

Supplementary Information

Specific types of male infertility are correlated with T cell exhaustion or senescence signatures

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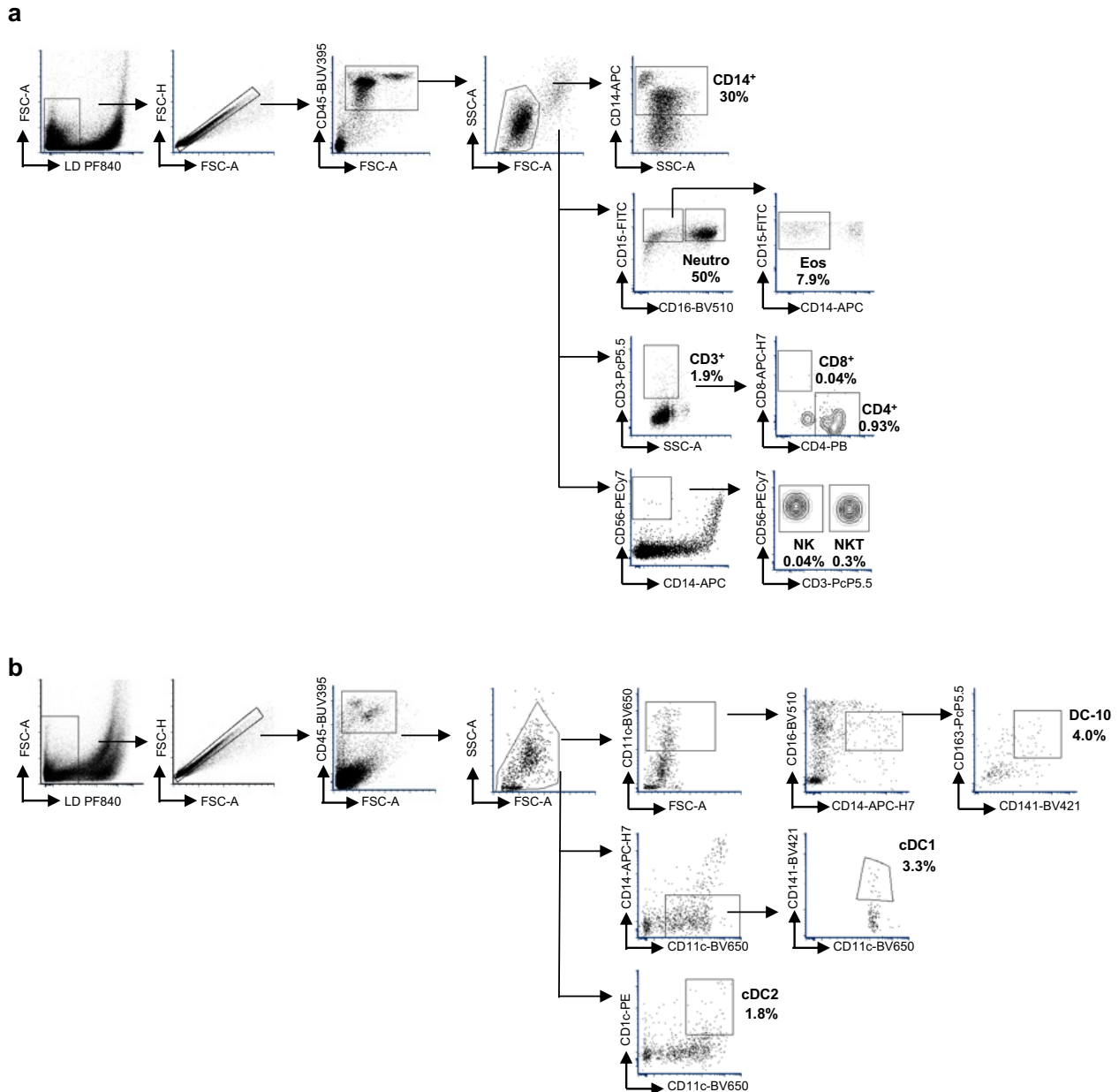
Supplementary Table 1. Descriptive statistics of fertile men grouped with normal vs. abnormal semen analyses.

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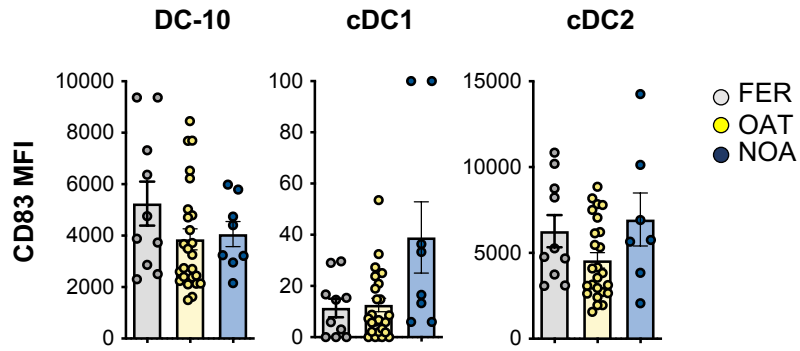
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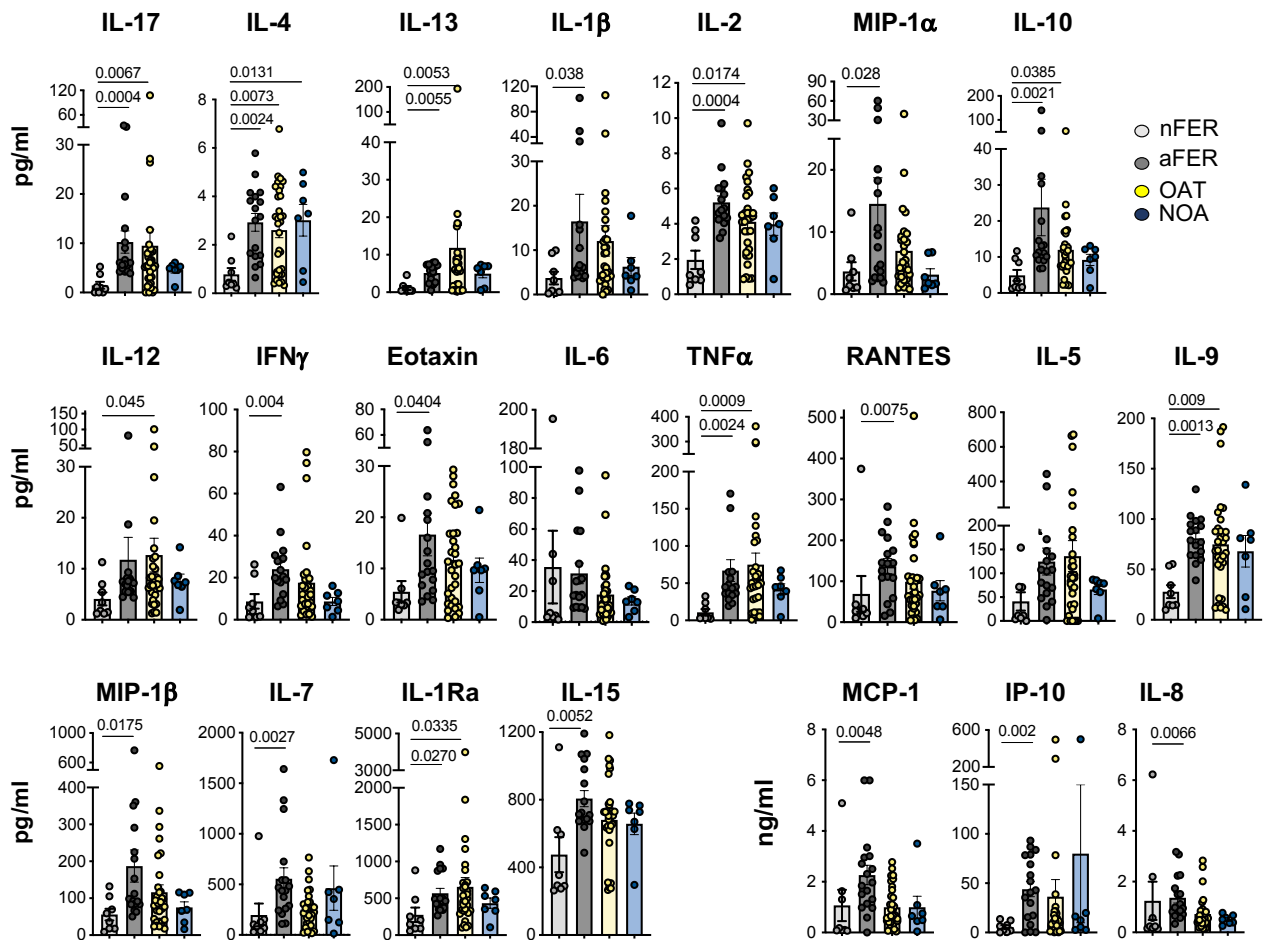
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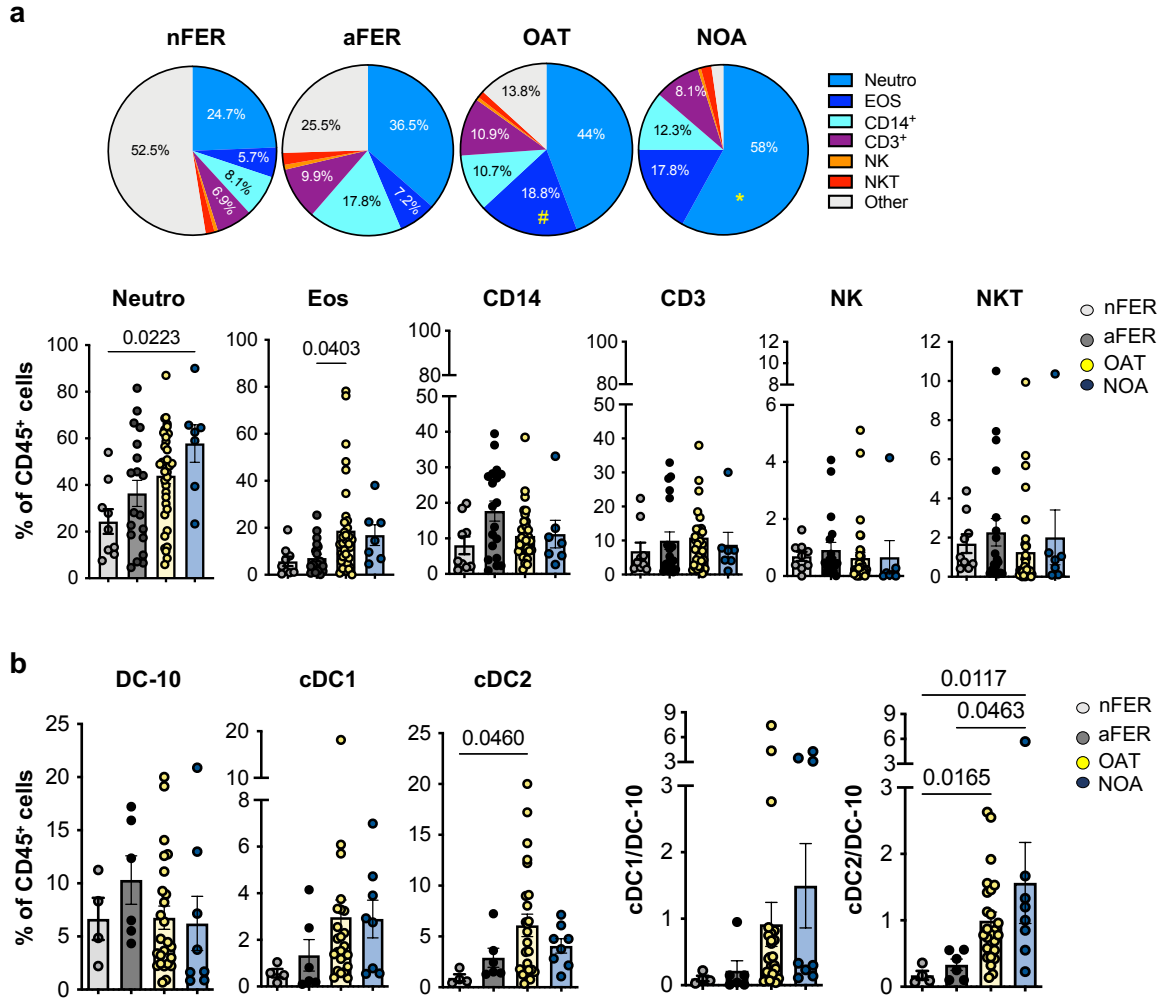
Supplementary Figure. 1. Gating strategies to identify leucocytes and dendritic cell subsets in seminal fluid. The proportion of the major leukocyte subsets, including dendritic cells (DC), in sperm fluids shown in Figure 1a and Figure 1b was analyzed by multiparametric flow cytometry. **a.** Representative gating strategy used to identify the frequencies of CD14⁺ monocytes, neutrophils (Neutro, CD15⁺CD16⁺), eosinophils (Eos, CD15⁺CD16⁻CD14⁻), CD3⁺CD8⁺ and CD3⁺CD4⁺ T cells, NK (CD3⁻CD56⁺), and NKT (CD3⁺CD56⁺) cells. **b.** Representative gating strategy used to identify DC-10 (CD11c⁺CD14⁺CD16⁺CD141⁺CD163⁺), cDC1 (CD14⁺CD11c⁺CD141⁺), and cDC2 (CD11c⁺CD1c⁺) cells. Numbers indicate the percentage relative to the FSC-A/SSC-A gate.



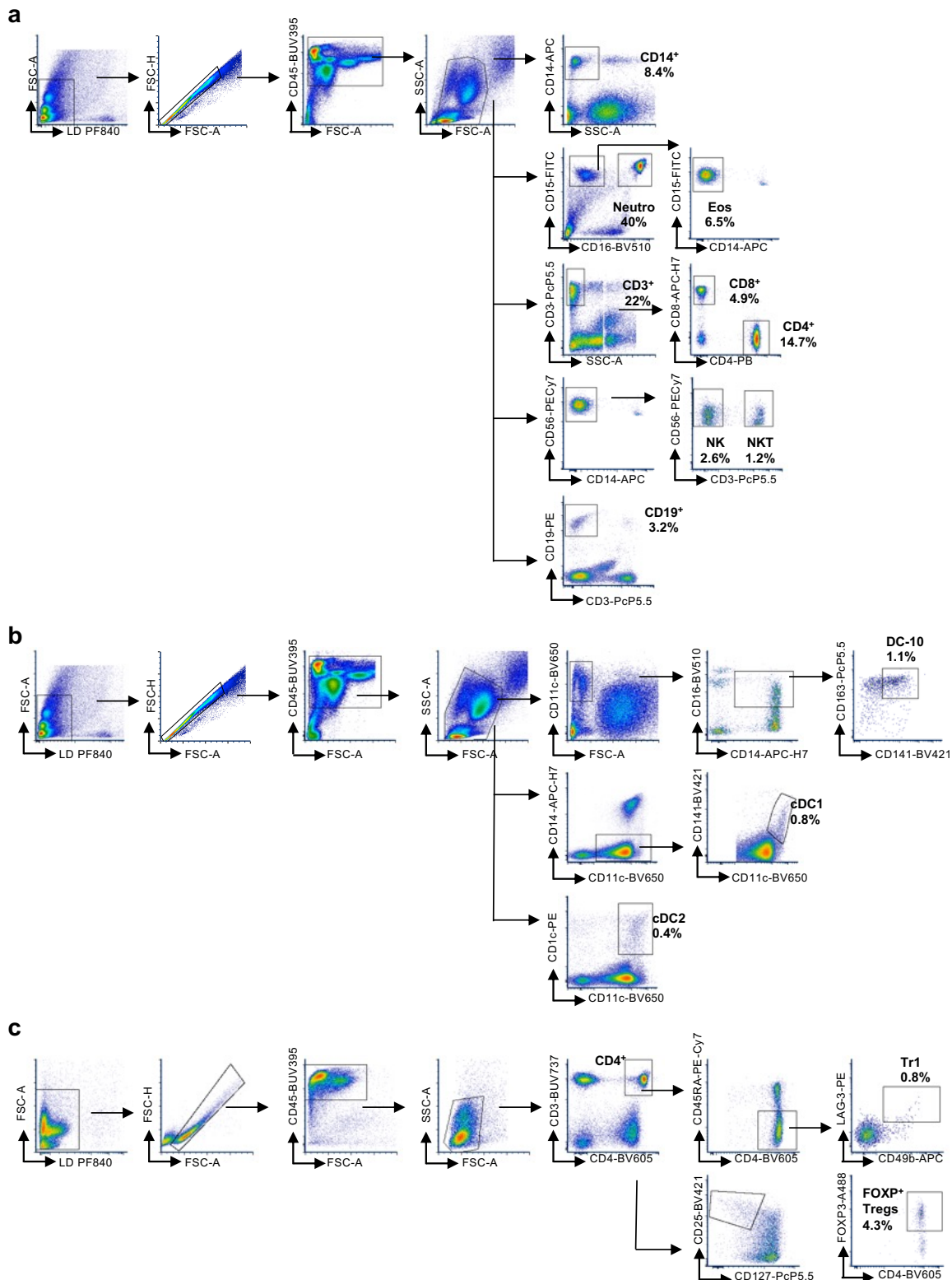
Supplementary Figure 2. Expression of CD83 on different sub-classes of dendritic cells in seminal fluid. The Mean Fluorescence Intensity (MFI) of CD83 expressed by DC-10 (CD11c⁺CD14⁺CD16⁺CD141⁺CD163⁺), cDC1 (CD14⁺CD11c⁺CD141⁺), and cDC2 (CD11c⁺CD1c⁺) in the semen of fertile, (FER, n=10), Oligo-Astheno-Terato spermia (OAT, n=25), and non-obstructive azoospermia (NOA, n=8) men, is shown. Each dot represents a single donor; scatter plots indicate mean±SEM; the Kruskal-Wallis test, in association with Dunn's multiple comparison test, has been used to determine the statistical significance of data. Source data are provided as a Source Data file.



Supplementary Figure 3. An abnormal spermogram is associated with increased levels of pro-inflammatory mediators in sperm fluid. The presence and concentration of the indicated cytokines and chemokines were evaluated using multi-beads array in the sperm plasma of fertile men without (nFER, n=8) or with one/two spermogram alterations (aFER, n=17), Oligo-Asthenoterato spermia (OAT, n=32), and non-obstructive azoospermia (NOA, n=7) men. Each dot represents a single donor; scatter plots indicate mean±SEM; Kruskal-Wallis test in association with Dunn's multiple comparison test performed comparing the mean rank of each group with the mean rank of FER group was used to determine the statistical significance of the data. Statistically significant P values are reported. Source data are provided as a Source Data file.

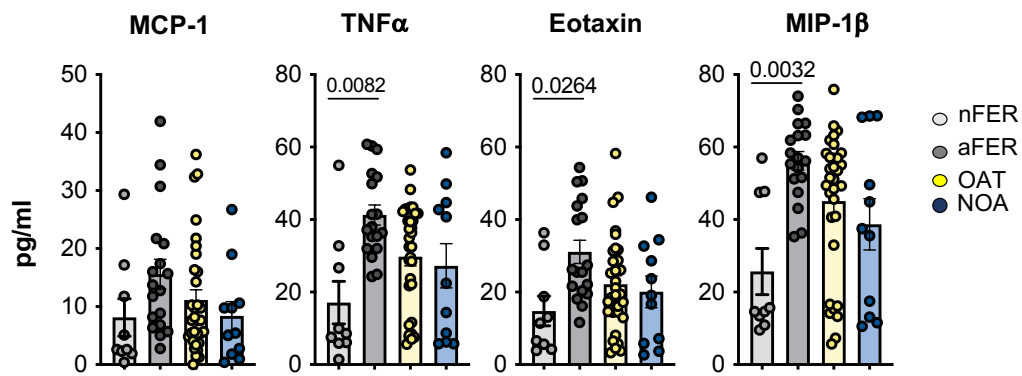


Supplementary Figure 4. Leucocyte composition in the semen of infertile and fertile men. The proportion of the major leucocyte subsets, including dendritic cells (DC), was analyzed by multiparametric flow cytometry in the sperm fluid of fertile men without (nFER) or with one/two spermiogram alterations (aFER), Oligo-Astheno-Terato spermia (OAT), and non-obstructive azoospermia (NOA) men. **a.** Pie charts showing the overall leucocyte subset distribution in nFER (n=9), aFER (n=19), OAT (n=35), and NOA (n=8) men; numbers in pie charts indicate the mean, * $P \leq 0.05$ vs nFER and # $P \leq 0.05$ vs aFER. Lower panels show the distribution of each leucocyte population in the different groups; each dot represents a single donor, scatter plots indicate mean $\pm$ SEM; Kruskal-Wallis test in association with Dunn's multiple comparison test performed comparing the mean rank of each group with the mean rank of FER group was used to determine the statistical significance of the data, statistically significant P values are reported. **b.** The proportion of DC-10 (CD11c⁺CD14⁺CD16⁺CD141⁺CD163⁺), cDC1 (CD14⁺CD11c⁺CD141⁺), and cDC2 (CD11c⁺CD1c⁺) in the sperm fluid of nFER (n=4), aFER (n=6), OAT (n=25), and NOA (n=8) men (left panels) and the ratio between the percentage of cDC1 and DC-10 and of cDC2 and DC-10 calculated for each donor (right panels) are shown. Each dot represents a single donor; scatter plots indicate mean $\pm$ SEM; Kruskal-Wallis test in association with Dunn's multiple comparison test performed comparing the mean rank of each group with the mean rank of FER group was used to determine the statistical significance of the data, statistically significant P values are reported. Source data are provided as a Source Data file.

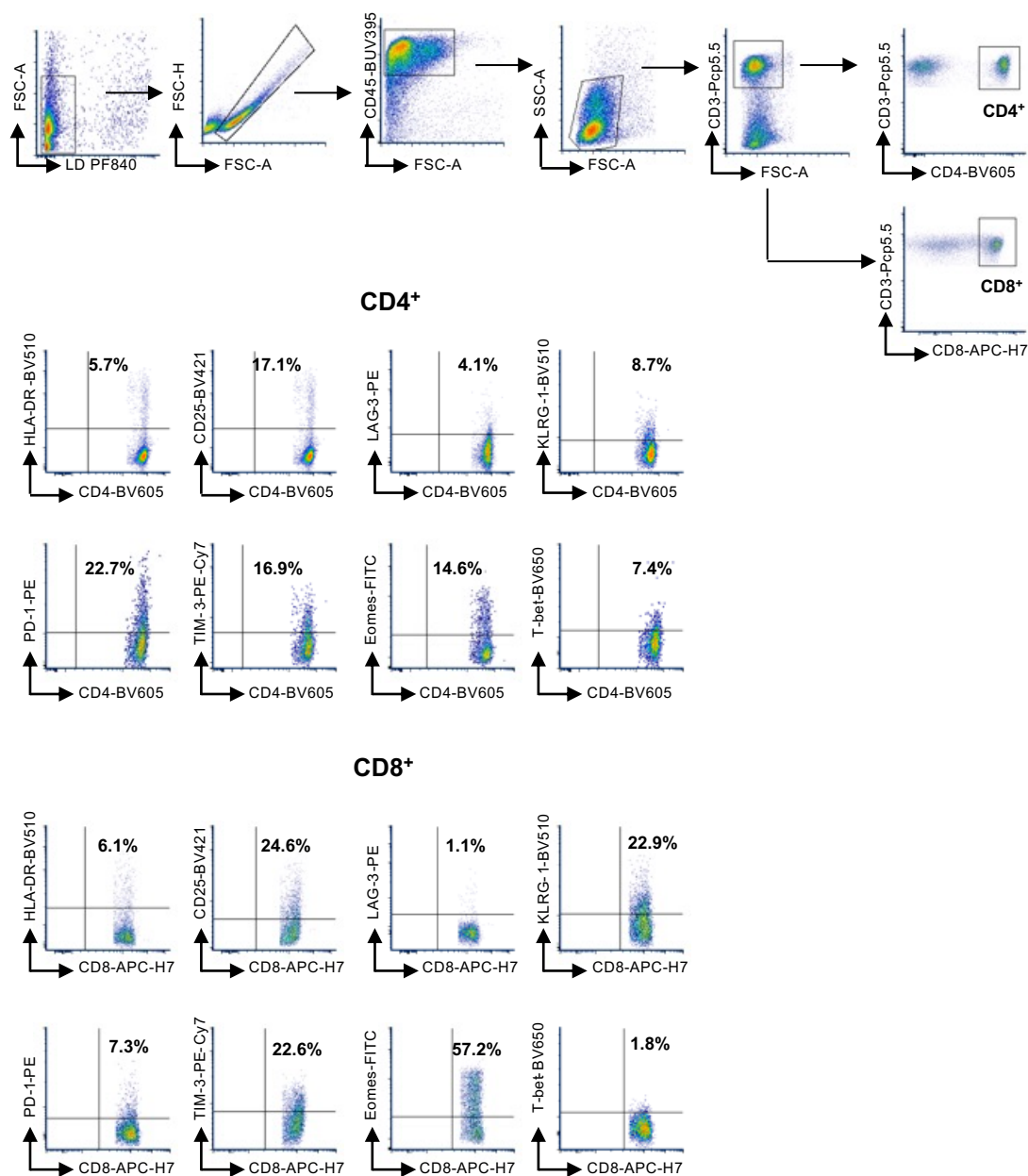


Supplementary Figure 5. Gating strategies to identify leucocytes, dendritic cells, and Treg subsets in the peripheral blood. The proportion of the major leukocyte subsets, including dendritic cells (DC) and regulatory T cells was analyzed by multiparametric flow cytometry in peripheral blood. **a.** Representative gating strategy used to identify the frequencies of CD14⁺ monocytes, neutrophils (Neutro, CD15⁺CD16⁺), eosinophils (Eos, CD15⁺CD16⁺CD14⁻), CD3⁺CD8⁺ and CD3⁺CD4⁺ T cells, NK (CD3⁺CD56⁺), NKT (CD3⁺CD56⁺), and CD19⁺ (CD19⁺CD3⁻) cells shown in Figure 2a and Figure 2b. Numbers indicate the percentage relative to the

FSC-A/SSC-A gate. **b.** Representative gating strategy used to identify DC-10 (CD11c⁺CD14⁺CD16⁺CD141⁺CD163⁺), cDC1 (CD14⁺CD11c⁺CD141⁺), and cDC2 (CD11c⁺CD1c⁺) cells shown in Figure 2a. Numbers indicate the percentage relative to the FSC-A/SSC-A gate. **c.** Representative gating strategy used to identify Tr1 cells (CD3⁺CD4⁺CD45RA⁻CD49b⁺LAG-3⁺) and FOXP3⁺Tregs (CD3⁺CD4⁺CD127⁻CD25⁺FOXP3⁺) that are shown in Figure 2c. Numbers indicate the percentage relative to the CD4⁺ gate.

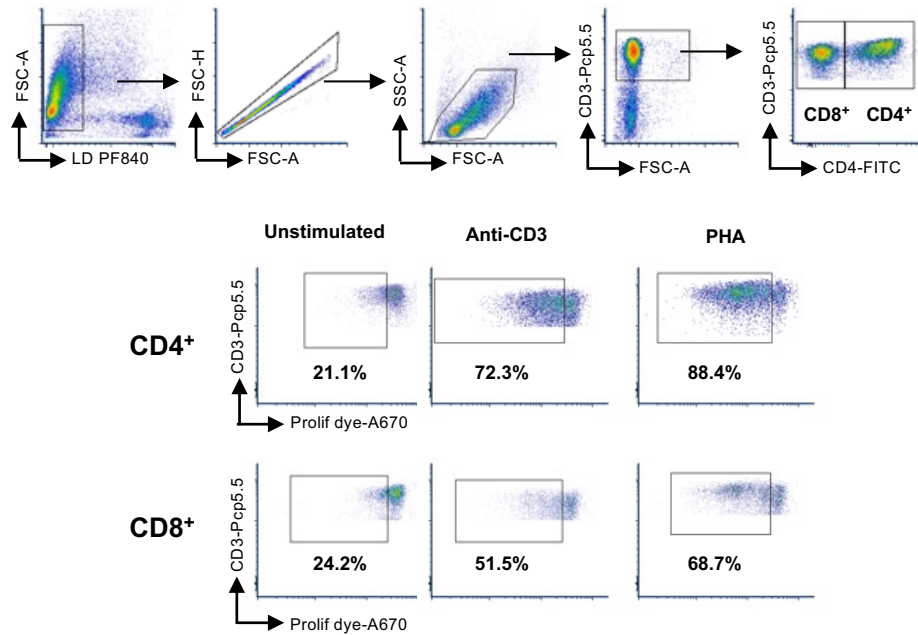


Supplementary Figure 6. An abnormal spermiogram is associated with increased levels of pro-inflammatory mediators in the peripheral blood. The presence and concentration of the indicated cytokines and chemokines were evaluated using multi-beads array in sperm plasma of fertile men without (nFER, n=9) or with one/two spermiogram alterations (aFER, n=18), Oligo-Asthenio-Terato spermia (OAT, n=32), and non-obstructive azoospermia (NOA, n=11) men. Each dot represents a single donor, scatter plots indicate mean±SEM; Kruskal-Wallis test in association with Dunn's multiple comparison test performed comparing the mean rank of each group with the mean rank of FER group was used to determine the statistical significance of the data. Statistically significant P values are reported. Source data are provided as a Source Data file.

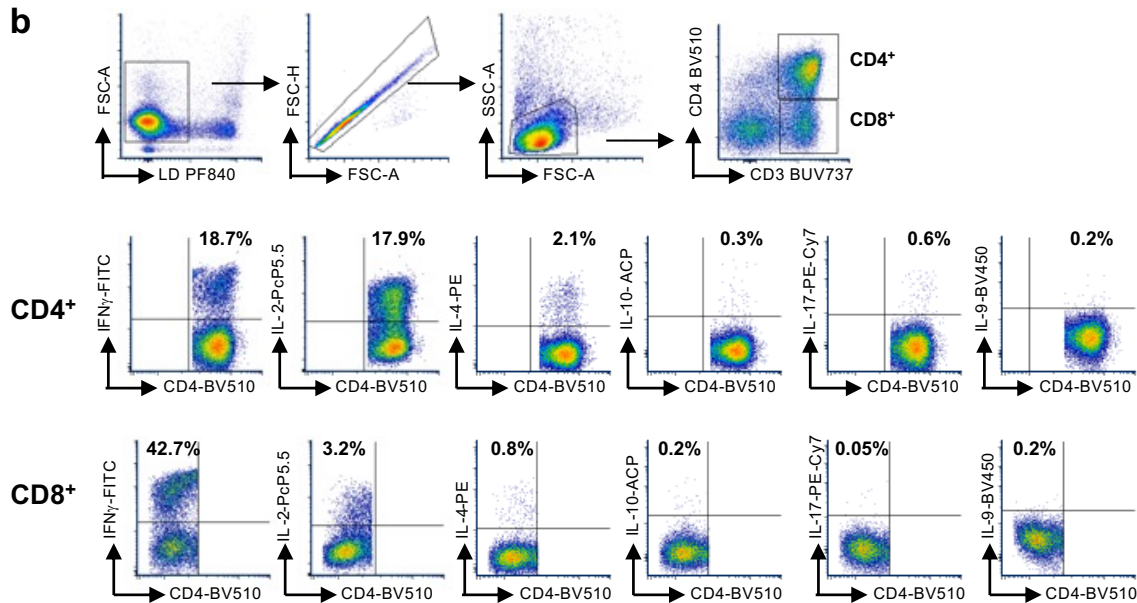


Supplementary Figure 7. Gating strategies to evaluate the expression of activation and inhibitory markers on circulating CD4⁺ and CD8⁺ T cells. CD4⁺ and CD8⁺ T cells were identified in peripheral blood mononuclear cells by multiparametric flow cytometry (upper panels). The expression of the indicated activation and inhibitory markers was evaluated on CD4⁺ (middle panels) and CD8⁺ (lower panels) T cells shown in Figure 3a. Numbers indicate the percentage of positive cells relative to the indicated parental gate.

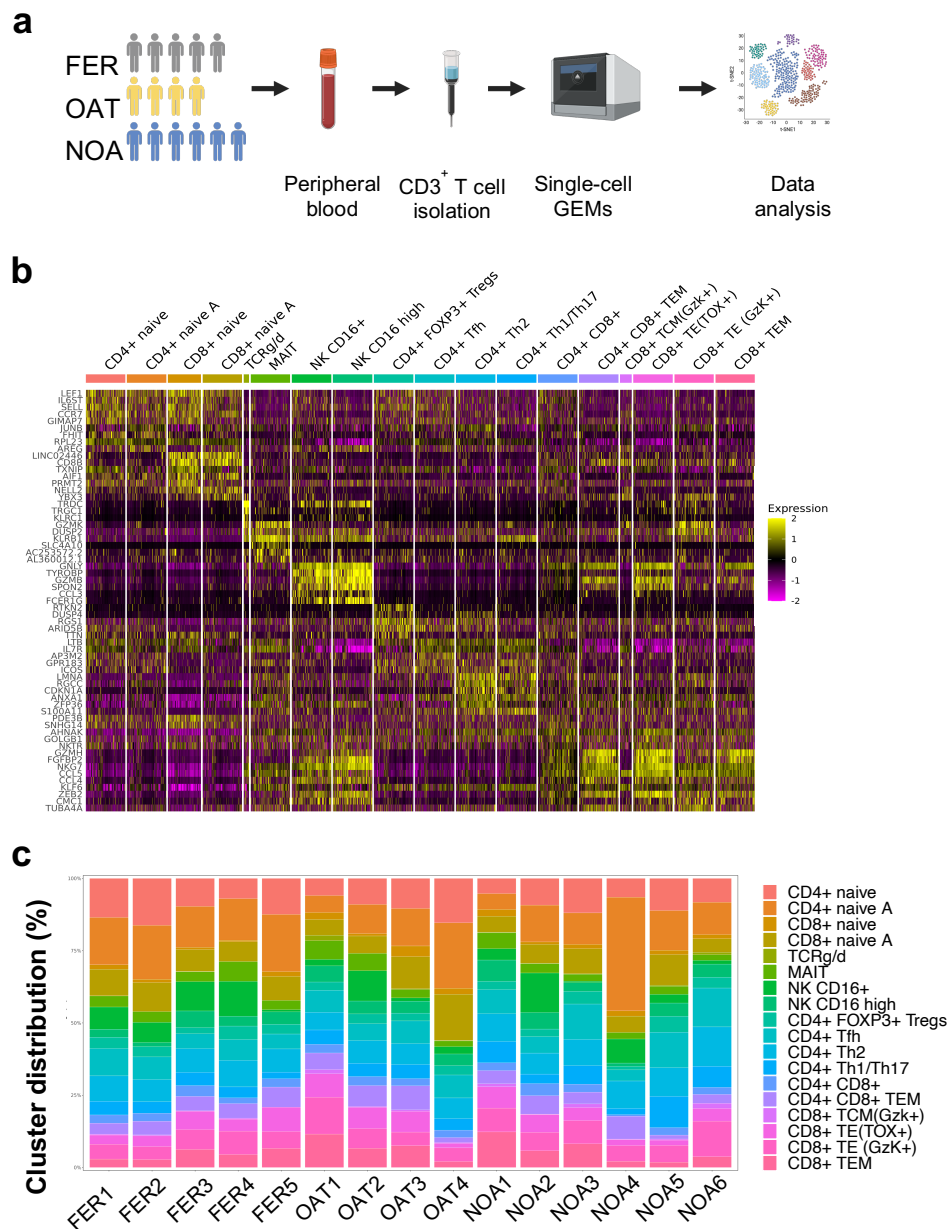
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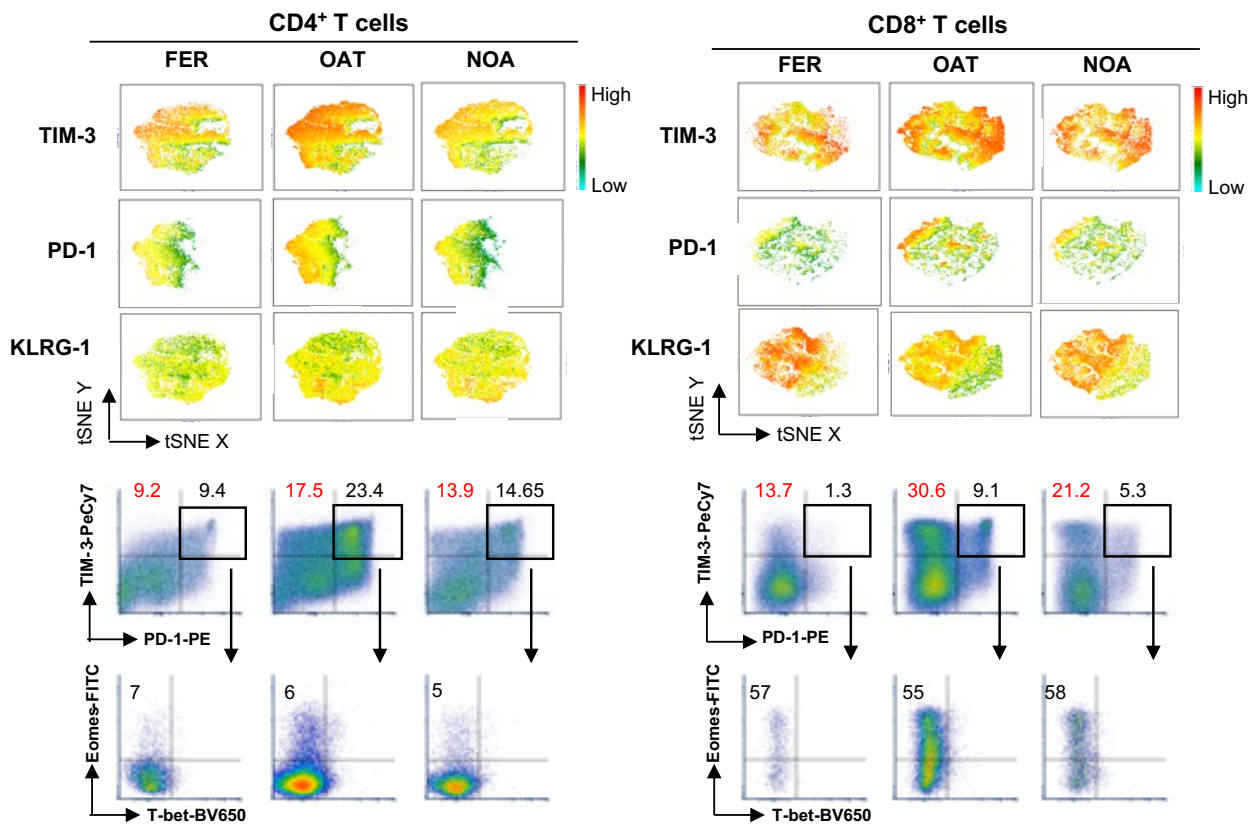
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Supplementary Figure 8. Gating strategies to evaluate the functional characteristics of peripheral T cells. **a.** PBMC were labelled with proliferation dye and left unstimulated or stimulated with anti-CD3 mAb or phytohaemagglutinin (PHA) for four days. CD4⁺ and CD8⁺ T cells were identified (upper panels) and the percentage of proliferated cells was evaluated as the percentage of cells that diluted the proliferation dye (lower panels). The proliferation index (PI), shown in Figure 3b, is the ratio between the percentage of proliferated CD4⁺ or CD8⁺ T cells upon anti-CD3 or PHA stimulation/percentage of proliferated CD4⁺ or CD8⁺ T cells in the unstimulated condition. **b.** PBMC were activated with phorbol 12-myristate 13-acetate/Ionomycin (PMA/IONO) for 6 hours. CD4⁺ and CD8⁺ T cells were identified (upper panels) and the percentage of CD4⁺ (middle panels) and CD8⁺ T (lower panels) cells producing the indicated cytokines, shown in Figure 3c and Figure 4a, was evaluated by multiparametric flow cytometry. Numbers indicate the percentage of positive cells relative to the indicated parental gate.



Supplementary Figure 9. Transcriptional profiling of T cells from infertile and fertile individuals. a. Experimental workflow for single-cell RNA-sequencing (scRNA-seq) analysis of T cells. scRNA-seq was applied to CD3⁺ T cells negatively isolated from the peripheral blood of fertile (FER, n=5), Oligo-Asthenoterato spermia (OAT, n=4), and non-obstructive azoospermia (NOA, n=6) men; cells were singularly encapsulated in Gel Beads in emulsion (GEMs) containing barcoded oligonucleotides. After GEMs disruption, an approximative target of 8000 cells for each sample was sent to deep RNA sequencing and the output data were used for expression analyses. Created in BioRender. Santoni de sio, F. (2025) <https://BioRender.com/j39n392>. **b.** The heatmap shows scaled gene expression of the top 5 genes across all the identified clusters. The heatmap color corresponds to expression levels, being the spectrum of purple to black to yellow, which corresponds to increasing values. **c.** The bar plot shows the distribution of each T-cell cluster at the single sample level.



Supplementary Figure 10. Co-expression on exhaustion markers on T cells from infertile and fertile individuals. The co-expression of exhaustion markers was evaluated by t-distributed stochastic neighbor embedding (tSNE) analysis performed on multiparametric flow cytometry data from fertile (FER, n=7), Oligo-Astheno-Terato spermia (OAT, n=21), and non-obstructive azoospermia (NOA, n=9) men. Dot plots of tSNE X and tSNE Y axes vs. the indicated antigens in CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells from the indicated cohort of donors; colour is based on the hyperlog transformed expression of the indicated markers, with the spectrum of light blue, blue to green to yellow to red corresponding to increasing levels of expression (upper panels). Dot plots obtained from tSNE analysis showing the co-expression of TIM-3 and PD-1, and of T-bet and Eomes in CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells from the indicated cohort of donors. Numbers indicate the percentage of positive cells in the relative quadrant (lower panels).

Supplementary Table 1. Descriptive statistics of fertile men with normal vs. abnormal semen analyses.

	nFER (n=12)	aFER (n=21)
Semen parameters		
Concentration (10 ⁶ /ml)	73.2±14.9	37.3±7.4**
Progressive motility (%)	46.4±3.4	39.0±2.7
Normal morphology (%)	4.8±0.5	2.2±0.2****
Clinical parameters		
Age, years	37.3±2.3	35.6±1.4
BMI (kg/m ²)	26.3±0.7	25.0±0.6
Waist circumference (cm)	93.4±2.1	88.4±2.3*
Regular physical activity (%)	16.6	57.1*
Active smokers (%)	66.7	33.3
CCI=0 (%)	91.7	90.5
CCI=1 (%)	8.3	9.5
CCI>1 (%)	0	0
HBP (%)	8.3	4.8
Comorbidities (n, type)	0	2 Hypothyroidism
Average testicular volume (ml)	19.9±0.9	18.9±1.1
PSA (ng/ml)	0.62±0.1	0.8±0.1
HbA1c (%)	33.4±1.0	32.6±0.5
Hormonal parameters		
tT (ng/ml)	3.8±0.3	4.6±0.3
SHBG (nmol/L)	26.3±3.7	33.0±2.3
E2 (pg/ml)	20.4±2.6	19.4±2.1
FSH (UI/ml)	3.2±0.7	4.5±0.5
LH (UI/ml)	3.8±0.4	5.0±0.3*
TSH (mU/ml)	2.0±0.2	2.9±0.6
AMH (ng/ml)	7.7±1.8	5.3±0.5
Prolactin (micro IU/L)	12.6±2.5	18.0±1.7*

Data are presented as Mean±SEM. For each variable, a two-sided Mann-Whitney test was used to test the difference between groups. * $P \leq 0.05$, ** $P \leq 0.01$, and **** $P \leq 0.0001$. nFER, fertile men without spermiogram alterations; aFER, fertile men with one/two spermiogram alterations. BMI, Body Mass Index; CCI, Charlson comorbidity index; HBP, high blood pressure; PSA, Prostate-Specific Antigen; HbA1c, glycosylated hemoglobin; tT, total testosterone; SHBG, sex hormone binding globulin; E2, 17 β -estradiol; FSH, follicle stimulating hormone; LH, luteinizing hormone; TSH, thyroid stimulating hormone; AMH, Anti-Müllerian Hormone.

Supplementary Table 2. Cluster composition for each group condition.

Number of cells (%)	FER	OAT	NOA	Total cell count
CD4 ⁺ naive	998 (13)	2062 (10.12)	1578 (8.98)	4638 (10.17)
CD4 ⁺ naive A	1292 (16.83)	2579 (12.66)	1994 (11.35)	5865 (12.86)
CD8 ⁺ naive	94 (1.22)	445 (2.18)	300 (1.71)	839 (1.84)
CD8 ⁺ naive A	682 (8.88)	1911 (9.38)	1313 (7.47)	3906 (8.56)
Naive cell count (%)	3066 (39.93)	6997 (34.34)	5185 (31.89)	15248 (33.43)
TCR γ/δ	2 (0.03)	96 (0.47)	40 (0.23)	138 (0.30)
MAIT	298 (3.88)	898 (4.41)	617 (3.51)	1813 (3.97)
NK CD16 ⁺	574 (7.48)	938 (4.6)	673 (3.83)	2185 (4.79)
NK CD16 ^{high}	242 (3.15)	919 (4.51)	859 (4.89)	2020 (4.43)
Cell count (%)	1116 (14.54)	2851 (13.99)	2158 (12.49)	6156 (13.49)
CD4 ⁺ FOXP3 ⁺ Tregs	268 (3.49)	624 (3.06)	654 (3.72)	1546 (3.39)
CD4 ⁺ Tfh	587 (7.65)	1464 (7.18)	1743 (9.92)	3794 (8.32)
CD4 ⁺ Th2	639 (8.32)	1469 (7.21)	1649 (9.39)	3757 (8.24)
CD4 ⁺ Th1/Th17	319 (4.16)	936 (4.59)	1214 (6.91)	2469 (5.41)
CD4 ⁺ CD8 ⁺	224 (2.92)	570 (2.8)	478 (2.72)	1272 (2.79)
CD4 ⁺ CD8 ⁺ TEM	360 (4.69)	1249 (6.13)	735 (4.18)	2344 (5.14)
CD8 ⁺ TCM(Gzk ⁺)	20 (0.26)	117 (0.57)	158 (0.90)	295 (0.65)
CD8 ⁺ TE(TOX ⁺)	345 (4.49)	1274 (6.25)	878 (5.0)	2497 (5.47)
CD8 ⁺ TE (Gzk ⁺)	427 (5.56)	1417 (6.95)	1330 (7.57)	3174 (6.96)
CD8 ⁺ TEM	305 (3.97)	1409 (6.91)	1356 (7.72)	3070 (6.73)
Memory cell count (%)	3226 (42.42)	9905 (48.59)	9541 (54.31)	22672 (49.71)
Total cell count (%)	7676 (100)	20377 (100)	17569 (100)	45622 (100)

Supplementary Table 3. Summary of the activation, exhaustion, and SASP scores.

Number of cells (%)	FER	OAT	NOA
Total cells	3226	9905	9541
Activation score >0.25	83 (2.57 %)	514 (5.19 %)	217 (2.27 %)
Exhaustion score >0.25	35 (1.08 %)	522 (5.27 %)	205 (2.14 %)
SASP score >0.25	495 (15.34 %)	1221 (12.32%)	1712 (17.95%)

Supplementary Table 4. List of antibodies used for flow cytometry

Antibody and clone	Fluorophore	Supplier	Cat. Number	Dilutions
CD1c (AD5-8E7)	PE	Miltenyi Biotec	130-113-864	1:100
CD3 (HIT3a)	PerCP/Cy5.5	BioLegend	300328	1:100
CD3 (SK7)	BUV737	BD Biosciences	612752	1:100
CD4 (SK3)	FITC	BD Biosciences	566320	1:100
CD4 (SK3)	PB	BioLegend	344620	1:100
CD4 (RPA-T4)	BV605	BD Horizon	562658	1:100
CD8 (SK1)	APC-H7	BD Biosciences	641400	1:100
CD11c (B-ly6)	BV650	BD Biosciences	563404	1:100
CD14 (M ϕ P9)	APC	BD Biosciences	345787	1:100
CD14 (M ϕ P9)	APC-H7	BD Biosciences	560180	1:100
CD15 (MMA)	FITC	BD Biosciences	332778	1:100
CD16 (3G8)	BV510	BD Biosciences	563830	1:100
CD19 (SJ25C1)	PE	BD Biosciences	345789	1:100
CD25 (M-A251)	BV421	BD Horizon	562442	1:25
CD45 (HI30)	BUV395	BD Biosciences	563792	1:200
CD49b (REA188)	APC	Miltenyi Biotec	130-100-348	1:50
CD56 (NCAM)	PE-Cy7	BioLegend	362510	1:100
CD83 (HB15e)	BUV737	BD Biosciences	612823	1:100
CD127 (A019D5)	PerCP/Cy5.5	BioLegend	351322	1:100
CD141 (1A4)	BV421	BD Biosciences	565321	1:200
CD163 (GH1/61)	PerCP/Cy5.5	BD Biosciences	563887	1:100
HLA-DR (G46-6)	BV510	BD Biosciences	563083	1:100
HLA-G (87G)	PE	EXBIO Praha	1P-437	1:100
KLRG1 (MAFA)	BV510	BioLegend	138421	1:50
LAG-3 (REA351)	PE	Miltenyi Biotec	130-120-470	1:200
LAG-3 (Polyclonal)	PE	R&D systems	FAB2319P	1:50
PD-1 (MIH4)	PE	BD Biosciences	557946	1:50
TIM-3 (F38-2E2)	PE-Cy7	BioLegend	345014	1:100
FOXP3 (259D)	AF488	BioLegend	320212	1:25
Eomes (WD1928)	FITC	Thermo Fisher Scientific	11-4877-42	1:25
T-bet (O4-46)	BV650	BD Biosciences	564142	1:50
IL-2 (MQ1-17H12)	PerCP-eFluor710	eBioscience	46-7029-42	1:100
IL-4 (MP4-25D2)	PE	BD Biosciences	554485	1:200
IL-9 (MH9D1)	BV421	eBioscience	554485	1:25
IL-10 (JES3-19F1)	APC	BD Biosciences	554707	1:50
IL-17 (eBio64DEC17)	PE-Cy7	eBioscience	25-7179-42	1:50
IFN γ (B27)	FITC	BD Biosciences	554700	1:100
FcR Blocking Agent		Miltenyi Biotec	130-059-901	1:50
PromoFluor-840 maleimide		PromoCell Inc.	PK-PF840-3-01	0.25 μ g/ μ l

Supplementary Table 5. List of commercial reagents used

Reagent	Supplier	Cat. number
Cell Proliferation Dye eFluor® 670	eBioscience	65-0840-90
Brilliant stain buffer	BD biosciences	566349
Foxp3/Transcription Factor Staining Buffer Set	eBioscience	00-5523-00
8-peak rainbow calibration particles	Spherotech	RCP-305-A
Paraformaldehyde Solution	Thermo Fisher Scientific	J19943.K2
Ionomycin calcium salt from <i>Streptomyces conglobatus</i>	Sigma-Aldrich	I0634-1MG
Phorbol-12-myristate-13-acetate (PMA)	Sigma-Aldrich	P8139
MACS® GMP CD3 pure (aCD3)	Miltenyi Biotec	170-076-116
Phytohemagglutinin (PHA)	Sigma-Aldrich	L2769-5MG
Brefeldin A	Sigma-Aldrich	B7651
Saponin from quillaja bark	Sigma-Aldrich	S7900
X-VIVO 15	Lonza	LOBE04418Q
Lympholyte-H	Cederlane	CL5020-R
Recombinant human IFN- γ	R&D Systems	285-IF100
Recombinant human IL-10	CellGeniX	1014-050
Recombinant human GM-CSF	Milteny Biotec	130-093-868
Purified Mouse Anti-Human IFN- γ	BD Pharmingen	551221
Biotin Mouse Anti-Human IFN- γ	BD Pharmingen	554550
Purified Rat Anti-Human and Viral IL-10	BD Pharmingen	554497
Biotin Anti-Human and Viral IL-10	BD Pharmingen	554499
Purified Rat Anti-Human GM-CSF	BD Pharmingen	554502
Biotin Rat Anti-Human GM-CSF	BD Pharmingen	554505
Chromium™ Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1,	10X Genomics	10X1000121
Chromium™ Next GEM Chip G Single Cell kit	10X Genomics	10X1000127
Single Index Kit T Set A	10X Genomics	10X1000213
SPRIselect	Beckman Coulter	B23317
Dynabeads™ MyOne™ Silane	Thermo Fisher Scientific	37002D
Qubit dsDNA HS assay kit	Thermo Fisher Scientific	Q32851
High Sensitivity D1000 Reagents	Agilent Technologies	5067-5585
Bio-Plex Pro™ Human Cytokine 27-plex Assay	Bio-Rad	M500KCAF0Y