Deep phenotyping of nodal T-cell lymphomas reveals immune alterations and therapeutic targets

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Supplementary material

- 8 supplementary Figures
- Supplementary figure legends
- 8 supplementary tables
- Supplementary methods

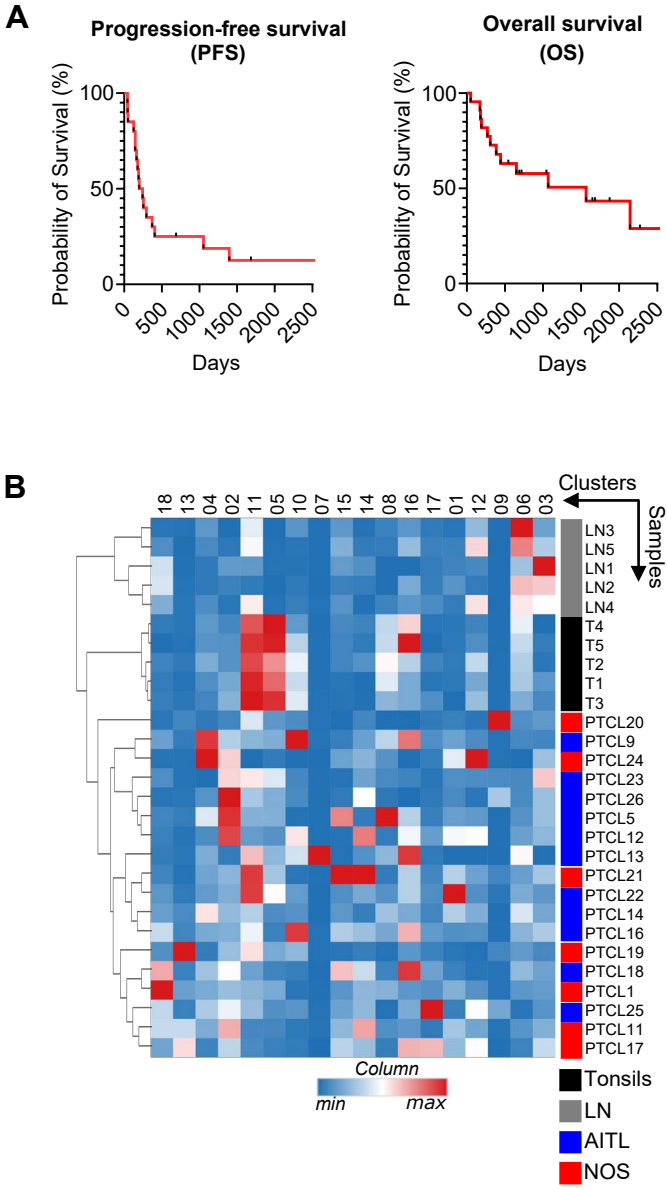
A
Progression-free survival
(PFS)
Probability of Survival (%)
100
50
0
0
500
1000
1500
2000
2500
Days
Overall survival
(OS)
Probability of Survival (%)
100
50
0
0
500
1000
1500
2000
2500
Days
B
Clusters
Samples
18
13
04
02
11
05
10
07
15
14
08
16
17
01
12
09
06
03
LN3
LN5
LN1
LN2
LN4
T4
T5
T2
T1
T3
PTCL20
PTCL9
PTCL24
PTCL23
PTCL26
PTCL5
PTCL12
PTCL13
PTCL21
PTCL22
PTCL14
PTCL16
PTCL19
PTCL18
PTCL1
PTCL25
PTCL11
PTCL17
Column
min
max
Tonsils
LN
AITL
NOS

Figure S1

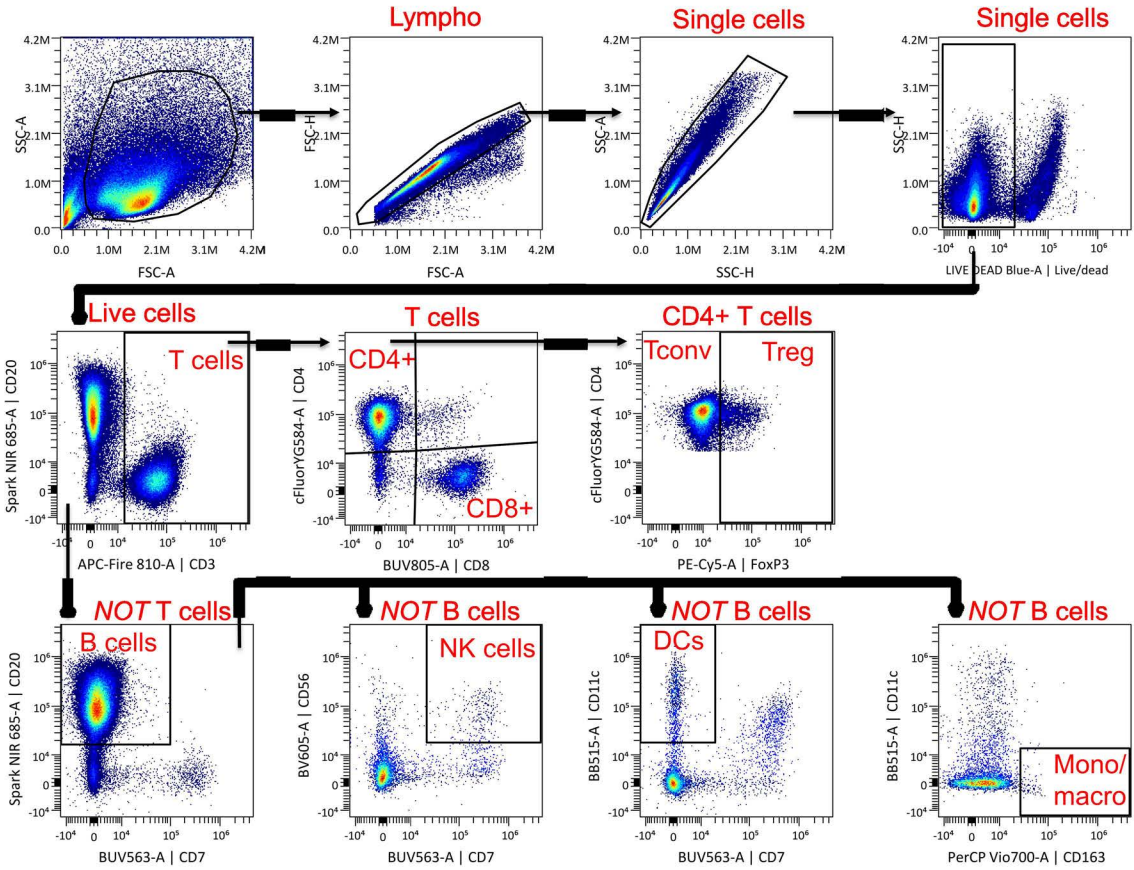
Lympho
Single cells
Single cells
SSC-A
FSC-A
FSC-H
FSC-A
SSC-A
SSC-H
SSC-H
LIVE DEAD Blue-A | Live/dead
Live cells
T cells
T cells
CD4+
CD8+
CD4+ T cells
Tconv
Treg
Spark NIR 685-A | CD20
APC-Fire 810-A | CD3
cFluorYG584-A | CD4
BUV805-A | CD8
cFluorYG584-A | CD4
PE-Cy5-A | FoxP3
NOT T cells
B cells
NOT B cells
NK cells
NOT B cells
DCs
NOT B cells
Mono/
macro
Spark NIR 685-A | CD20
BUV563-A | CD7
BV605-A | CD56
BUV563-A | CD7
BB515-A | CD11c
BUV563-A | CD7
BB515-A | CD11c
PerCP Vio700-A | CD163

Figure S2

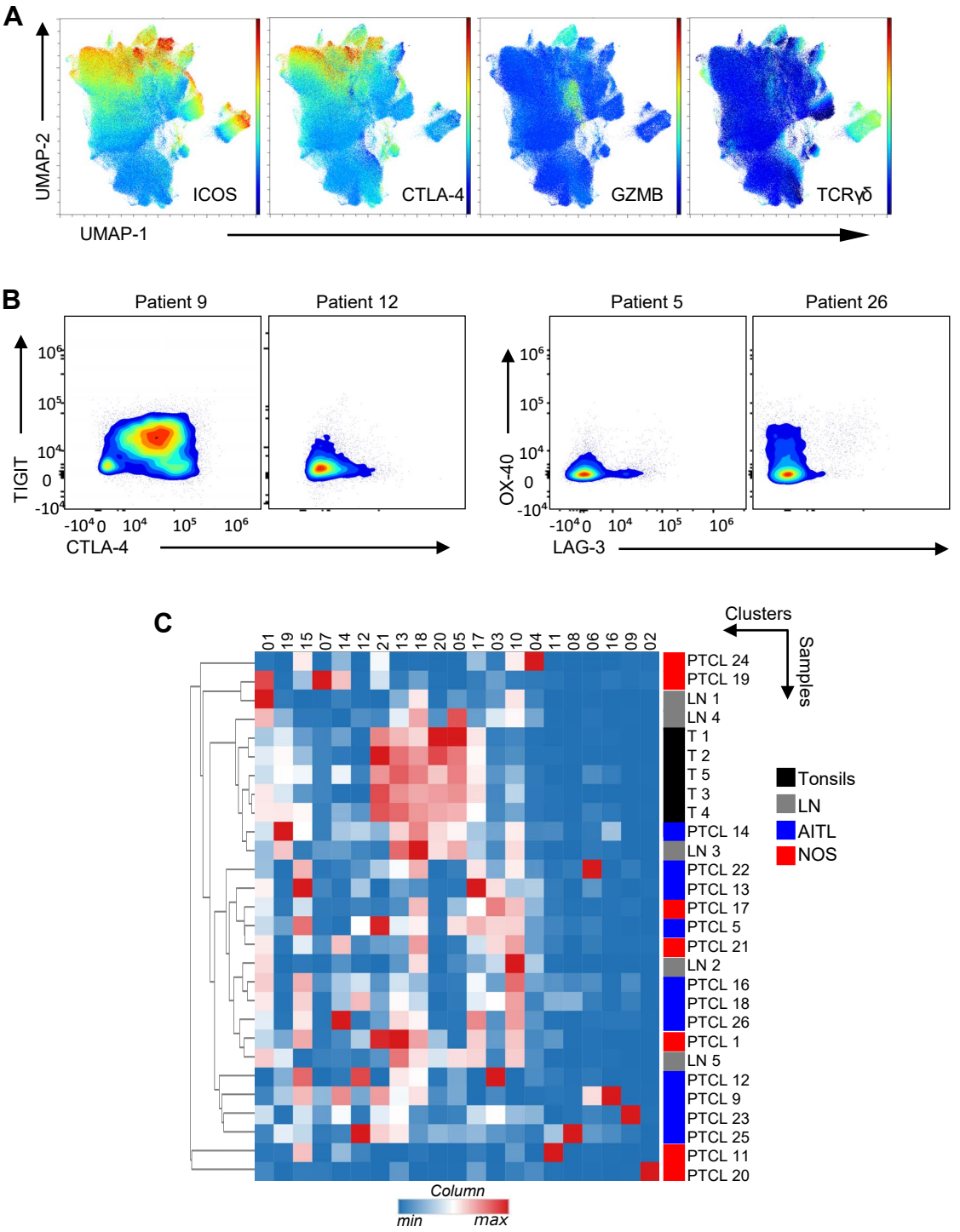
A
UMAP-2
UMAP-1
ICOS
CTLA-4
GZMB
TCRγδ
B
Patient 9
Patient 12
Patient 5
Patient 26
TIGIT
CTLA-4
OX-40
LAG-3
C
Clusters
Samples
01 19 15 07 14 12 21 13 18 20 05 17 03 10 04 11 08 06 16 09 02
PTCL 24
PTCL 19
LN 1
LN 4
T 1
T 2
T 5
T 3
T 4
PTCL 14
LN 3
PTCL 22
PTCL 13
PTCL 17
PTCL 5
PTCL 21
LN 2
PTCL 16
PTCL 18
PTCL 26
PTCL 1
LN 5
PTCL 12
PTCL 9
PTCL 23
PTCL 25
PTCL 11
PTCL 20
Tonsils
LN
AITL
NOS
Column
min
max

Figure S3

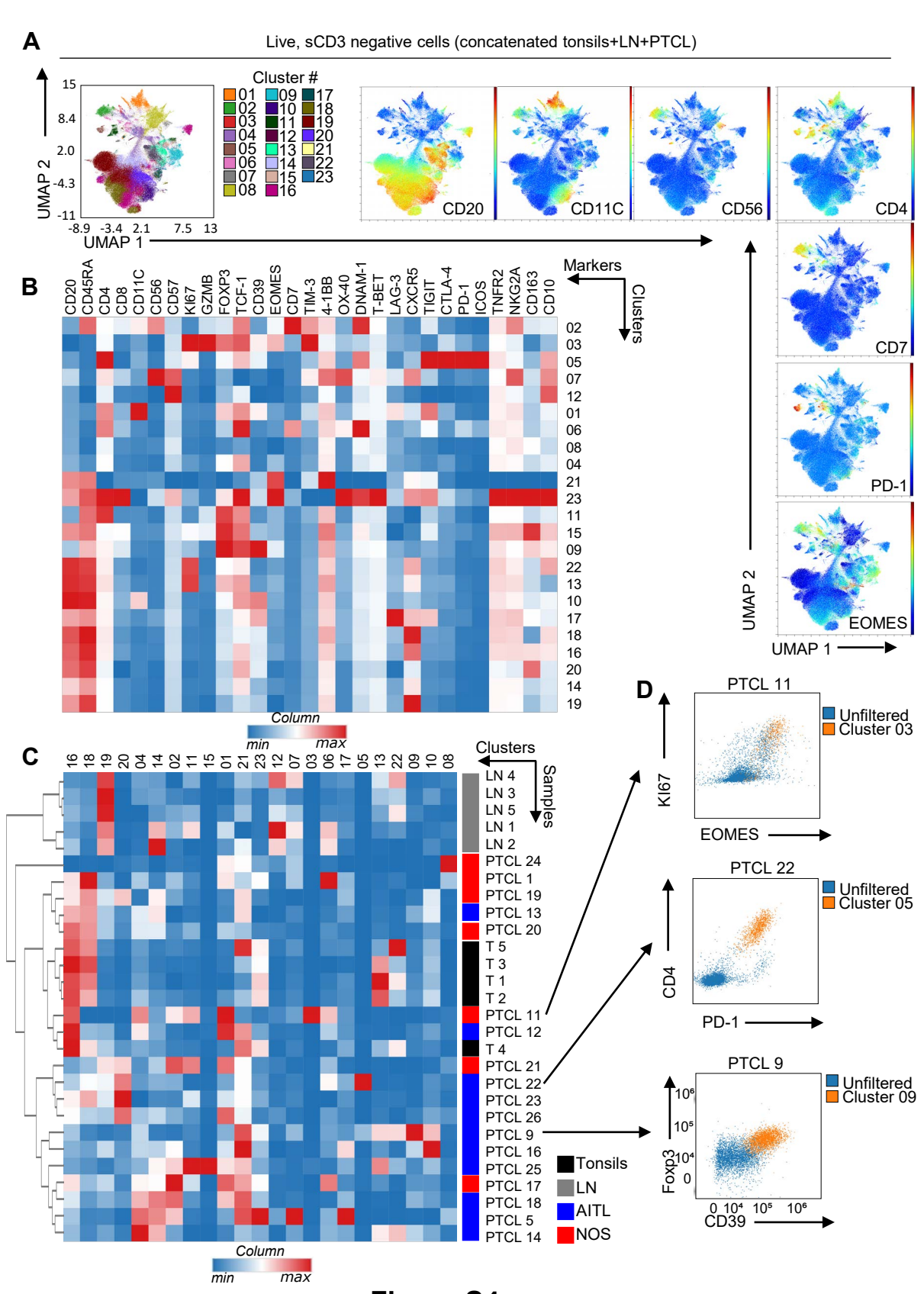
A
Live, sCD3 negative cells (concatenated tonsils+LN+PTCL)
Cluster #
UMAP 1
UMAP 2
CD20
CD11C
CD56
CD4
CD7
PD-1
EOMES
B
Markers
Clusters
C
Clusters
Samples
Tonsils
LN
AITL
NOS
Column
min
max
D
PTCL 11
Unfiltered
Cluster 03
KI67
EOMES
PTCL 22
Unfiltered
Cluster 05
CD4
PD-1
PTCL 9
Unfiltered
Cluster 09
Foxp3
CD39

Figure S4

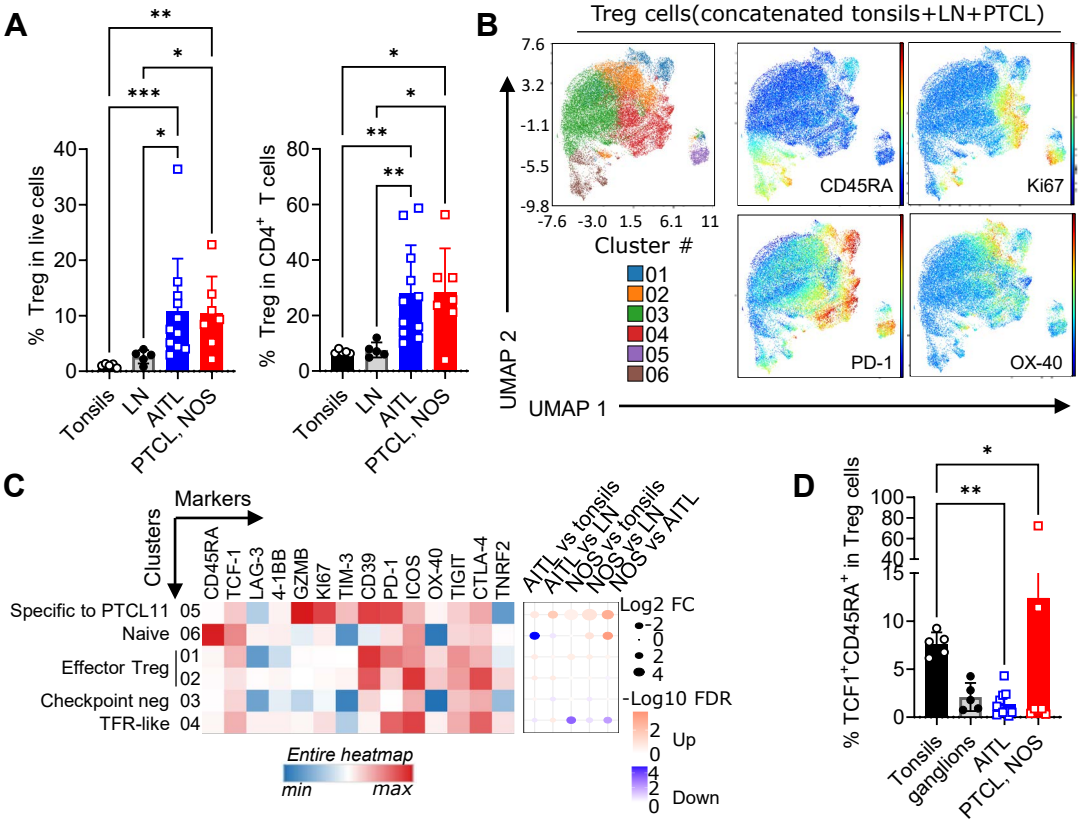
A
% Treg in live cells
% Treg in CD4+ T cells
Tonsils
LN
AITL
PTCL, NOS
B
Treg cells(concatenated tonsils+LN+PTCL)
UMAP 2
UMAP 1
Cluster #
01
02
03
04
05
06
CD45RA
Ki67
PD-1
OX-40
C
Markers
Clusters
CD45RA
TCF-1
LAG-3
4-1BB
GZMB
KI67
TIM-3
CD39
PD-1
ICOS
OX-40
TIGIT
CTLA-4
TNRF2
Specific to PTCL11 05
Naive 06
Effector Treg 01
02
Checkpoint neg 03
TFR-like 04
AITL vs tonsils
AITL vs LN
NOS vs tonsils
NOS vs LN
NOS vs AITL
Log2 FC
-Log10 FDR
Up
Down
Entire heatmap
min
max
D
% TCF1+CD45RA+ in Treg cells
Tonsils
ganglions
AITL
PTCL, NOS

Figure S5

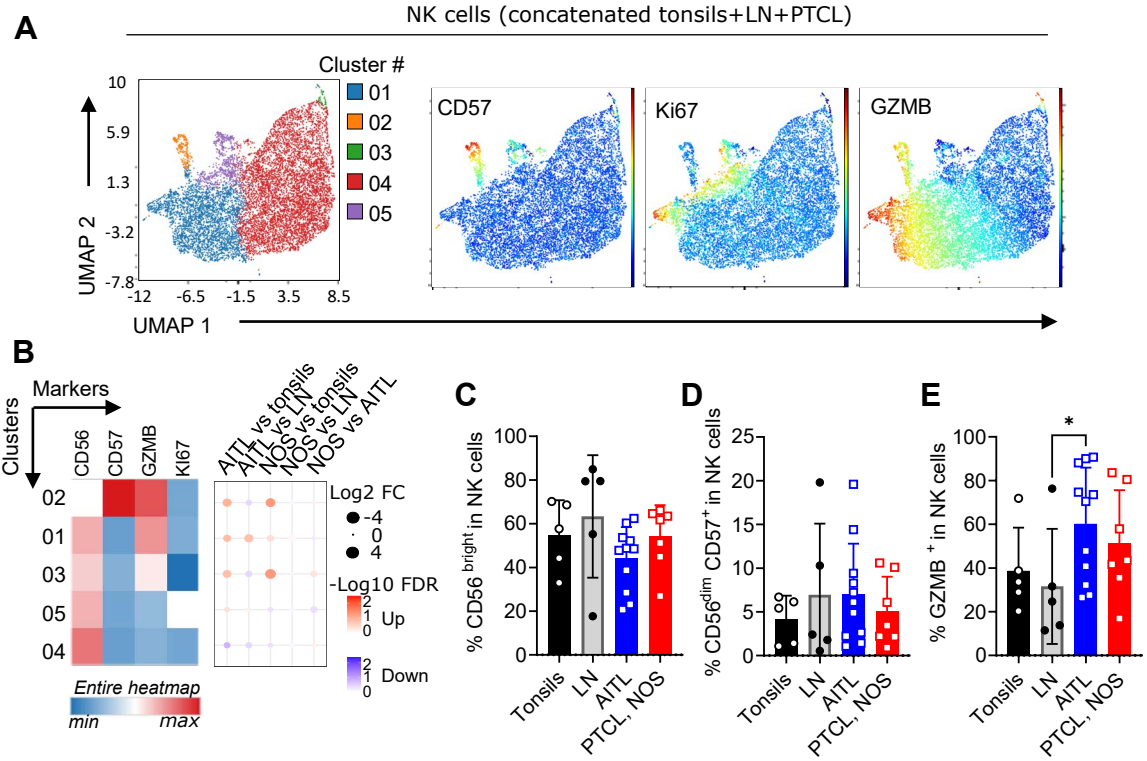
A
NK cells (concatenated tonsils+LN+PTCL)
Cluster #
01
02
03
04
05
UMAP 2
UMAP 1
CD57
Ki67
GZMB
B
Markers
Clusters
CD56
CD57
GZMB
KI67
AITL vs tonsils
AITL vs LN
NOS vs tonsils
NOS vs LN
NOS vs AITL
Log2 FC
-Log10 FDR
Up
Down
Entire heatmap
min
max
C
% CD56bright in NK cells
D
% CD56dim CD57+ in NK cells
E
% GZMB + in NK cells
Tonsils
LN
AITL
PTCL, NOS

Figure S6

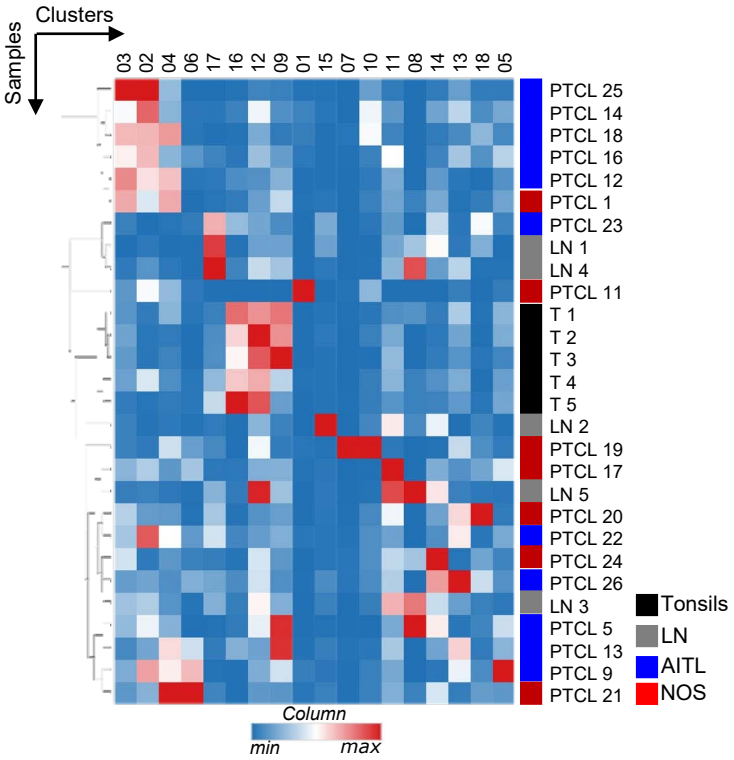
Clusters
Samples
03
02
04
06
17
16
12
09
01
15
07
10
11
08
14
13
18
05
PTCL 25
PTCL 14
PTCL 18
PTCL 16
PTCL 12
PTCL 1
PTCL 23
LN 1
LN 4
PTCL 11
T 1
T 2
T 3
T 4
T 5
LN 2
PTCL 19
PTCL 17
LN 5
PTCL 20
PTCL 22
PTCL 24
PTCL 26
LN 3
PTCL 5
PTCL 13
PTCL 9
PTCL 21
Tonsils
LN
AITL
NOS
Column
min
max

Figure S7

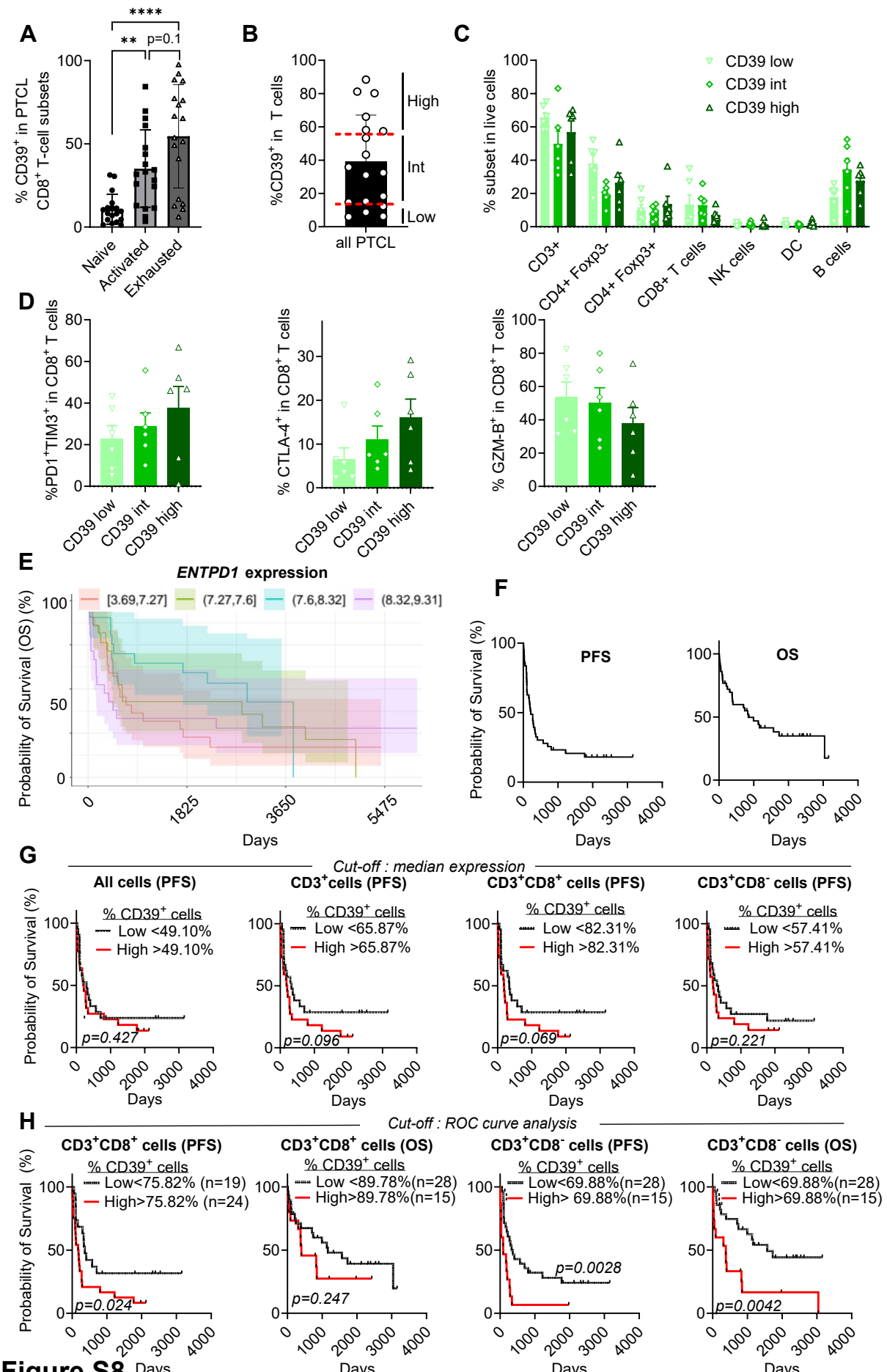
A
****
** p=0.1
% CD39+ in PTCL CD8+ T-cell subsets
Naive
Activated
Exhausted
B
%CD39+ in T cells
High
Int
Low
all PTCL
C
CD39 low
CD39 int
CD39 high
% subset in live cells
CD3+
CD4+ Foxp3-
CD4+ Foxp3+
CD8+ T cells
NK cells
DC
B cells
D
%PD1+TIM3+ in CD8+ T cells
% CTLA-4+ in CD8+ T cells
% GZM-B+ in CD8+ T cells
CD39 low
CD39 int
CD39 high
E
ENTPD1 expression
[3.69,7.27]
(7.27,7.6]
(7.6,8.32]
(8.32,9.31]
Probability of Survival (OS) (%)
Days
F
PFS
OS
Probability of Survival (%)
Days
G
Cut-off : median expression
All cells (PFS)
CD3+cells (PFS)
CD3+CD8+ cells (PFS)
CD3+CD8- cells (PFS)
% CD39+ cells
Low <49.10%
High >49.10%
p=0.427
Low <65.87%
High >65.87%
p=0.096
Low <82.31%
High >82.31%
p=0.069
Low <57.41%
High >57.41%
p=0.221
Probability of Survival (%)
Days
H
Cut-off : ROC curve analysis
CD3+CD8+ cells (PFS)
CD3+CD8+ cells (OS)
CD3+CD8- cells (PFS)
CD3+CD8- cells (OS)
% CD39+ cells
Low<75.82% (n=19)
High>75.82% (n=24)
p=0.024
Low <89.78%(n=28)
High>89.78%(n=15)
p=0.247
Low<69.88%(n=28)
High> 69.88%(n=15)
p=0.0028
Low<69.88%(n=28)
High>69.88%(n=15)
p=0.0042
Probability of Survival (%)
Days

Figure S8

Supplementary figure legends

Figure S1. Spectral FACS analyses of PTCL samples (A) Kaplan-Meier curves of PFS and OS in the 18-patient flow cytometry cohort. (B) Samples were stained for FACS and analyzed using unsupervised clustering after manual gating on total live cells. The heatmap shows unsupervised hierarchical distribution of clusters between samples (normalized by column).

Figure S2. Manual gating strategy. An example of gating strategy in an AITL sample is shown. This strategy was used for both supervised and unsupervised analyses.

Figure S3. Perturbations of Tconv cell phenotype in PTCL patients. FACS analyses following manual gating on live $CD3^+CD4^+Foxp3^-$ Tconv cells. (A) Expression of selected markers projected on UMAP. (B) Dot plots showing heterogeneous expression of checkpoint molecules in selected patients, following manual gating on live Tconv cells. (C) Heatmap showing unsupervised hierarchical separation of clusters between samples (normalized by column).

Figure S4. Analysis of surface CD3-negative live cells. Unsupervised clustering analyses following manual gating on live $CD3^-$ cells in tonsils, reactive LN and AITL samples, and concatenation using the same number of cells in each sample. (A) FlowSOM distribution of clusters and expression of selected markers projected on UMAP. (B) Heatmap showing hierarchical clustering and expression (normalized by column) of indicated markers in FlowSOM clusters. (C) Heatmap showing unsupervised hierarchical separation of clusters between samples (normalized by column). (D) Dot plots showing expression of the indicated markers in selected patients.

Figure S5. Analysis of Treg cells. (A) Proportion of $Foxp3^+$ regulatory T cells (Treg cells) among live cells (left) and among $CD4^+$ T cells (right) following manual gating. (B) UMAP visualization, FlowSOM distribution of clusters and projection of selected markers in concatenated samples. (C) Heatmap showing hierarchical clustering and expression (normalized across markers) of indicated markers in FlowSOM clusters, and their differential

enrichment between groups. (D) Proportion of CD45RA$^+$TCF1$^+$ naïve Treg cells among total Treg cells. In A, mean +/- SEM is shown; each dot represents a sample. Kruskal-Wallis tests were used. * $p < 0.05$, ** $p < 0.005$.

Figure S6. Analysis of NK cells. (A) UMAP visualization, FlowSOM distribution of clusters and projection of selected markers in concatenated samples upon gating on live CD7$^+$CD56$^+$ Natural Killer (NK) cells. (B) Heatmap showing hierarchical clustering and expression (normalized across markers) of indicated markers in FlowSOM clusters, and their differential enrichment between groups. No statistical difference in cluster representation between samples was found. (C-E) Proportions of CD56^{bright} (B), CD56^{dim} (C) and GzmB$^+$ (E) NK cells upon manual gating. Kruskal-Wallis tests were used. * $p < 0.05$.

Figure S7. CD8$^+$ T cells in PTCL. FACS analyses following manual gating on live CD8$^+$ T cells and random selection of an equal number of cells between each group. The heatmap shows unsupervised hierarchical separation of clusters between samples (normalized by column).

Figure S8. CD39 expression and prognosis value in PTCL. (A) Proportion of CD39$^+$ cells among naïve (CD45RA$^+$), activated (CD45RA$^-$PD-1$^-$TIM3$^-$) and exhausted (PD-1$^+$TIM3$^+$) CD8$^+$ T cells in the 18 PTCL samples. (B) Proportion of CD39$^+$ cells among live T cells. Dashed lines denote the 3 groups of patients according to their level of CD39 expression (low, intermediate, high). (C) Distribution of immune cell subsets in the 3 groups of patients. (D) Proportion of PD-1$^+$TIM3$^+$ exhausted CD8$^+$ T cells, CTLA-4$^+$ CD8$^+$ T cells and GzmB$^+$ CD8$^+$ T cells. (E) Kaplan-Meier curves of OS in patients from the TENOMIC AITL cohort (n=85). *ENTPD1* (CD39) mRNA expression was split in quartiles. (F) Kaplan-Meier curves of PFS and OS in the overall 43-AITL patient cohort used for multi-IF analyses. (G) Kaplan-Meier curves of PFS in different cell populations. CD39 high and low populations were split based on median expression across samples. (H) Kaplan-Meier curves of PFS and OS in CD3$^+$CD8$^+$ and CD3$^+$CD8$^-$ populations. CD39 *high* and *low* samples were split following ROC curve analyses.

	N patients with available data	n	%
Age median [range] Age <60y Age ≥60y Age ≥70y	18 67 [39-82]	 6 5 7	 33 28 39
Sex Male Female	18	 8 10	 44 56
Type of PTCL AITL PTCL-NOS	18	 11 7	 61 39
Ann Arbor stage I II III IV	17	 0 0 7 10	 0 0 41 59
ECOG performance status 0 1 2 3 4	15	 6 3 2 1 3	 40 20 13 7 20
B symptoms No Yes	16	 4 12	 25 75
Extranodal sites 0 1 ≥2	15	 6 3 6	 40 20 40
Bone marrow involvement No Yes	11	 9 2	 82 8
LDH ≤ Upper limit of normal > Upper limit of normal	14	 3 11	 21 79
Hemoglobin ≥ 12g/dL <12g/dL	13	 7 6	 54 46
IPI 0 1 2 3 4 5 IPI (simplified) <3 ≥3	15 17	 0 1 0 10 3 1 1 16	 0 7 0 67 20 7 6 94

PIT	15		
0		1	7
1		2	13
2		8	53
3		4	27
4		0	0
PIT (simplified)	16		
<2		3	19
≥2		13	81
PIAI (simplified)			
<2	16	0	0
≥2		16	100

Supplementary Table 1. Clinical characteristics of the PTCL cohort used in spectral flow cytometry analyses

Sample ID	Sex	Age (years)	Diagnosis	Clinical phenotying data	Institutional source	Year of collection	Storage time (month)	Tissue Origin
PTCL 1	F	67	NOS	Missing Data	Lyon-Sud hospital	1996	300	LN
PTCL 5	M	67	AITL	CD2+ CD3+ CD7+ CD4+ CD8- CD10- BCL6low PDL1low CXCL13-	Lyon-Sud hospital	2019	19	LN
PTCL 9	F	62	AITL	CD2+ CD3+CD5+ CD4+ CD8- CD7+ PD-1+ ICOS+ CXCL13+ CD10- BCL6- CD30+(15%)	Lyon-Sud hospital	2019	28	LN
PTCL 11	M	55	NOS	CD2+ CD3+ CD5- CD7- CD4- CD8- CD30low ICOS +/- PD-1 +/- EMA- CD10- ALK1- GZMB+ Perforin+ TiA1+	CEVI group	2014	91	LN
PTCL 12	M	49	AITL	CD2+ CD3+ CD5+ CD7+/- CD4+ CD8- TCRb1+ CD10- BCL6- PD1+ ICOS+ CXCL13- CD30- CD25- CD103+ CD56- Perforine- TiA1-	CEVI group	2015	76	LN
PTCL 13	M	64	AITL	CD20+ CD79a- PAX5- CD3+ CD2+ CD5+ CD7- CD4+ CD8- TCRb1+ CD10- ICOS+ PD1+ BCL6+(heterogeneous) CXCL13+ CD30(10%) CD25-	CEVI group	2017	53	LN
PTCL 14	M	57	AITL	CD3+ CD4+/- CD8- CD5+ CD2+ CD7+ PD1+ CXCL13+(partial) ICOS+(heterogeneous) CD15-	CEVI group	2019	30	LN
PTCL 16	F	75	AITL	CD3+ CD4+ CD5+ PD1+ CD7low CD10- CXCL13-	CEVI group	2015	79	LN
PTCL 17	F	69	NOS	CD3+ CD4+ CD8- CD5+ CD7- CD30- CD10- PD-1 +/- CXCL13-	CEVI group	2016	56	LN
PTCL 18	F	71	AITL	CD3+ CD4+ CD8- CD7low PD1+ CXCL13+ CD10+(heterogeneous) BCL6-	CEVI group	2018	37	LN
PTCL 19	M	80	NOS	CD3+ CD4+ CD7+ Ki67+	CEVI group	2019	44	LN
PTCL 20	M	74	NOS	CD3+ CD4+ CD7+ CD10-	CEVI group	2017	56	LN
PTCL 21	F	82	NOS	CD3+ CD5low CD4+ CD8- PD1+(heterogeneous) CXLC13+(heterogeneous) CD30-	CEVI group	2021	9	LN
PTCL 22	F	46	AITL	CD3+ CD4+ CD10+ PD1+ CD7low	CEVI group	2019	44	LN
PTCL 23	M	39	AITL	CD3+ CD4+ CD10+ PD1+ CD7-	CEVI group	2015	78	LN
PTCL 24	F	58	NOS	CD3+ CD4+ CD7- PD1- CD10-	CEVI group	2019	25	LN
PTCL 25	F	75	AITL	CD3+ CD4+ CD7- PD1+ CD10+ Bcl6+	CEVI group	2016	62	LN
PTCL 26	F	73	AITL	CD3+ CD4+ CD7+ PD1+ CD10- Bcl6-	CEVI group	2017	53	LN
LN 1	F	50	Reactive LN	Follicular hyperplasia, no T/B clonality	Lyon-Sud hospital	2019	49	LN
LN 2	M	77	Reactive LN	No abnormalities, no inflammation.	Lyon-Sud hospital	2020	42	LN
LN 3	M	57	Reactive LN	Follicular hyperplasia in a patient cured (complete response) from Hodgkin lymphoma.	Lyon-Sud hospital	2017	78	LN

LN 4	F	51	Reactive LN	Follicular hyperplasia, no T/B clonality. Suggestive of lupus lymphadenopathy	Lyon-Sud hospital	2019	56	LN
LN 5	F	71	Reactive LN	Lymphadenopathy, benign, no tumor cells, no B clonality	Lyon-Sud hospital	2023	9	LN
Tonsil 1	F	8	Tonsil	NA	Clinique du parc, Lyon	2020	16	Tonsil
Tonsil 2	M	7	Tonsil	NA	Clinique du parc, Lyon	2020	16	Tonsil
Tonsil 3	M	7	Tonsil	NA	Clinique du parc, Lyon	2020	8	Tonsil
Tonsil 4	M	6	Tonsil	NA	Clinique du parc, Lyon	2021	2	Tonsil
Tonsil 5	F	5	Tonsil	NA	Clinique du parc, Lyon	2021	1	Tonsil

Supplementary Table 2. Individual clinical immunophenotyping data of the spectral flow cytometry cohort

	N patients with available data	n	%
Age median [range] Age <60y Age ≥60y Age ≥70y	43	 69 [30-87] 11 11 21	 26 26 49
Sex Male Female	43	 24 19	 56 44
Type of PTCL AITL PTCL-NOS	43	 43 0	 100 0
Ann Arbor stage I II III IV	42	 0 2 19 21	 0 5 45 50
ECOG performance status 0 1 2 3 4	41	 4 13 17 4 3	 10 32 41 29 7
B symptoms No Yes	43	 9 34	 21 79
Extranodal sites 0 1 ≥2	42	 20 12 10	 48 28 24
Bone marrow involvement No Yes	33	 16 17	 49 51
LDH ≤ Upper limit of normal > Upper limit of normal	41	 5 36	 12 88
Hemoglobin ≥ 12g/dL <12g/dL	40	 17 23	 42 58
Platelets ≥ 150 000/mm^3 <150 000/mm^3	39	 34 5	 87 13
Monocytes ≥800/mm^3 <800/mm^3	34	 14 20	 41 59

IPI	41		
0		0	0
1		1	2
2		6	15
3		13	32
4		18	44
5		3	7
IPI (simplified)	41		
<3		7	17
≥3		34	83
PIT	32		
0		0	0
1		4	13
2		10	33
3		13	38
4		5	15
PIT (simplified)	41		
<2		4	10
≥2		37	90
PIAI	39		
0		2	7
1		6	16
2		10	27
3		14	33
4		7	18
5		0	0
PIAI (simplified)	43		
<2		8	19
≥2		35	81

Supplementary Table 3. Clinical characteristics of the PTCL cohort used in multi-IF analyses

AITL Patient ID	Sex	Age	Diagnosis	Year of collection	Source of the sample	Tissue origin
AITL 1	M	82	AITL	2012 to 2021	Lyon	Lymph node
AITL 2	M	58	AITL			
AITL 3	F	68	AITL			
AITL 4	M	53	AITL			
AITL 5	F	30	AITL			
AITL 6	M	70	AITL			
AITL 7	M	86	AITL			
AITL 8	F	77	AITL			
AITL 9	F	48	AITL			
AITL 10	M	86	AITL			
AITL 11	M	73	AITL			
AITL 12	M	86	AITL			
AITL 13	M	75	AITL			
AITL 14	F	76	AITL			
AITL 15	F	81	AITL			
AITL 16	M	76	AITL			
AITL 17	F	70	AITL			
AITL 18	M	53	AITL			
AITL 19	F	75	AITL			
AITL 20	F	67	AITL			
AITL 21	M	62	AITL			
AITL 22	M	80	AITL			
AITL 23	F	78	AITL			
AITL 24	M	67	AITL			
AITL 25	M	69	AITL/TFH			
AITL 26	M	48	AITL			
AITL 27	F	62	AITL/TFH			
AITL 28	M	68	AITL			
AITL 29	M	75	AITL			
AITL 30	M	64	AITL			
AITL 31	F	57	AITL			
AITL 32	M	54	AITL			
AITL 33	F	49	AITL			
AITL 34	M	71	AITL			
AITL 35	F	80	AITL			
AITL 36	F	75	AITL			
AITL 37	F	50	AITL			
AITL 38	M	61	AITL			
AITL 39	M	53	AITL			
AITL 40	F	87	AITL/TFH			
AITL 41	F	84	AITL			
AITL 42	F	68	AITL			
AITL 43	M	66	AITL/TFH			

Supplementary Table 4. Individual sample data from the PTCL cohort used in multi-IF analyses

Reagent	Source	Identifier	Working concentration
Live/Dead Blue	Thermo Fisher Scientific	L23105	1/1000
BV711 Rat Anti-Human CXCR5 (CD185) (RF8B2)	BD Biosciences	740737	1/80
cFluor® YG584 Anti-Human CD4 (SK3)	Cytek	SKU R7-20041	1/100
Spark NIR™ 685 anti-human CD20 Antibody (2H7)	BioLegend	302366	1/40
APC/Fire™ 750 anti-human CD39 Antibody (A1)	BioLegend	328230	1/80
Pacific Blue™ anti-human CD57 Antibody (HNK-1)	BioLegend	359608	1/80
APC/Fire™ 810 anti-human CD3 Antibody (SK7)	BioLegend	344858	1/80
Vio® Bright FITC anti-human CD120b (TNF-RII) Antibody,REAfinity™ (REA520)	Miltenyi Biotec	130-119-777	1/50
PE/Dazzle™ 594 anti-human TIGIT (VSTM3) Antibody (A15153G)	BioLegend	372716	1/40
PE/Cy5.5 LAG-3 Antibody (17B4)	Novus Biologicals	NBP1-97657PECY55	1/3200
PerCP-Vio® 700 anti-human CD163 Antibody, REAfinity™ (REA812)	Miltenyi Biotec	130-112-133	1/100
PerCP-Cy™5.5 Mouse Anti-Human TCR γδ (B1)	BD Biosciences	564157	1/20
CD137 Antibody, anti-human, APC, REAfinity™ (REA765)	Miltenyi Biotec	130-110-764	1/50
BUV563 Mouse Anti-Human CD7 (M-T701)	BD Biosciences	741355	1/80
Brilliant Violet 570™ anti-human CD45RA Antibody (HI100)	BioLegend	304132	1/40
BV605 Mouse Anti-Human CD56 (NCAM16.2)	BD Biosciences	562780	1/40
BUV805 Mouse Anti-Human CD8 (SK1)	BD Biosciences	612889	1/80
BB515 Mouse Anti-Human CD11c (B-ly6)	BD Biosciences	564490	1/40
BV510 Mouse Anti-Human NKG2A (CD159a) (131411)	BD Biosciences	747922	1/20
BUV661 Mouse Anti-Human CD226 (DX11)	BD Biosciences	749934	1/40
BUV737 Mouse Anti-Human CD134 (ACT35)	BD Biosciences	749286	1/40
BV750 Mouse Anti-Human CD278 (ICOS) (DX29)	BD Biosciences	746858	1/40
BUV615 Mouse Anti-Human TIM-3 (CD366) (7D3)	BD Biosciences	752363	1/20
BV786 Mouse Anti-Human CD279 (PD-1) (EH12.1)	BD Biosciences	563789	1/40
BUV496 Mouse Anti-Human CD10 (MEM-78)	BD Biosciences	750190	1/40
Alexa Fluor® 647 Mouse Anti-TCF-7/TCF-1 (S33-966)	BD Biosciences	566693	1/20
PE-Cyanine5 anti-human FOXP3 Monoclonal Antibody (PCH101)	Thermo Fisher Scientific	15-4776-42	1/40
Alexa Fluor® 700 Mouse anti-Human Granzyme B (GB11)	BD Biosciences	560213	1/80
PE-Cyanine7 anti-human CD152 (CTLA-4) (14D3)	Thermo Fisher Scientific	25-1529-42	1/40
PE Mouse Anti-EOMES (X4-83)	BD Biosciences	566749	1/20
BV650 Mouse Anti-T-bet (O4-46)	BD Biosciences	564142	1/40
BUV395 Mouse Anti-Ki-67 (B56)	BD Biosciences	564071	1/40
BV421 Mouse Anti-Bcl-6 (K112-91)	BD Biosciences	563363	1/40

Reagent	Source	Identifier	Working concentration
Mouse anti-human CD3 (polyclonal)	Agilent	A052	1/100
Mouse anti-human CD8 (C8/144B)	Agilent	M7103	1/40
Rabbit anti-human CD39 (polyclonal)	Sigma Aldrich	HPA014067	1/50

Supplementary Table 5. FACS and multi-IF Abs used in this study

Sample ID	Diagnosis	Number of cells in the sample	Number of labeled cells	Number of acquired events	% of live cells	Number of live cells processed
PTCL 1	NOS	25,000,000	1,000,000	237,714	67.0%	159,353
PTCL 5	AITL	3,000,000	1,000,000	141,138	35.9%	50,690
PTCL 9	AITL	4,000,000	1,000,000	263,894	82.1%	216,700
PTCL 11	NOS	2,000,000	1,000,000	43,133	48.5%	20,935
PTCL 12	AITL	2,000,000	1,000,000	281,939	44.6%	125,630
PTCL 13	AITL	10,000,000	1,000,000	247,175	81.2%	200,599
PTCL 14	AITL	9,000,000	1,000,000	81,989	85.7%	70,293
PTCL 16	AITL	3,000,000	1,000,000	273,702	67.0%	183,471
PTCL 17	NOS	7 500,000	1,000,000	228,216	88.1%	201,156
PTCL 18	AITL	5,000,000	1,000,000	360,963	43.2%	155,963
PTCL 19	NOS	3,000,000	1,000,000	406,876	68.9%	280,518
PTCL 20	NOS	4,000,000	1,000,000	524,588	76.8%	402,729
PTCL 21	NOS	4,000,000	1,000,000	344,937	64.4%	222,123
PTCL 22	AITL	6,000,000	1,000,000	153,189	65.5%	100,366
PTCL 23	AITL	8,000,000	1,000,000	370,228	79.9%	295,805
PTCL 24	NOS	1,000,000	1,000,000	154,338	63.5%	98,070
PTCL 25	AITL	7,000,000	1,000,000	394,985	66.1%	261,057
PTCL 26	AITL	3,000,000	1,000,000	305,753	65.7%	200,795
LN 1	Reactive LN	8,230,000	1,000,000	106,181	89.4%	94,952
LN 2	Reactive LN	6,070,000	1,000,000	188,014	78.1%	146,884
LN 3	Reactive LN	9,100,000	1,000,000	194,612	87.5%	170,287
LN 4	Reactive LN	467,000	467,000	32,533	90.6%	29,489
LN 5	Reactive LN	8,230,000	1,000,000	155,779	91.9%	143,159
Tonsil 1	Tonsil	26,000,000	1,000,000	156,041	92.3%	144,077
Tonsil 2	Tonsil	13,000,000	1,000,000	149,960	90.9%	136,332
Tonsil 3	Tonsil	43,000,000	1,000,000	223,181	89.5%	199,819
Tonsil 4	Tonsil	44,000,000	1,000,000	140,042	86.0%	120,410
Tonsil 5	Tonsil	120,000,000	1,000,000	226,043	86.8%	196,244

Supplementary Table 6. Individual cell counts before and after staining for spectral flow cytometry analyses

Sample ID	Live cells		T conv cells		CD8+ T cells	
	Total count	Included in unsupervised analysis	Total count	Included in unsupervised analysis	Total count	Included in unsupervised analysis
PTCL 1	159,353	83,538	26,664	16,740	55,500	4,424
PTCL 5	50,690	50,690	16,857	10,653	2,667	2,667
PTCL 9	216,700	53,161	33,258	10,653	17,059	2,815
PTCL 11	20,935	20,935	6,553	6,553	2,892	2,892
PTCL 12	125,630	53,161	56,625	10,653	7,798	2,815
PTCL 13	200,599	53,161	19,182	10,653	7,331	2,815
PTCL 14	70,293	53,161	16,431	10,653	5,349	2,815
PTCL 16	183,471	53,161	37,869	10,653	28,570	2,815
PTCL 17	201,156	83,538	54,418	16,740	33,907	4,424
PTCL 18	155,963	53,161	27,635	10,653	40,488	2,815
PTCL 19	280,518	83,538	30,368	16,740	18,433	4,424
PTCL 20	402,729	83,538	180,200	16,740	13,469	4,424
PTCL 21	222,123	83,538	47,824	16,740	11,964	4,424
PTCL 22	100,366	53,161	18,438	10,653	8,170	2,815
PTCL 23	295,805	53,161	150,710	10,653	17,058	2,815
PTCL 24	98,070	83,538	50,996	16,740	2,994	2,994
PTCL 25	261,057	53,161	93,769	10,653	71,063	2,815
PTCL 26	200,795	53,161	57,203	10,653	6,126	2,815
LN 1	94,952	94,952	49,643	23,436	13,761	6,194
LN 2	146,884	116,954	50,197	23,436	21,358	6,194
LN 3	170,287	116,954	26,505	23,436	5,556	5,556
LN 4	29,489	29,489	9,941	9,941	2,986	2,986
LN 5	143,159	116,954	33,121	23,436	8,086	6,194
Tonsil 1	144,077	116,954	27,352	23,436	5,191	5,191
Tonsil 2	136,332	116,954	19,087	19,087	6,934	6,194
Tonsil 3	199,819	116,954	37,128	23,436	10,299	6,194
Tonsil 4	120,410	116,954	10,478	10,478	4,288	4,288
Tonsil 5	196,244	116,954	23,139	23,139	4,258	4,258

Supplementary Table 7. Number of cells of each indicated subset used for unsupervised analyses following down-sampling and concatenation

Sample ID	Treg cells		NK cells	
	Total count	Included in unsupervised analysis	Total count	Included in unsupervised analysis
PTCL 1	8,277	1,243	1,196	447
PTCL 5	8,156	791	1,385	284
PTCL 9	11,144	791	695	284
PTCL 11	1,767	1,243	323	323
PTCL 12	12,029	791	224	224
PTCL 13	27,226	791	264	264
PTCL 14	3,109	791	491	284
PTCL 16	13,840	791	3,339	284
PTCL 17	18,761	1,243	4,068	447
PTCL 18	19,005	791	5,618	284
PTCL 19	39,055	1,243	1,197	447
PTCL 20	91,788	1,243	691	447
PTCL 21	24,211	1,243	12,503	447
PTCL 22	2,957	791	990	284
PTCL 23	20,316	791	680	284
PTCL 24	2,108	1,243	296	296
PTCL 25	10,480	791	3,381	284
PTCL 26	72,999	791	1,003	284
LN 1	3,778	1,741	503	503
LN 2	4,344	1,741	232	232
LN 3	1,359	1,359	1,129	626
LN 4	634	634	29	29
LN 5	4,499	1741	1,326	626
Tonsil 1	1,840	1,741	276	276
Tonsil 2	1,377	1,377	702	626
Tonsil 3	2,746	1,741	488	488
Tonsil 4	709	709	645	626
Tonsil 5	2,034	1,741	1,019	626

Supplementary Table 7 (continued). Number of cells of each indicated subset used for unsupervised analyses following down-sampling and concatenation

Sample ID	Diagnosis	Putative malignant aberrant phenotype 1	Putative malignant aberrant phenotype 2
PTCL 1	NOS	**Not identified**	Not identified
PTCL 5	AITL	CD3+CD4+CD8-CD7-CD10+/-PD1+TIM3+ICOS+/-LAG3+/-	Not identified
PTCL 9	AITL	**Not identified** (all T cells are PD1+ICOS+CTLA4+TIGIT+)	Not identified
PTCL 11	NOS	CD3+CD4-CD8-CD7-CD10+PD1+TIM3+ICOS-GZMB+	Not identified
PTCL 12	AITL	CD3+CD4+CD8-CD7-PD1+ICOS+CTLA4+EOMES-	CD3+CD4+CD8-CD7-PD1+ICOS+CTLA4-EOMES+
PTCL 13	AITL	CD3+CD4+CD8-CD7-PD1+ICOS+CTLA4+	CD3+CD4-CD8-CD7- PD1+ICOS+CTLA4bright
PTCL 14	AITL	CD3+CD4+CD8-CD7+CD10-PD1+ICOS+CTLA4+OX40+Ki67+	CD3+CD4+CD8-CD10+PD1+ICOS+CTLA4-OX40-Ki67- **AND** Ki67+ cells, not other specified
PTCL 16	AITL	CD3+CD4+CD8-CD7-CD10+PD1+ICOS+CTLA4+OX40+	Not identified
PTCL 17	NOS	CD3+CD4+CD8-CD7-CD10+PD1+ICOS+/-CTLA4+/-EOMES+/-	CD3+CD4-CD8-CD7-CD10-PD1+ICOS+/-CTLA4+/-EOMES+/-
PTCL 18	AITL	CD3+CD4+CD7+PD1+ICOS+CTLA4+OX40+	Not identified
PTCL 19	NOS	CD3+CD4+CD8-CD7+PD1+ICOS+Ki67+	CD3+CD4-CD8-CD7-PD1+ICOS+Ki67-
PTCL 20	NOS	CD3+CD4+CD8-CD7-TCRgd+PD1+/-ICOS+/-	Not identified
PTCL 21	NOS	CD3+CD4+CD8-CD7-PD1+ICOS+/-EOMES+/-	CD3-CD4+CD8-CD7- PD1-GZMB+EOMES+FOXP3+
PTCL 22	AITL	CD3+CD4+CD8-CD7-CD10-PD1+ICOS+CTLA4+	CD3-CD4+CD8-CD7-CD10+PD1+ICOS+CTLA4+
PTCL 23	AITL	CD3+CD4+CD8-CD7-CD10+PD1+ICOS+	Not identified
PTCL 24	NOS	CD3+CD4+CD8-CD7-PD1-ICOS+LAG3-	CD3+CD4+CD8-CD7-PD1-ICOS+LAG3+
PTCL 25	AITL	CD3+CD4+CD8-CD7-CD10+PD1+ICOS+CTLA4+OX40+	CD3-CD4-CD8-FOXP3+CD39+
PTCL 26	AITL	CD3+CD4+CD8-CD7-CD10+PD1+ICOS+CTLA4+OX40+EOMES+	CD3+CD4+CD8-CD7+CD10+PD1+ICOS+CTLA4+OX40+EOMES-

Supplementary Table 8. Putative identification of neoplastic phenotypes in PTCL samples.

Supplemental methods

Flow cytometry staining

After thawing in a water bath at 37°C, the cell suspensions were washed in RPMI 1640 W/HEPES W/GLUTAMAX-I (supplemented with 10% FBS; Penicillin/Streptomycin; Non-Essential Amino Acids; Sodium Pyruvate and β-Mercaptoethanol, all from Thermo Fisher). 1 million cells per sample were stained with viability dye for 15 min at room temperature (RT), then incubated with Human FC block for 10 min at RT. Cells were then incubated with CXCR5 antibody mix in FACS buffer (PBS1X with 2.5 mM EDTA and 3% FBS) for 20 min at 37°C, then with surface marker antibodies mixed in FACS buffer and brillant stain buffer (BD) for 30 min at RT in the dark. Cells were then fixed and permeabilized using the eBioscience Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to manufacturer's instructions. Cells were finally incubated with intracellular marker antibodies mix for 20 min at 4°C.

Flow cytometry unsupervised analyses

Briefly, each cell subset was gated manually (Live cells, sCD3-negative cells, Tconv cells, Treg cells, CD8+ T cells and NK cells). Prior to concatenation, we used down-sampling to avoid over-representation of one group over the others, which could lead to the identification of poorly representative clusters. The total number of cells in each tissue (tonsils, reactive LN, AITL and PTCL, NOS) was therefore made identical by reducing the number of events to that of the smaller sample. Details on the number of events processed in each case can be found in Tables S3 and S4. Following concatenation, 2-dimensional reduction through Uniform Manifold Approximation and Projection (UMAP) and hierarchical clustering through FlowSOM were used.

Tissue micro-arrays and multi-IF protocol

TMA blocks were generated by punching 1 mm cores from FFPE tissue samples using a Tissue Arrayer MiniCore instrument with TMADesigner® 2 Software from ALPHELYS. Three

1 mm cores were taken from each patient sample. The three-plex mIF assay was first optimized as previously described [23]. The standard seven-color TSA protocol template on the BOND RXm was used with modifications. TMAs underwent an initial antigen retrieval step of ER1 at 100°C for 20 min, a dispensing of the TSA reagents (incubation time of 30 min), and DAPI staining at a volume of 150 µL for 5 min. The following sequence was used: anti-CD3+OPAL 570 (position 3), anti-CD8+OPAL 520 (position 4), anti-CD39+OPAL 690 (position 5). All antibodies were diluted using Akoya's antibody diluent/blocking buffer. Slides were imaged using the Vectra Polaris spectral imaging system (Perkin Elmer) at 20X or 40X for TMA. Scans were visualized with the Phenochart software where autofluorescence can be directly removed.

Multi-IF data processing

TMA cores were excluded if one of the following criteria was present: large part or whole of core lost; poor quality staining; or widespread necrosis. Cell phenotypes could be analyzed in the 43 patients. In 6 of them, it was evaluated in one TMA core. For the others, the results retained for each patient were the median of the 2 or 3 TMA cores. Images were spectrally separated with a synthetic algorithm in InForm version 2.4.8 (Akoya Biosciences). Cell phenotypes were identified and counted using image analysis in InForm. Six TMA cores representing the heterogeneous nature of AITL, were selected to train machine learning algorithms for tissue segmentation, cell segmentation and cell phenotyping. First, the tissue was divided into tumor or non-tumor compartments using the tissue segmentation setting by drawing different areas as different categories. Then, cell segmentation was performed using DAPI counterstaining, using the adaptive cell segmentation setting in InForm software. The splitting parameter was adjusted to segment crowded and overlapping cells. Membrane staining was selected to assist in nuclear segmentation. Seven cell phenotypes were analyzed: total T lymphocytes ($CD3^+$), $CD8^+$ T lymphocytes ($CD3^+CD8^+$), non-$CD8^+$ T lymphocytes ($CD3^+CD8^-$), total cells expressing CD39 ($CD39^+$), total T lymphocytes expressing CD39

($CD3^+CD39^+$), $CD8^+$ T lymphocytes expressing CD39 ($CD3^+CD8^+CD39^+$) and non-$CD8^+$ T lymphocytes expressing CD39 ($CD3^+CD8^-CD39^+$). When the training was completed, it was applied to a set of cases to verify that it was working properly. These parameters were then applied to all TMA cores and the percentage of cells according to their phenotypes was calculated using the R version 4.2.1 software.