86 monocytes expressing CD57 median expression is lower in patients with ALS compared to HC ($p < 0.05$). P-values were determined via quasi-likelihood negative binomial generalized linear models. HC: Healthy Control. CD4 T EFF: Effector CD4 T cells.



eFigure 5: Standard pre-processing of cytometry FCS files.

Data was normalised, debris and doublets were removed, and gating was performed to obtain live CD45+ cells.



eFigure 6. B cells subset frequencies and survival: Kaplan-Meier analysis.

(A) Kaplan-Meier survival analysis from baseline (V1) shows shorter survival for ALS patients with higher LM-B blood frequencies (above median; p=0.0306). **(B)** Kaplan-Meier survival analysis from V1 shows shorter survival for ALS patients with higher M-B blood and S-B frequencies (above median; p=0.0274).

Red lines indicate ALS subgroups with higher analyte levels (above median or Q3) and black lines ALS subgroups with lower level (below median or Q3). Log rank Chi-square and p values as well as survival in months calculated

for each subset of ALS patients are reported for each Kaplan Mayer figure. *p*-value was obtained from Log-rang test chi square; $p \leq 0.05$ was considered statistically significant.

Supplementary references

e1. *Senescent cell turnover slows with age providing an explanation for the Gompertz law.* **Omer Karin, Amit Agrawal, Ziv Porat, Valery Krizhanovsky & Uri Alon.** s.l. : Nature Communications , 2019, Vol. Article number: 5495.

e2. *T-Cell Avidity and Tuning: The Flexible Connection Between Tolerance and Autoimmunity.* **Jasper G. van den Boorn, I. Caroline Le Poole, and Rosalie M. Luiten.** s.l. : Int Rev Immunol. , 2006 May-Aug; 25(3-4): 235–258.

e3. *Correlation of Peripheral Immunity With Rapid Amyotrophic Lateral Sclerosis Progression.* **Benjamin J Murdock, Tingting Zhou, Samy R Kashlan, Roderick J Little, Stephen A Goutman, Eva L Feldman.** s.l. : JAMA Neurol. 2017 Dec 1;74(12):1446-1454. doi: 10.1001/jamaneurol.2017.2255.

e4. *Premature B-cell senescence as a consequence of chronic immune activation.* **Paolo Palma, Stefano Rinaldi, Nicola Cotugno, Veronica Santilli, Savita Pahwa, Paolo Rossi, and Alberto Cagigi.** s.l. : Hum Vaccin Immunother., 2014, Vols. 10(7): 2083–2088.