TUMOR INFILTRATING B-CELLS SIGNAL FUNCTIONAL HUMORAL IMMUNE RESPONSES IN BREAST CANCER

Authors and affiliations

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Supplementary Figure S1

A. Gating strategies for fresh breast tissue homogenates
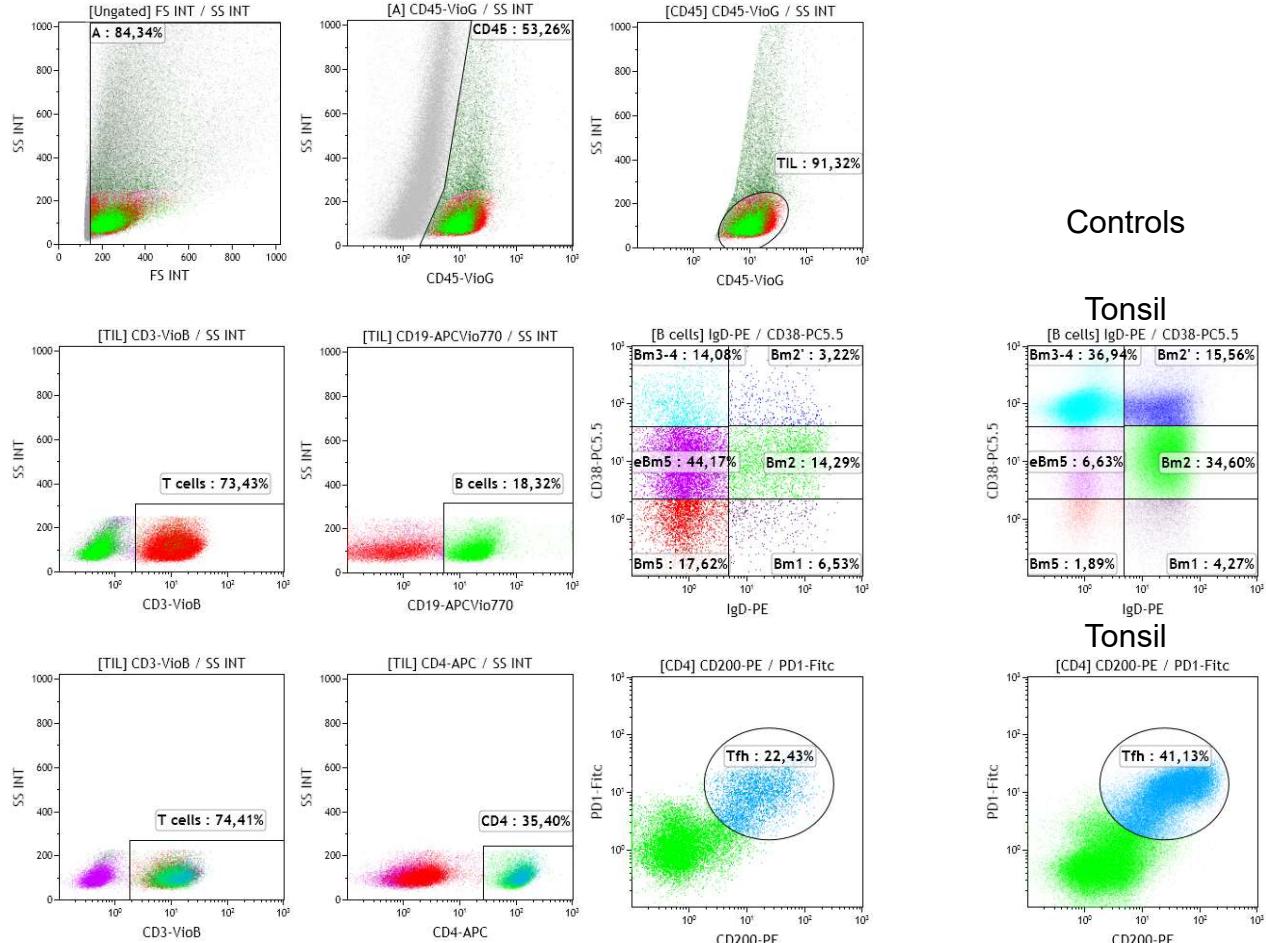
B. Gating strategies for blood (PBMC)
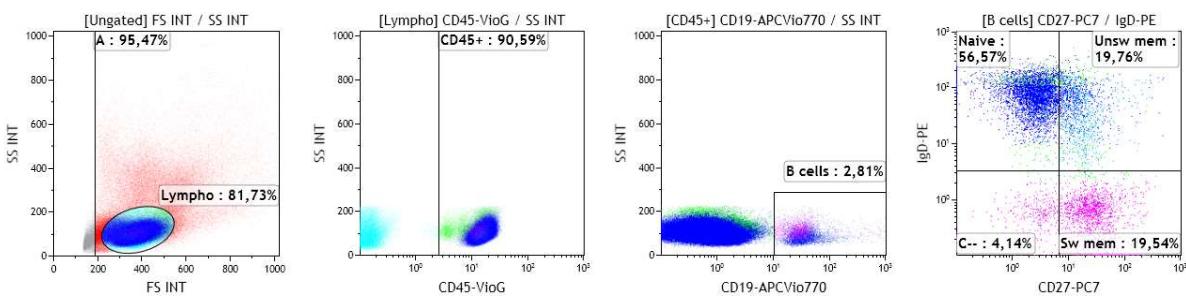
**Supplementary figure S1: Gating strategies for lymphocyte subpopulations.**

(A) TIL and tonsillar mononuclear cells were labelled with antibodies specific for CD45, CD3, CD4, CD19, IgD, CD38, CD200 and PD1. (B) PBMC were labelled with antibodies specific for CD45, CD19, CD27, and IgD.

Supplementary Figure S2

A. CD19+ B cells in Blood (PBMC)
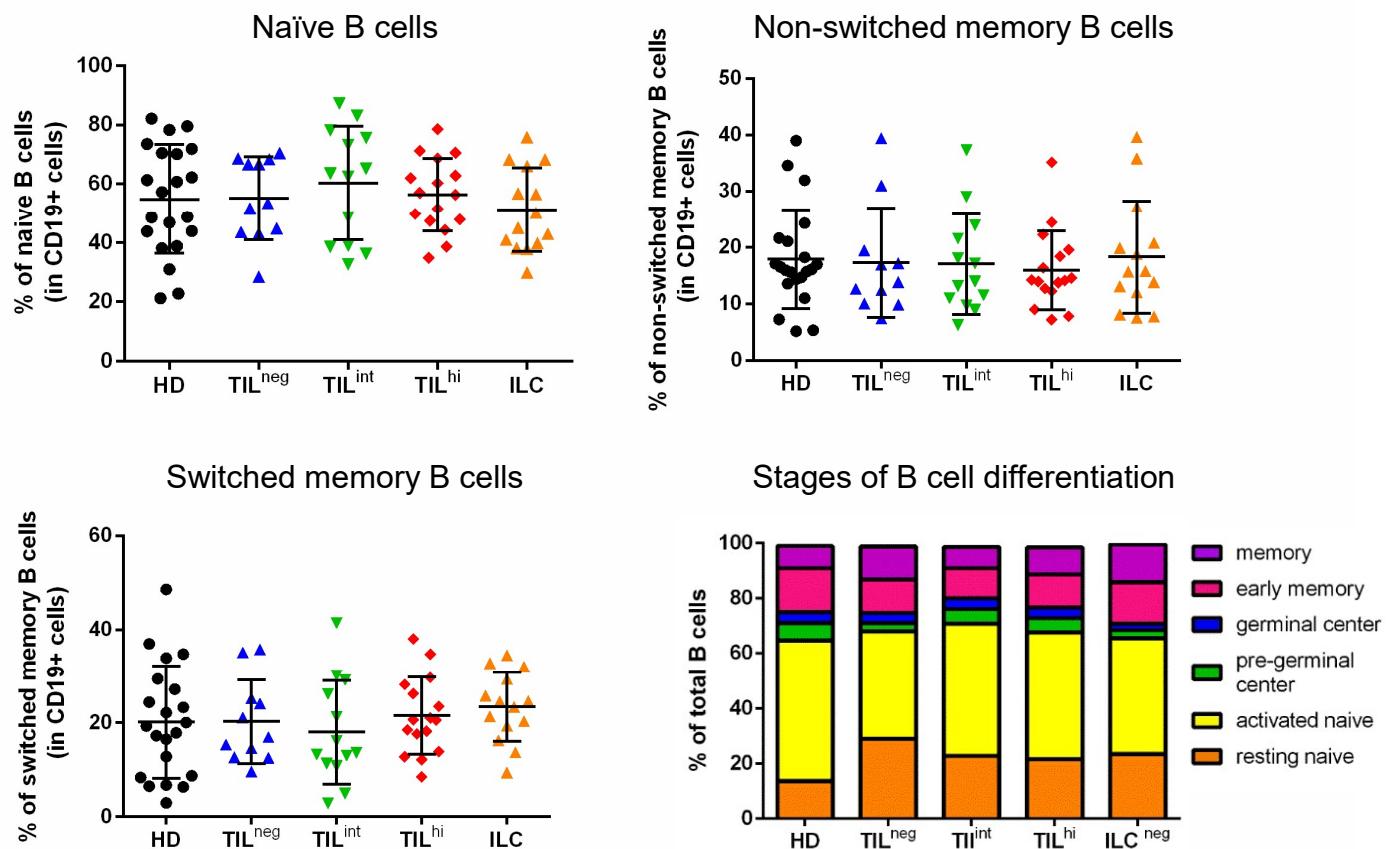
B. CD19+ B cells in Tonsil and involved Lymph Node Tissues
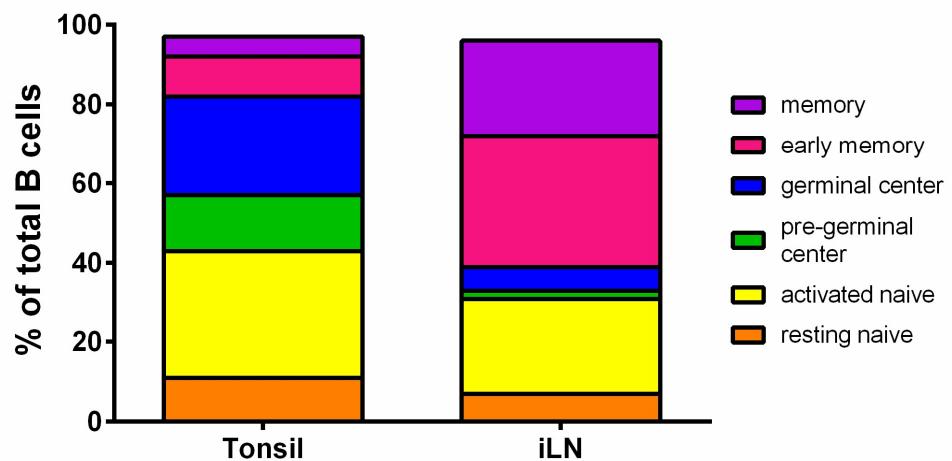
C. CD19+ B cells in Breast Tissues
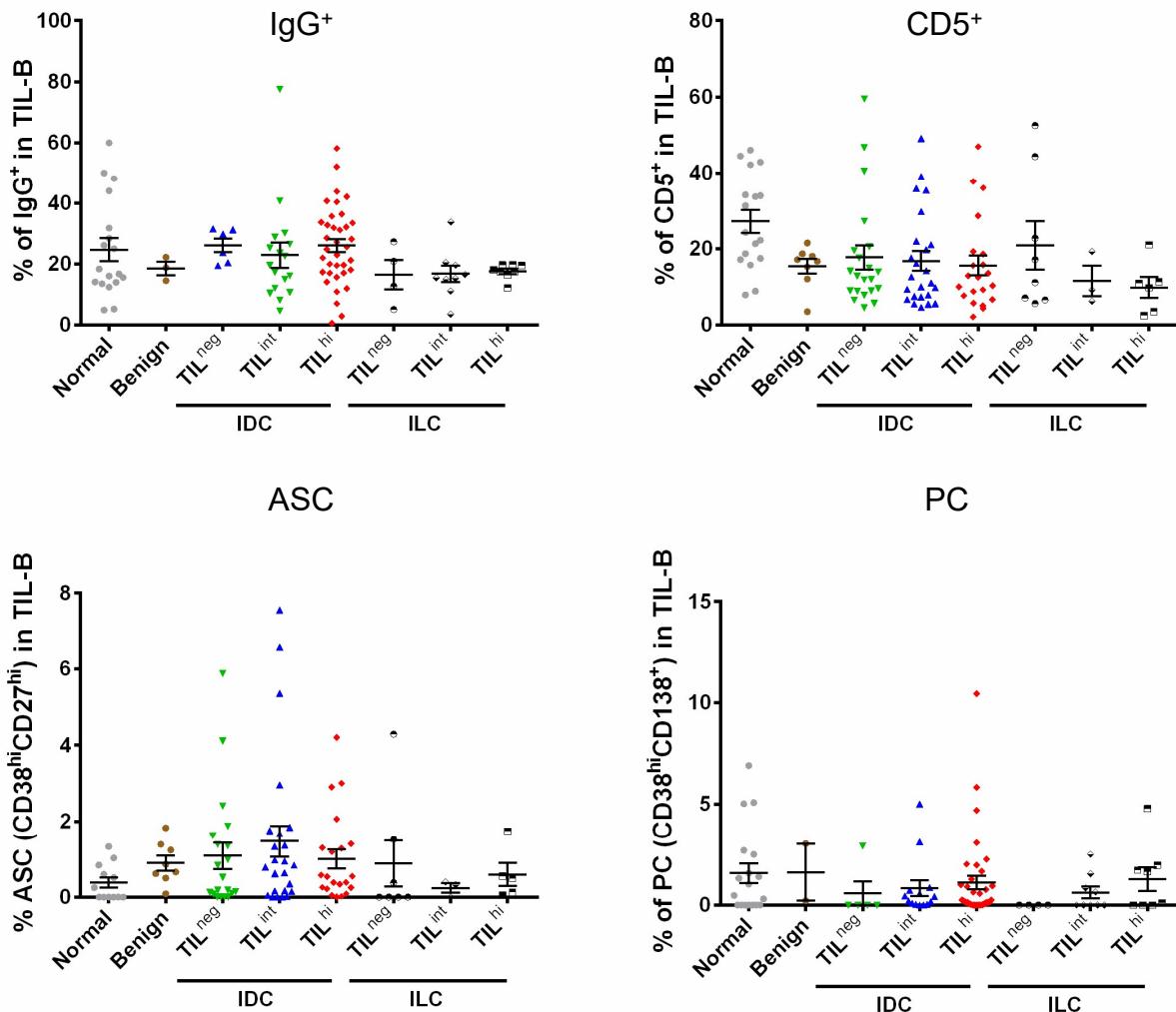
Supplementary figure S2: Phenotypic characterization of B-cells in blood, lymph nodes, and breast cancer tissues.

(A) B-cell subsets were determined as a percentage of CD45+ leukocytes in the blood by FACS. (B) Stacked bars show the percentage of TIL-B subsets in total B-cells according to the Bm classification (CD38 and IgD) in tonsil and involved lymph node tissues. (C) B-cell subsets were determined as a percentage of CD19+ TIL-B in the breast tissues by FACS. Data represent a combination of experiments involving individual patients. Abbreviations: HD, healthy donors; TIL, tumor-infiltrating lymphocytes; LN, lymph nodes; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; ASC, antibody-secreting B-cells; PC, plasma cells.

Supplementary Figure S3

A.   H&E and IF-stained human tonsil tissues

Hematoxylin and Eosin

Immunofluorescence (confocal microscopy)

Overlay

CD20/**CD8**/**CD4**

CD20/**IgD**/**PD-1**
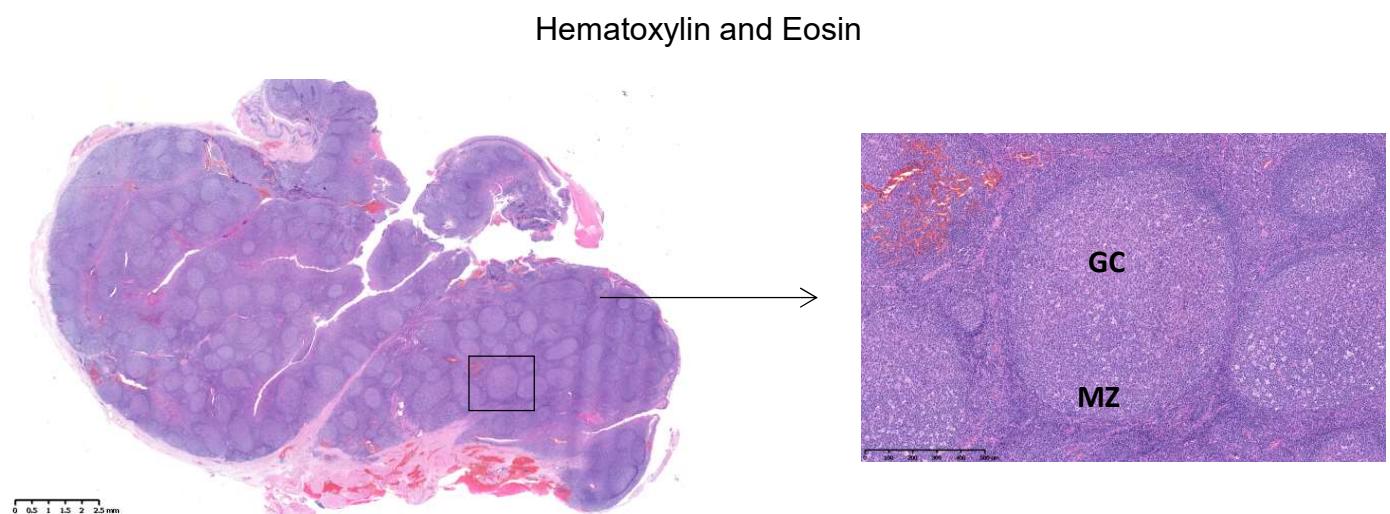
B. IF and mIHC-stained human tonsil tissues

Immunofluorescence (confocal microscopy)

Overlay

CD20/CD21

CD20/CD35

CD20/IgM

CD20/Ki67

CD20/CD138

Multiplex IHC

CD4
CD8
CD20
FOXP3
CD68
panCK
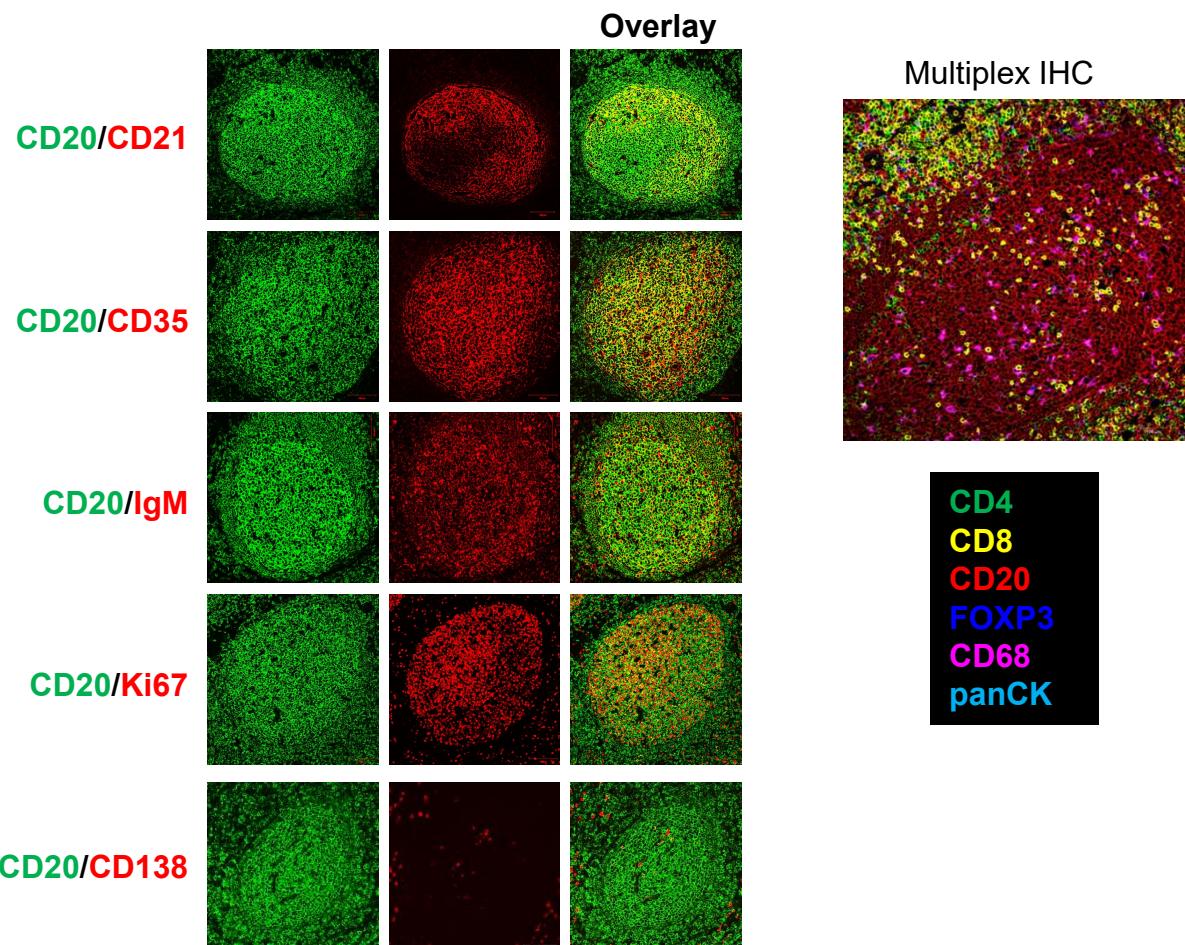
Supplementary figure S3: B-cell organization in tonsil.

(A) Hematoxylin and eosin and immunofluorescent staining of tonsil (20X). Lymphocytes were revealed by CD20 (green), CD8 (red) and CD4 (blue). Germinal center were identified by the absence of naïve IgD+ B-cells (red) and the presence of PD-1+ cells (blue) (B), CD21+ CD35+ (red) follicular dendritic cells, IgM+ (red) memory B-cells, Ki67+ (red) proliferating cells, and CD138+ (red) plasma cells. (B) Representative multiplexed IHC images of tonsil. Lymphocytes were revealed by CD20 (red), CD8 (yellow) and CD4 (green), FOXP3 (blue), CD68 (magenta), and tumor cells by panCK (cyan).

Supplementary Figure S4

A. Composite view
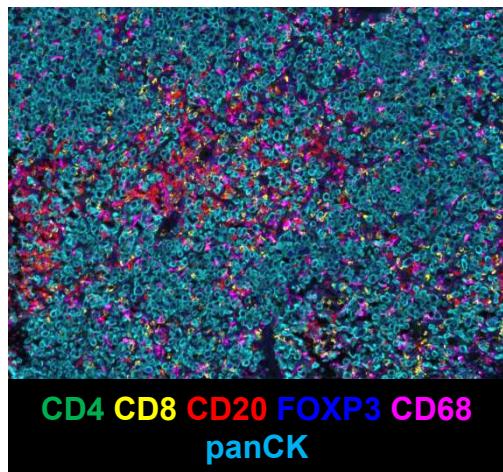
B. View of TIL-B touching tumor cells

TIL-B/Tumor cells

40,58%   59,42%
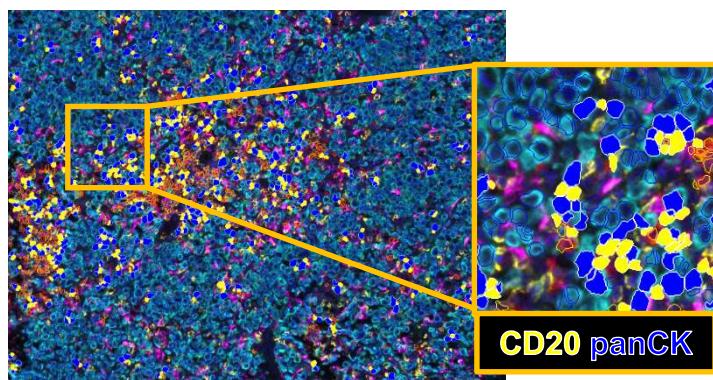
■ Non-touching ■ Touching
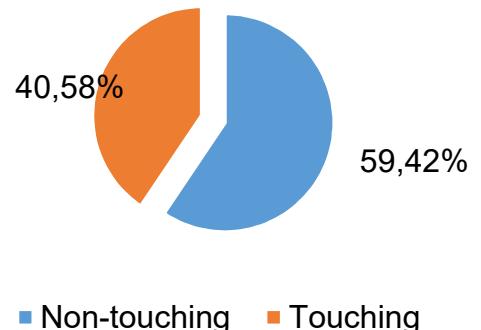
C. View of TIL-B touching CD8+ TIL

TIL-B/CD8-TIL

28,88%   71,12%

■ Non-Touching ■ Touhing
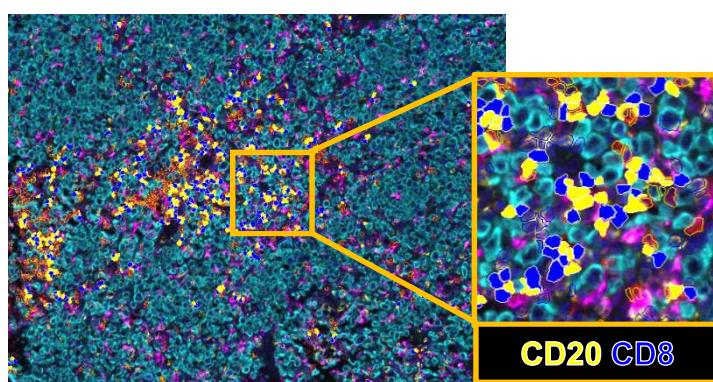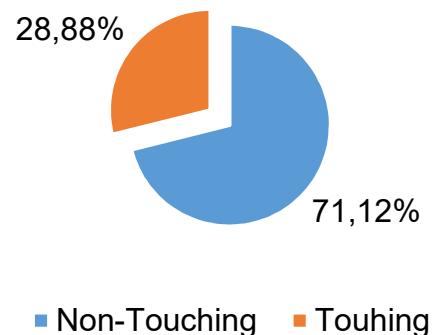
Supplementary figure S4: Spatial distribution of TIL-B in human breast cancer.
Pseudo colored composite of "unmixed" component planes (A). Cells were phenotyped in inForm®, and interactions assessed with R and phenoptr ("*Find and count touching cells*"). Pie graphs denote the percentage of TIL-B touching tumor cells (CK+) (B) and CD8+ TIL (C) in the tumor microenvironment. Touching TIL-B and tumor cells/CD8+ TIL were labelled in yellow and blue, respectively.

Supplementary Figure S5

A. Cytokine expression (protein) in plasma
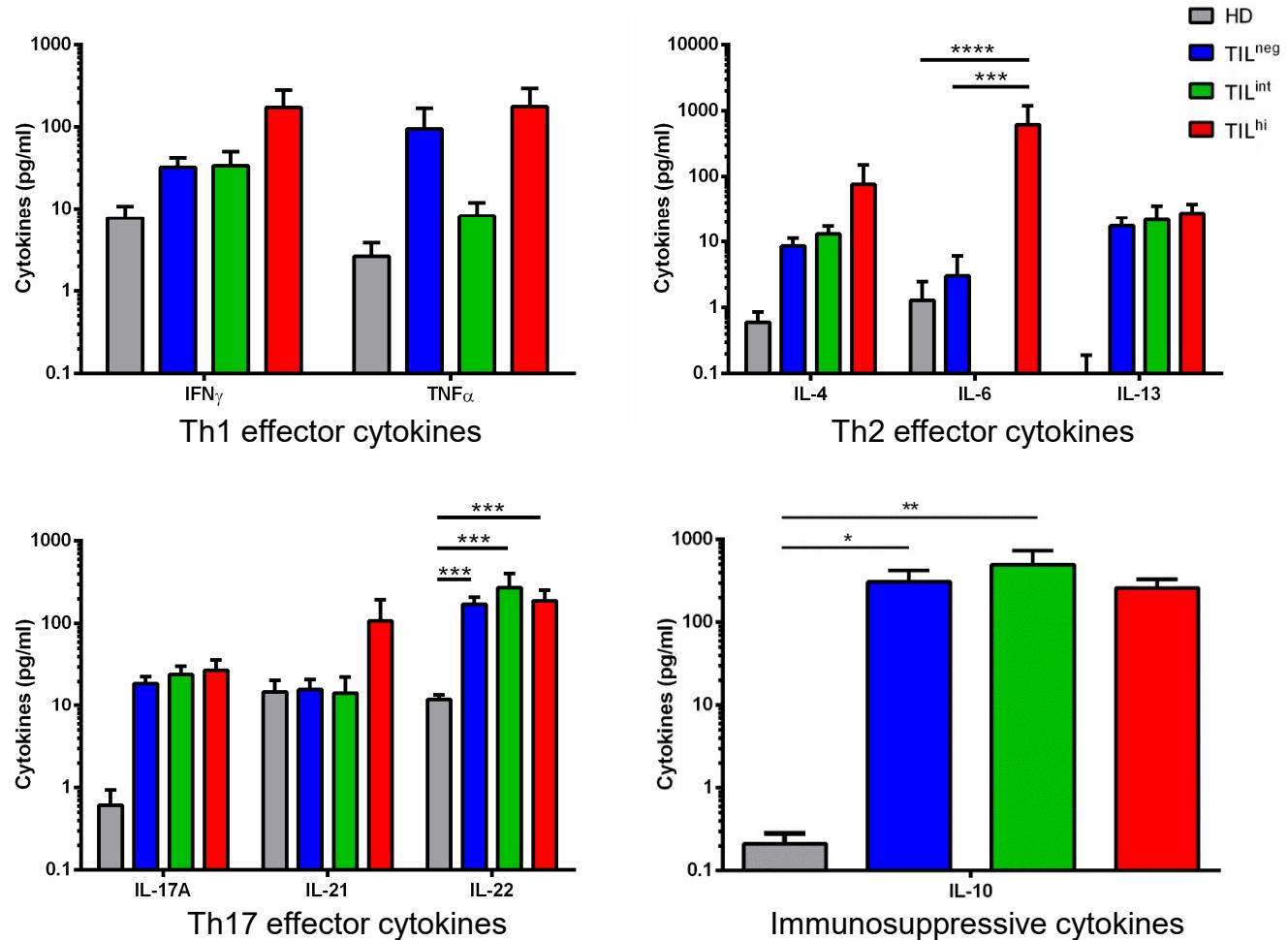
B. Cytokine expression (protein) in tissue supernatants
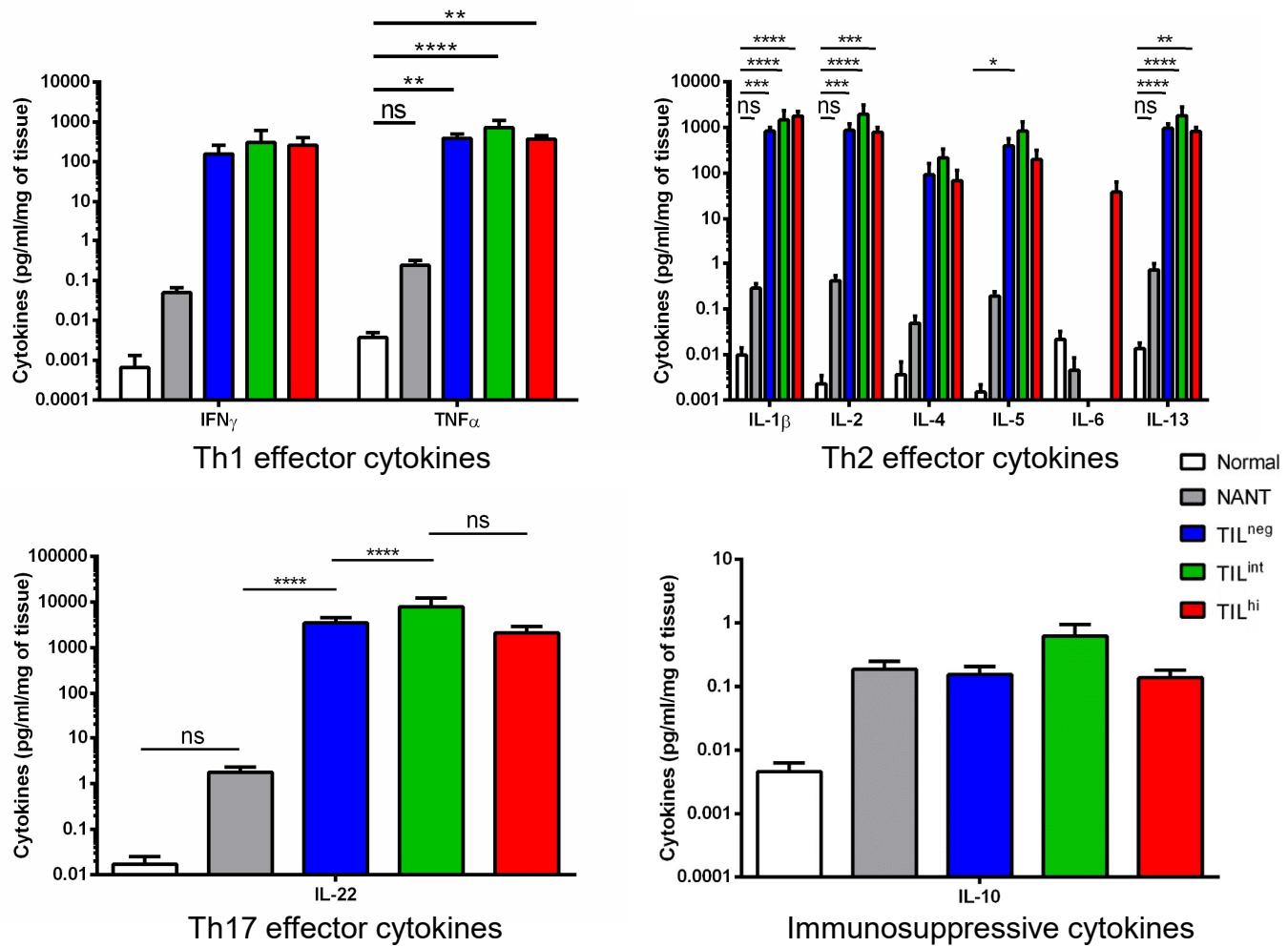
Supplementary figure S5: Cytokine profile in plasma and fresh breast tissue supernatants.

Interleaved bars show the protein levels of cytokines in plasma from healthy donors and BC patients (A) together with fresh breast tissue supernatants (mammary reductions, NANT and tumors) (B). Data represent a combination of experiments involving individual patients and are displayed as mean ± SEM by One-Way ANOVA with Tukey multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P< 0.0001. Abbreviations: IL, interleukin; TIL, tumor-infiltrating lymphocytes.

Table S1: Association between clinicopathological parameters and TIL-B of patients from the BIG 02-98 clinical trial.

	Number	Association with TIL-B (IHC)		
		Median	IQR	*p* Value
Age (years)				
<50	127	2.0	0.78 to 4.50	0.11
≥50	121	2.5	1.00 to 5.50	
Surgery				
Mastectomy	144	2.25	0.89 to 4.88	0.56
Tumorectomy	104	2.28	0.94 to 5.50	
Histology				
Ductal	232	2.25	0.89 to 5.08	0.68
Lobular	7	2.65	0.75 to 3.00	(ductal vs other)
Mixed (ductal+lobular)	3	1.80	1.50 to 2.25	
Other	6	2.69	2.25 to 3.50	
Node status				
1-3	127	2.45	1.00 to 5.50	0.40
4-9	79	2.00	0.80 to 5.00	
≥10	42	2.10	0.85 to 3.25	
Stage (AJCC staging)				
II	121	2,5	1,0 to 5,5	0,13
III	127	2	0,85 to 4,5	
Histological grade				
1	3	0.85	0.25 to 1.75	**0.02**
2	55	1.75	0.75 to 4.25	(grade 3 vs other)
3	187	2.50	1.00 to 5.50	
Ki67-Proliferation index				
<20%	36	1.50	0.75 to 2.88	**0.04**
≥20%	205	2.50	1.00 to 5.25	
Hormonal receptors (IHC)				
ER negative	51	2.50	1.00 to 6.00	**0.01**
ER positive	84	1.90	0.75 to 3.25	
PR negative	70	2.25	1.00 to 5.25	**0.04**
PR positive	64	1.75	0.75 to 3.25	
HER-2 receptor (FISH)				
HER-2 negative	113	2,5	1,00 to 6,25	0,09
HER-2 positive	135	2	0,88 to 4,50	
Tumor size				
<20	58	2.48	0.75 to 7.50	0.12
20-49	152	2.45	1.00 to 5.00	
≥50	38	1.73	0.78 to 2.50	
Laterality				
Left	126	2.25	1.00 to 5.00	0.46
Right	122	2.50	0.85 to 5.15	
Treatment arm				
A1	45	2.50	1.30 to 5.25	0.79
A2	47	2.13	0.75 to 6.25	(A1, A2 vs B, C)
B	80	2.25	1.00 to 5.00	
C	76	2.23	1.00 to 4.38	
Radiotherapy				
Node status	52	2,83	1,13 to 6,75	0,14
Yes	196	2,06	0,88 to 5,00	

The p value was determined using the chi-square or Fisher exact test when appropriate. The confidence limits were calculated using Fisher's z transformation. Bold type indicates significant (p<0.05).

Table S2: Added value of TIL-B, T cell TIL and stromal TIL scores as continuous variables for predicting DFS and OS.

	HER2+ BC				TNBC			
	iDFS		OS		iDFS		OS	
Analysis	Likelihood Ratio (χ2)	*P*-Value	Likelihood Ratio (χ2)	*P*-Value	Likelihood Ratio (χ2)	*P*-Value	Likelihood Ratio (χ2)	*P*-Value
Univariate								
Tumor size	2.20	0.33	4.35	0.11	5.13	0.08	2.03	0.36
Histological grade	0.03	0.85	1.16	0.28	1.49	0.22	0.23	0.63
Lymph nodes involvment	21.46	<0.001	25.21	<0.001	5.54	0.06	6.14	0.046
CD20	**5.79**	0.02	**5.78**	0.02	**10.03**	0.002	**6.39**	0.01
CD3	**1.42**	0.23	**0.81**	0.37	**19.78**	<0.001	**14.56**	<0.001
Stromal TIL	**3.08**	0.08	**2.58**	0.11	**14.96**	<0.001	**12.51**	<0.001
Multivariate								
Base model = tumor size, grade, lymph nodes	**25.16**	<0.001	**30.60**	<0.001	**10.79**	0.06	**7.91**	0.16
Base + CD20 vs base	**4.18**	0.04	**2.62**	0.11	**5.89**	0.02	**4.24**	0.04
Base + CD3 vs base	**0.71**	0.40	**0.93**	0.33	**15.11**	<0.001	**11.82**	<0.001
Base + stromal TIL vs base	**1.55**	0.21	**1.18**	0.28	**9.49**	0.002	**9.37**	0.002
CD20 vs CD20 + CD3	**0.09**	0.76	**0.01**	0.92	**9.92**	**0.002**	**8.48**	**0.004**
CD3 vs CD20 + CD3	**4.46**	**0.03**	**4.98**	**0.03**	**0.17**	0.68	**0.31**	0.58
CD20 vs CD20 + stromal TIL	**0.40**	0.53	**0.3**	0.58	**5.03**	0.02	**7.05**	0.008
Stromal TIL vs CD20 + stromal TIL	**3.11**	0.08	**3.5**	0.06	**0.09**	0.76	**0.93**	0.33
CD3 vs CD3 + stromal TIL	**1.71**	0.19	**2**	0.16	**0.05**	0.82	**0.42**	0.52
Stromal TIL vs CD3 + stromal TIL	**0.05**	0.82	**0.23**	0.63	**4.87**	0.03	**2.47**	0.12

Table S3A: Correlation between B cell TIL and other TIL subsets in BC

				TIL density (TIL subsets/mg)			
				CD45	CD3	CD4	CD8
IDC	Spearman's rho	TIL-B density (CD19+ cells/mg)	Correlation coefficient	0.798**	0.778**	0.819**	0.775**
			Sig. (2-tailed)	0.000	0.000	0.000	0.000
			N	250	157	121	117
ILC	Spearman's rho	TIL-B density (CD19+ cells/mg)	Correlation coefficient	0.879**	0.809**	0.865**	0.740**
			Sig. (2-tailed)	0.000	0.000	0.000	0.000
			N	54	33	24	24

**. Correlation is significant at the 0.01 level (2-Tailed).

†. Data from flow cytometric analysis.

Table S3B: TIL subset correlations between FACS and IHC in IDC

				FACS					IHC									
				TIL density (TIL subset/mg)					TIL density (% of TIL subset/tumor area)									
				CD45	CD3	CD4	CD8	CD19	Global TIL	Intrastromal TIL	Intratumoral TIL	CD3	CD4	CD8	CD20	Agregates nb	TLS nb	TLS nb/surface
Spearman's rho	FACS	TIL-B density (CD19+ cells/mg)	Correlation coefficient	0.849**	0.844**	0.775**	0.806**	1.000	0.629**	0.625**	0.543**	0.515**	0.433	0.452	0.736**	0.546**	0.592**	0.576**
			Sig. (2-tailed)	0.000	0.000	0.003	0.005		0.001	0.001	0.005	0.008	0.082	0.079	0.000	0.005	0.002	0.003
			N	25	19	12	10	25	25	25	25	25	17	16	25	25	25	25
	IHC	TIL-B density (% of CD20/tumor area)	Correlation coefficient	0.713**	0.701**	0.710**	0.705*	0.736**	0.920**	0.923**	0.784**	0.791**	0.452	0.513*	1.000	0.418*	0.720**	0.694**
			Sig. (2-tailed)	0.000	0.001	0.010	0.023	0.000	0.000	0.000	0.000	0.000	0.060	0.035		0.034	0.000	0.000
			N	25	19	12	10	25	26	26	26	26	18	17	26	26	26	26

**. Correlation is significant at the 0.01 level (2-Tailed).

*. Correlation is significant at the 0.05 level (2-Tailed).

Supplemental Table 4. TIL subsets correlations by FACS.

Correlations

Gated	Variable	Statistic	% CD19	% CD3	% CD4	% CD8	% NK	% NKT	% Resting Naive	% Activated Naive	% Pre-Germinal Center	% Germinal Center	% Early Memory	% Memory	% TLM	% IgG+ B	% Naive CD4	% Memory CD4	% PD1+CD4+	% TFH	% Naive CD8	% Memory CD8	% PD1+CD8+
Gated on TIL	% CD19	Pearson Correlation	1																				
		Sig. (2-tailed)																					
		N	350																				
	% CD3	Pearson Correlation	-,691**	1																			
		Sig. (2-tailed)	,000																				
		N	225	229																			
	% CD4	Pearson Correlation	-,049	,156*	1																		
		Sig. (2-tailed)	,521	,047																			
		N	176	163	180																		
	% CD8	Pearson Correlation	-,373**	,293**	-,257**	1																	
		Sig. (2-tailed)	,000	,000	,001																		
		N	171	159	173	174																	
	% NK	Pearson Correlation	-,386*	-,369*	-,168	,008	1																
		Sig. (2-tailed)	,035	,049	,384	,965																	
		N	30	29	29	29	31																
	% NKT	Pearson Correlation	-,356*	,239	-,441*	-,293	-,001	1															
		Sig. (2-tailed)	,050	,204	,015	,117	,997																
		N	31	30	30	30	31	32															
Gated on TIL-B	% Resting Naive	Pearson Correlation	-,004	-,178	-,266*	-,032	,429	,502	1														
		Sig. (2-tailed)	,967	,068	,012	,775	,288	,205															
		N	140	106	88	83	8	8	140														
	% Activated Naive	Pearson Correlation	,362**	-,236*	,280**	-,178	-,503	-,792*	-,188*	1													
		Sig. (2-tailed)	,000	,015	,008	,106	,204	,019	,026														
		N	140	106	89	84	8	8	140	141													
	% Pre-Germinal Center	Pearson Correlation	,013	,084	,290**	,046	-,540	-,801*	-,320**	,499**	1												
		Sig. (2-tailed)	,877	,392	,006	,677	,167	,017	,000	,000													
		N	140	106	88	83	8	8	140	140	140												
	% Germinal Center	Pearson Correlation	-,119	,102	,089	,245*	-,186	-,760*	-,416**	-,034	,472**	1											
		Sig. (2-tailed)	,161	,300	,408	,026	,660	,029	,000	,690	,000												
		N	140	106	88	83	8	8	140	140	140	140											
	% Early Memory	Pearson Correlation	-,133	,247*	,265*	,172	,313	,849**	-,547**	-,314**	-,057	,321**	1										
		Sig. (2-tailed)	,116	,011	,012	,119	,451	,008	,000	,000	,507	,000											
		N	140	106	89	84	8	8	140	140	140	140	141										
	% Memory	Pearson Correlation	-,215*	,103	-,191	,036	,509	,732*	,174*	-,621**	-,556**	-,417**	-,189*	1									
		Sig. (2-tailed)	,011	,292	,073	,748	,198	,039	,040	,000	,000	,000	,025										
		N	140	106	89	84	8	8	140	141	140	140	141	141									
	% TLM	Pearson Correlation	-,328**	,301*	-,275*	,575**	,431	,252	-,050	-,433**	-,127	,450**	,216*	,139	1								
		Sig. (2-tailed)	,001	,023	,044	,000	,393	,630	,643	,000	,235	,000	,042	,193									
		N	92	57	54	50	6	6	89	89	89	89	89	89	92								
	% IgG+ B	Pearson Correlation	-,204*	,143	-,071	,156	-,181	-,013	-,182	-,176	,021	,235*	,177	,051	,018	1							
		Sig. (2-tailed)	,049	,288	,607	,275	,732	,981	,092	,103	,845	,028	,100	,637	,865								
		N	93	57	55	51	6	6	87	87	87	87	87	87	89	93							
Gated on CD4+ TIL	% Naive CD4	Pearson Correlation	,231	-,193	,170	-,298	-,430	-,531	-,037	,376	-,158	,062	,086	-,341	-,586	-,645	1						
		Sig. (2-tailed)	,246	,344	,396	,132	,570	,469	,889	,137	,545	,812	,744	,181	,299	,240							
		N	27	26	27	27	4	4	17	17	17	17	17	17	17	5	27						
	% Memory CD4	Pearson Correlation	-,301	,142	-,430*	,445*	,707	,334	,155	-,380	,034	,102	-,127	,248	,906*	,312	-,526**	1					
		Sig. (2-tailed)	,127	,489	,025	,020	,293	,666	,553	,132	,896	,698	,627	,338	,034	,610	,005						
		N	27	26	27	27	4	4	17	17	17	17	17	17	17	5	27	27					
	% PD1+CD4+	Pearson Correlation	-,021	,002	-,073	,000	-,188	-,351	-,167	-,076	,418**	,511**	,289	-,330**	,481**	,149	-,533	,599	1				
		Sig. (2-tailed)	,827	,980	,452	,998	,604	,320	,161	,524	,000	,000	,014	,005	,000	,303	,174	,116					
		N	108	109	109	105	10	10	72	72	72	72	72	72	72	49	50	8	8	109			
	% TFH	Pearson Correlation	,023	-,001	-,033	,245*	-,359	-,320	-,230*	-,068	,248*	,629**	,295*	-,336**	,288*	,173	-,261	,216	,340**	1			
		Sig. (2-tailed)	,807	,991	,728	,011	,278	,337	,048	,562	,032	,000	,010	,003	,038	,216	,497	,577	,000				
		N	112	113	112	107	11	11	75	75	75	75	75	75	75	52	53	9	9	108	113		
Gated on CD8+ TIL	% Naive CD8	Pearson Correlation	-,156	,238	,093	,085	-1,000**	1,000**	,369	-,182	-,450	-,255	-,155	,067	-,079	-,078	,525**	-,525**	,342	-,451	1		
		Sig. (2-tailed)	,455	,252	,659	,687			,176	,517	,092	,360	,582	,812	,950	,951	,008	,008	,407	,262			
		N	25	25	25	25	2	2	15	15	15	15	15	15	3	3	24	24	8	8	25		
	% Memory CD8	Pearson Correlation	,156	-,238	-,093	-,085	1,000**	-1,000**	-,369	,182	,450	,255	,155	-,067	,079	,078	-,525**	,525**	-,342	,451	-1,000**	1	
		Sig. (2-tailed)	,455	,252	,659	,687			,176	,517	,092	,360	,582	,812	,950	,951	,008	,008	,407	,262	0,000		
		N	25	25	25	25	2	2	15	15	15	15	15	15	3	3	24	24	8	8	25	25	
	% PD1+CD8+	Pearson Correlation	-,121	,104	-,006	,292**	-,072	-,411	-,264*	-,049	,311*	,548**	,322**	-,321**	,632**	-,031	-,590	,656	,144	,481**	-,503	,503	1
		Sig. (2-tailed)	,229	,298	,954	,003	,866	,312	,031	,694	,010	,000	,008	,008	,000	,837	,218	,157	,149	,000	,309	,309	
		N	101	102	102	102	8	8	67	67	67	67	67	67	46	47	6	6	102	101	6	6	102

**. Correlation is significant at the 0.01 level (2-Tailed).

*. Correlation is significant at the 0.5 level (2-Tailed).

Supplemental Table 5. The antibodies used for flow cytometry.

Name		Clone	Conjugation	Firme	Reference	Applications
CD197	CCR7		PE	BD	552176	Flow cytometry
CD10		ALB1	APC-AlexaFluor700	Beckman Coulter	A86353	Flow cytometry
CD138		B-B4	APC	Miltenyi	130-091-250	Flow cytometry
CD14		TUK4	APC-Vio770	Miltenyi	130-096-622	Flow cytometry
CD19		4G7	PE	BD	345777	Cell sorting
CD19		LT19	APC-Vio770	Miltenyi	130-096-643	Flow cytometry
CD1d		51.1	PerCP-eFluor710	eBioscience	46-0016	Flow cytometry
CD20		2H7	AlexaFluor700	eBioscience	56-0209	Flow cytometry
CD200		OX104	PC7	eBioscience	25-9200-42	Cell sorting/Flow cytometry
CD21		BL13	FITC	Beckman Coulter	IM0473U	Flow cytometry
CD24		ML5	FITC	BD	555427	Flow cytometry
CD25		B1.49.9	APC-AlexaFluor700	Beckman Coulter	A86356	Flow cytometry
CD27		1A4CD27	PC7	Beckman Coulter	A54823	Flow cytometry
CD278	ICOS	ISA-3	PC7	eBioscience	25-9948-42	Flow cytometry
CD279	PD1	MIH4	FITC	eBioscience	nov-69	Cell sorting/Flow cytometry
CD3		UCHT1	ECD	Beckman Coulter	A07748	Cell sorting
CD3		BW264/56	VioBlue	Miltenyi	130-094-363	Flow cytometry
CD38		LS198.4.3	PC5.5	Beckman Coulter	A70205	Flow cytometry
CD4		M-T466	APC	Miltenyi	130-091-232	Cell sorting/Flow cytometry
CD40		HB14	APC	Miltenyi	130-094-137	Flow cytometry
CD45		5B1	VioGreen	Miltenyi	130-096-906	Flow cytometry
CD45RA		T6D11	PE-Vio770	Miltenyi	130-097-577	Flow cytometry
CD45RO		UCHL1	FITC	Miltenyi	130-095-462	Flow cytometry
CD5		UCHT2	PE	Miltenyi	130-096-575	Flow cytometry
CD56		N901 (HLDA6)	PE	Beckman Coulter	A07788	Flow cytometry
CD8		SFCI21Thy2D3	ECD	Beckman Coulter	737659	Flow cytometry
CD8		BW135/80	PerCP	Miltenyi	130-094-972	Flow cytometry
CD80		2D10	PE	Miltenyi	130-097-202	Flow cytometry
CD86		FM95	FITC	Miltenyi	130-094-878	Flow cytometry
CD185	CXCR5	TG2/CXCR5	PC5.5	Biolegend	335001	Flow cytometry
HLA-DR			ECD	Beckman Coulter		Flow cytometry
IgD		IgD26	PE	Miltenyi	130-094-539	Flow cytometry
IgG		IS11-3B2.2.3	VioBlue	Miltenyi	130-099-483	Flow cytometry
IgM		PJ2-22H3	APC	Miltenyi	130-093-076	Flow cytometry