CXCL13-producing T_{FH} cells link immune suppression and adaptive memory in human breast cancer

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Supplemental Methods

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Supplemental Figures

Supplemental Methods

Patient population and clinical samples

Tumor and blood samples were obtained from patients who underwent surgical resection for BC or benign breast tumors at the Institut Jules Bordet. Fresh tonsils (*n* = 10) and mammary reduction tissues (*n* = 5) were obtained from CHU Saint-Pierre. Prospectively collected fresh tumor tissues (*n* = 42; Table S3) and peripheral blood (PB; *n* = 9; Table S3) from BC patients were immediately analyzed by flow cytometry ex vivo. PB samples from 5 healthy female adults were used as controls or for CXCL13 induction experiments. Formalin-fixed paraffin embedded (FFPE) tumor tissues from 60 BC patients were used for survival analysis (retrospective set; whole genome gene expression microarray data available in Dedeurwaerder *et al*., 2011)(Table S1). These samples were analyzed by quantitative PCR (qPCR) and IHC. In addition, FFPE tumor tissues from 10 BC patients were immunofluorescent (IF)- and IHC-stained.

Flow cytometry

Tissue fragments from fresh surgical specimens were processed as previously described (Garaud *et al*., 2014; Gu-Trantien *et al*., 2013). Briefly, dissected tissues were directly transferred into 3 ml of X-VIVO 20 (Lonza) before two 30s rounds of mechanical dissociation with the GentleMACS™ Dissociator (Program A.01; Myltenyi Biotec). The resulting cell suspension was filtered following each dissociator run using a 40 µm cell strainer (BD Falcon), washed with X-VIVO 20, centrifuged 15 min at 600 g and resuspended in X-VIVO 20 before flow cytometric analysis or cell sorting. For flow cytometric analysis, samples were surface stained for 35 min at 4 °C followed by dead cell staining using Fixable Viability Dye (eBioscience) before intracellular staining with FoxP3 Staining Buffer Set (eBioscience). For control D-PB and P-PB, surface staining was performed in 100 µl of fresh whole blood followed by downstream steps as above without using red blood cell lysis reagents. CXCL13 staining was performed directly ex vivo without using Golgi-stop reagents and the entire staining procedure was completed within 6-hours of tumor resection. Brefeldin A (BD Biosciences; see **In vitro stimulation** below) was added for experiments where IFNγ was detected. Ab used are listed in the table below. Data were acquired on a Navios Flow Cytometer and analyzed using Kaluza (Beckman Coulter) and FlowJo softwares. B cell subpopulations are determined using criteria in Bohnhorst *et*

al., 2001, Green *et al*., 2012, Perez-Andres *et al*., 2010 and Zirakzadeh *et al*. 2013.

Ab used for flow cytometric analysis				
Name	**Color**	**Company**	**Reference**	**Clone**
BCL6	PE	BD Biosciences	561522	K112-91
CCR7	AC7	Biolegend	353211	G043H7
CCR7	BV510	Biolegend	353231	G043H7
CD3	VioBlue	Miltenyi Biotec	130-094-363	BW264/56
CD4	AF700	eBioscience	56-0049	RPA-T4
CD8	BV510	BD Biosciences	563919	SK1
CD19	APC	R&D Systems	FAB4867A	4G7-2E3
CD25	PF710	eBioscience	46-0257	CD25-4E3
CD27	PC7	Beckman Coulter	A54823	1A4CD27
CD38	PF710	eBioscience	46-0388	HB7
CD45RA	PTR	Life Technologies	MHCD45RA17	MEM-56
CD56	PE	eBioscience	12-0567	CMSSB
CD200	PE	Biolegend	329206	OX-104
CTLA-4	PE	BD Biosciences	555853	BNI3
CTLA-4	BV421	BD Biosciences	562743	BNI3
CXCL13	APC	R&D Systems	IC801A	53610
CXCR5	PE	R&D Systems	FAB190P	51505
CXCR5	PF610	eBioscience	61-9185	MU5UBEE
CXCR5	BV421	BD Biosciences	562747	RF8B2
Fixable Viability Dye	eF780	eBioscience	65-0865	
FoxP3	PF610	eBioscience	61-4776	PCH101
FoxP3	PC5.5	eBioscience	45-4776	PCH101
ICOS	PC7	eBioscience	25-9948	ISA-3
IFNγ	PE	eBioscience	12-7319	4S.B3
IFNγ	PC7	BD Biosciences	557643	B27
IgD	PE	Miltenyi Biotec	130-094-539	IgD26
Ki67	FITC	eBioscience	11-5699	20Raj1
Ki67	PF710	eBioscience	46-5699	20Raj1
PD-1	FITC	eBioscience	11-9969	MIH4
PD-1	PF710	eBioscience	46-9969	MIH4
T-bet	BV421	BD Biosciences	563318	O4-46
TIGIT	PF710	eBioscience	46-9500	MBSA43

IHC and Immunofluorescence

FFPE sections (4 µm) from available tumor tissues were deparaffinized and demasked prior to Ab labeling. Ab are listed in the table below. FFPE tumor blocs from 60 patients of the retrospective set (Table S1) were labeled with αCD20 (*n* = 51), αCD23 or αCD45 Ab. Four select tumors from the prospective patient set (Table S3) were dual-labeled with αCD3/αCD20 or with αCD4/αCD8. IHC Ab staining on full-face sections was performed using the iVIEW DAB Detection Kit and a BenchMark XT IHC/ISH automated slide stainer (Discovery XT, Tucson, USA). Slides were read by 2 trained pathologists. IHC images were acquired at Lens 20x with a Nanozoomer 2.0-RS Digital slide scanner (Hamamatsu) and analyzed using NDP.view2 software. These methods were standardized as described in Buisseret *et al*., *in press*.

FFPE blocs from 10 BC tumors and 4 tonsils were analyzed using IF. Mounted sections were deparaffinized with xylene, pretreated with citrate 10 mM, pH 6.0 at 95 °C for 30 min, blocked with 1% BSA for 30 min prior to incubation with a mixture of 3 primary Ab including αCXCL13 in a moist chamber at 4 °C overnight. After washing, a mixture of 3 secondary Ab was added for 2-hours at room temperature followed by mounting in a Prolong Gold Antifade Reagent with DAPI (Life Technologies). Slides were analyzed using a Zeiss 710 confocal microscope (Carl Zeiss). Images were acquired at 20X Magnification and analyzed using ZEN software. Two different magnifications from the same original images are presented for each boxed region shown in the Figures.

Ab used for IHC staining					
Antigens	Dilution	Company	Reference	Host	Clone
CD3	prediluted	Dako	IR503	Rabbit	polyclonal
CD4	prediluted	BioSB	BSB5150	Rabbit	RBT-CD4
CD8	prediluted	Dako	IR623	Mouse	C8/144B
CD20	prediluted	Dako	IR604	Mouse	L26
CD23	prediluted	Ventana	790-4408	Rabbit	SP23
CD45	prediluted	Dako	IR751	Mouse	2B11 + PD7/26
Primary Ab used for IF staining					
Antigens	Dilution	Company	Reference	Host	Clone
CD3	prediluted	Dako	IS503	Rabbit	polyclonal
CD4	1/50	Cell Marque	104R-14	Rabbit	SP35
CD8	1/50	Thermo	RM-9116-S1	Rabbit	SP16
CD20	1/50	Abcam	ab9475	Mouse	L26
CD21	1/500	Abcam	ab75985	Rabbit	EP3093
CXCL13	1/200	R&D Systems	AF801	Goat	polyclonal
IFN-γ	1/100	R&D Systems	MAB2851	Mouse	25723
IgA	1/100	Cell Marque	267A-14	Rabbit	polyclonal
IgG	1/100	Cell Marque	269A-14	Rabbit	polyclonal
Ki67	1/50	Abcam	ab16667	Rabbit	SP6
T-bet	1/400	R&D Systems	MAB5385	Mouse	525803
Secondary Ab used for IF staining					
Antigens	Dilution	Company	Reference	Host	Reactivity
DyLight 488	1/200	Abcam	ab98514	Donkey	Goat
DyLight 550	1/200	Abcam	ab98767	Donkey	Mouse
DyLight 650	1/200	Abcam	ab98491	Donkey	Rabbit

Cell sorting and isolation

For molecular analyses, $CD4^+$ T cell subpopulations were sorted using a MoFlo Astrios Cell Sorter (Beckman Coulter) from 5 tonsils (mononuclear cells frozen in DMSO) and 1 fresh BC homogenate. The cell purification protocols were controlled by flow cytometry with >95% purity observed. Total $CD4^+$ TIL were positively purified from 21 fresh BC homogenates using Dynabeads CD4 (Life Technologies) with the remaining non-CD4 cells also included for analysis (Gu-Trantien *et al*., 2013).

Quantitative PCR

RNA was extracted using RNeasy FFPE kit (Qiagen) or TRIzol Reagent (Invitrogen) for FFPE samples and sorted cells, respectively, before reverse transcription to cDNA using High Capacity RNA-to-cDNA kit (Life Technologies). Dr. Christos Sotiriou and his group, Breast Cancer Translational Research Laboratory, Institut Jules Bordet, Brussels, Belgium, kindly provided cDNA samples from some patients in the retrospective series (Table S1; Dedeurwaerder *et al*., 2011). FFPE samples were preamplified using Taqman PreAmp Master Mix (Life Technologies) before real-time PCR reactions using commercially available Gene Expression Assays (listed in the table below) and TaqMan Universal Master Mix II with UNG (Life Technologies). Sorted cells were analyzed without preamplification using the same primers as previously described (Gu-Trantien *et al*., 2013; in the table below) and iTaq SYBR Green Supermix with ROX (Bio-Rad). PCR reactions were run on a 7900HT Fast Real-Time PCR System (Life Technologies). House-keeping genes included: *POLR2A*, *TBP* and *TMBIM4* for FFPE samples, *SDHA*, *TBP* and *TMBIM4* for CD4-depleted fresh BC cells or *CASC3* for purified $CD4^+$ T cell and subpopulations.

Taqman Gene Expression Assays used for FFPE samples			
Gene Name	**Company**	**Assay ID**	**Amplicon Length (bp)**
POLR2A	Life technologies	Hs00172187_m1	61
TBP	Life technologies	Hs00427621_m1	65
TMBIM4	Life technologies	Hs00211390_m1	63
CXCL13	Life technologies	Hs00757930_m1	70

SyBR Green Gene Expression Assays used for fresh samples		
Gene Name	**Company**	**Assay ID**
TBX21	Qiagen	QT00042217
TMBIM4	Qiagen	QT00070329

Primers used for fresh samples		
Gene	**Forward**	**Reverse**
BCL6	AGCAAGGCATTGGTGAAGACA	ATGGCGGGTGAACTGGATAC
CASC3	CAAGGAAGGTCGTGCTGGTT	ACCAGACCGGCCACCAT
CXCL13	GAGGCAGATGGAACTTGAGC	CTGGGGATCTTCGAATGCTA
FOXP3	CTGCCCCTAGTCATGGTGG	CTGGAGGAGTGCCTGTAAGTG
IFNG	GTTTTGGGTTCTCTTGGCTGTTA	AAAAGAGTTCCATTATCCGCTACATC
IL10	GCTGGAGGACTTTAAGGGTTACCT	CTTGATGTCTGGGTCTTGGTTCT
IL21	CATGGAGAGGATTGTCATCTGTC	CAGAAATTCAGGGACCAAGTCAT
PDCD1	GCCTGGTGCTGCTAGTCTG	GGCATACTCCGTCTGCTCAG
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
TBP	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA

In vitro stimulation

Total PBMC from 5 healthy controls were stimulated with plate-bound αCD3/αCD28 Ab (10 µg/ml each) or Staphylococcal Enterotoxin B (SEB; 500 ng/ml) in X-VIVO 20 (Lonza) for 3-days before analysis by flow cytometry. An αIL2 Ab (1 µg/ml for αCD3/αCD28 and 10 µg/ml for SEB stimulation conditions, respectively) or TGFβ1 (5 ng/ml) were added at time zero. For IFNγ detection, Brefeldin A (dose recommended by BD Biosciences) was added 4-hours before staining. Reagents are listed in the table below. Fresh BC tumor tissues were stimulated with SEB ± αIL2 Ab for 18-hours in the presence of 0.5x dose of Brefeldin A before mechanical dissociation to obtain a single cell suspension for flow cytometric analysis. The tumor supernatant (SN) equals the initial 3 ml of X-VIVO 20 recovered after the first round of tumor tissue dissociation (ex vivo samples), which was subsequently clarified by centrifugation for 15 min at 13000 g (Gu-Trantien *et al*., 2013). Frozen tonsil mononuclear cells (*n* = 3) were briefly B cell-depleted using CD19 MicroBeads (Miltenyi Biotec) before treatment with tumor SN (*n* = 12) or CXCL13 (10 ng/ml) for 24-hours.

Products used for in vitro stimulation experiments			
Product	**Company**	**Reference**	**Clone**
αCD3	eBioscience	16-0037	OKT3
αCD28	BD Biosciences	555725	CD28.2
Staphylococcal enterotoxin B (SEB)	Sigma-Aldrich	S4881	
αIL2	R&D Systems	MAB202	5334
CD19 MicroBeads	Miltenyi Biotec	130-050-301	
CXCL13	R&D Systems	801-CX-025	
TGFβ1	R&D Systems	240-B	
Brefeldin A	BD Biosciences	555029	

Public data analyses

RNA sequencing expression data for 1058 BC patients (TCGA, 2012) were downloaded from the TCGA website (https://tcga-data.nci.nih.gov/tcga/) in July 2015. Expression intensities in RPKM were log-2 normalized before the data were used in the frequency plots. Pearson correlation coefficients for each gene with *CXCL13* for the BC subtypes (determined using PAM50 gene signature) were estimated by linear regression analysis.

Statistics

Statistical analysis (except for Cox analysis in Table S1) was performed using GraphPad Prism 6 software. *P* values for 2-group comparisons were generated using a 2-tailed student *t* test with unequal variance, either paired or unpaired depending upon the samples. One-way ANOVA was applied for multiple- (more than 2-) group comparisons, either matched or unmatched depending upon the samples. Tukey's or Sidak's tests were used for *P* value correction depending upon the comparisons performed across all or between side-by-side groups, respectively, within each data set. Data associated with the above analyses are represented as mean ± SEM in all Figures except for paired representations. The linear regression method was used for most of the correlation analyses. When appropriate, nonlinear fits were applied to reach the best fit. *P* values were calculated using correlation coefficients and sample sizes. The log-rank (Mantel-Cox) test was used for calculating *P* values for the survival analyses. Multivariate Cox analysis was performed using R software. *P* values <0.05 are considered significant. Degrees of significance: $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) and $P<0.0001$ (****).

Supplemental References:

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Perez-Andres M, *et al*. Primary Health Care Group of Salamanca for the Study of MBL. Human peripheral blood B-cell compartments: a crossroad in B-cell traffic. Cytometry B Clin Cytom. 2010;78.

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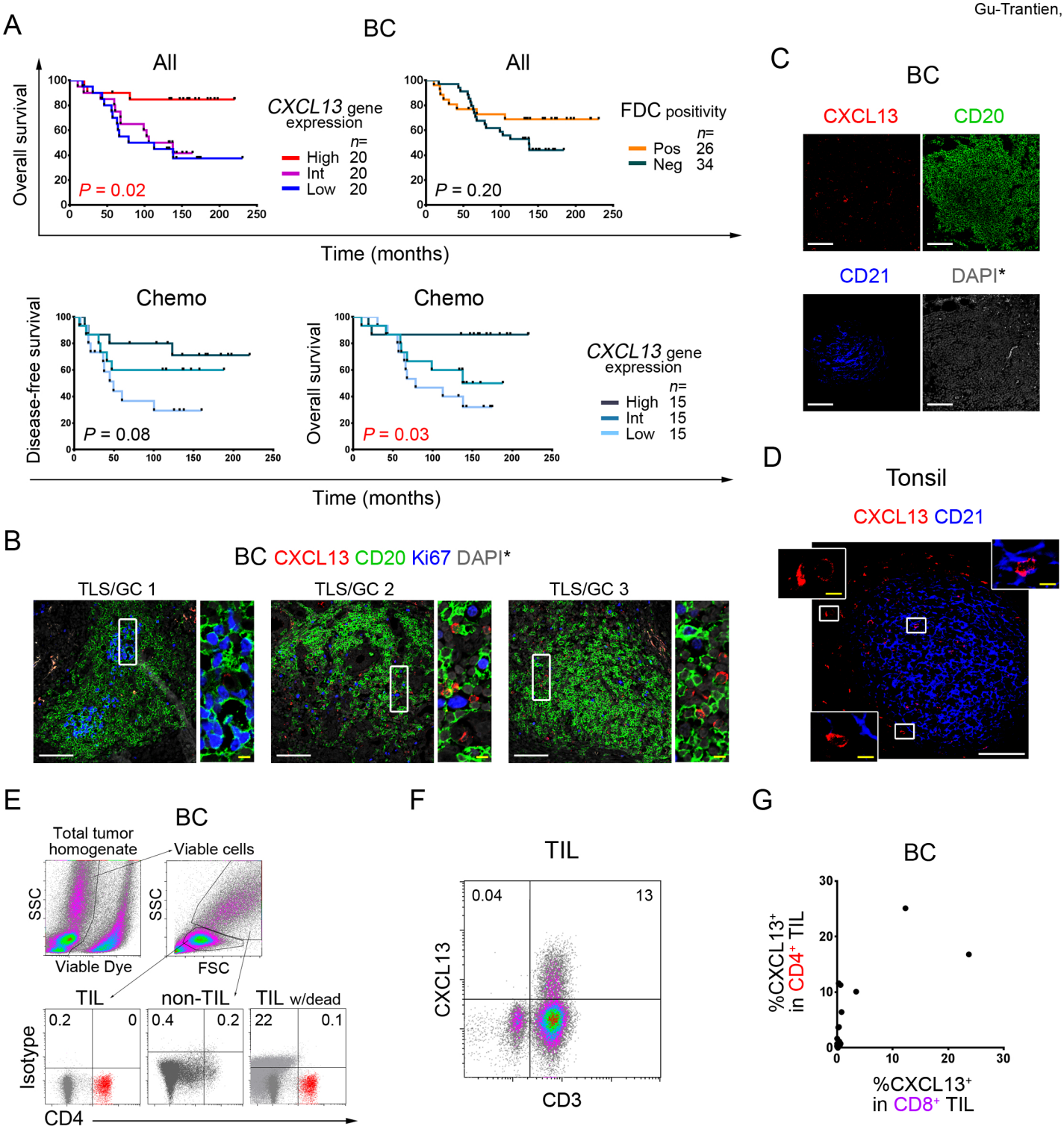
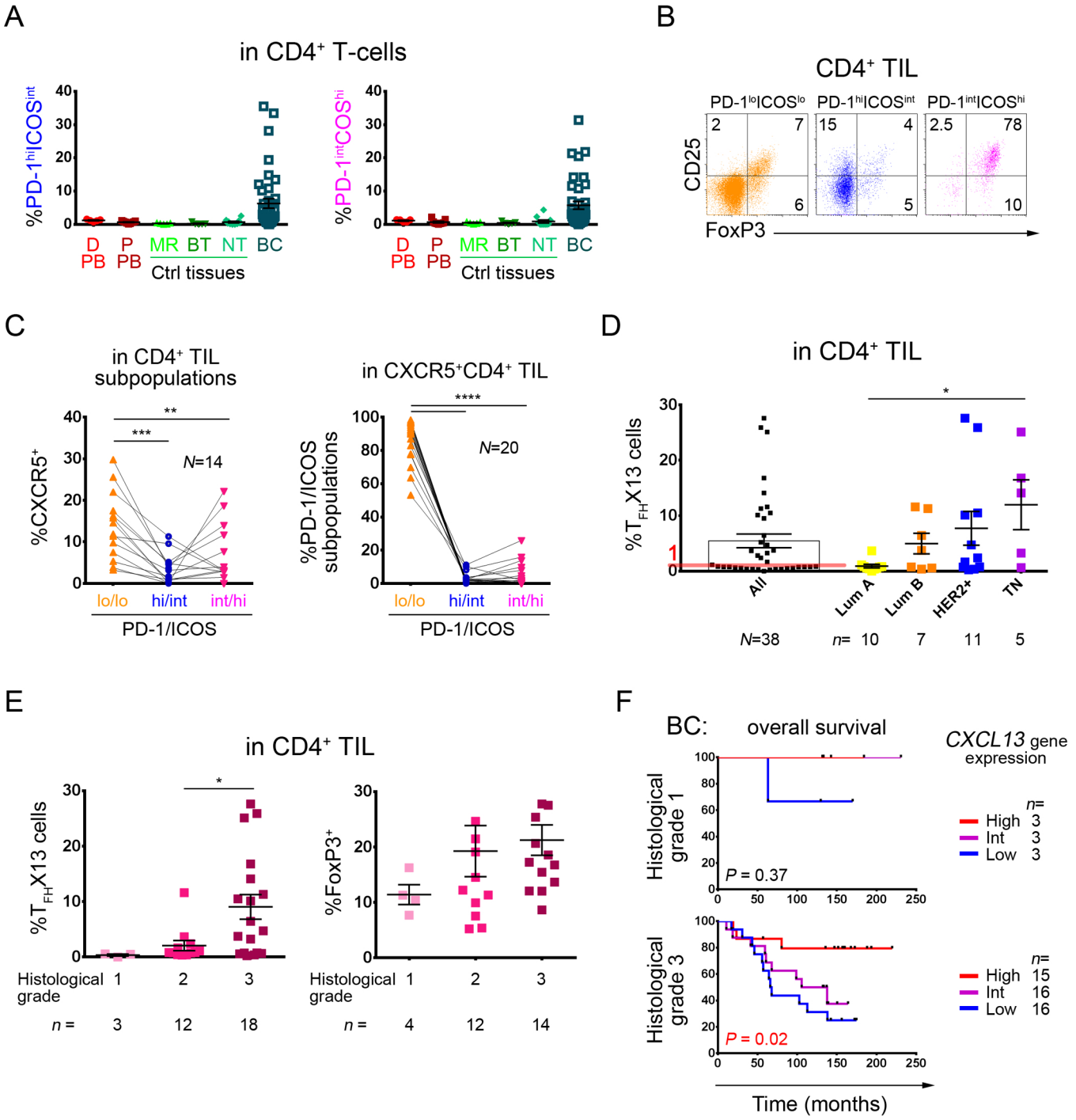
Supplemental Figures


A
BC
All
Overall survival
P = 0.02
CXCL13 gene expression
n=
High 20
Int 20
Low 20
All
P = 0.20
FDC positivity
n=
Pos 26
Neg 34
Time (months)
Chemo
Disease-free survival
P = 0.08
Chemo
Overall survival
P = 0.03
CXCL13 gene expression
n=
High 15
Int 15
Low 15
Time (months)
C
BC
CXCL13
CD20
CD21
DAPI*
B
BC CXCL13 CD20 Ki67 DAPI*
TLS/GC 1
TLS/GC 2
TLS/GC 3
D
Tonsil
CXCL13 CD21
E
BC
Total tumor homogenate
Viable cells
SSC
Viable Dye
FSC
TIL
non-TIL
TIL w/dead
Isotype
CD4
0.2
0
0.4
0.2
22
0.1
F
TIL
0.04
13
CXCL13
CD3
G
BC
%CXCL13+ in CD4+ TIL
%CXCL13+ in CD8+ TIL
H
BC
CXCL13
CD3
Merge

Supplemental Figure 1 (Supporting data for Figure 1).

(**A**) Kaplan-Meier analysis: (top) breast cancer (BC) overall survival rates stratified on *CXCL13* gene expression or follicular DC (FDC) positivity; (bottom) BC disease-free and overall survival rates stratified on *CXCL13* gene expression for the subgroup of patients receiving adjuvant chemotherapy. (**B-D**, **H**) Immunofluorescence (IF) staining of BC tissue sections; the zoomed areas are defined by white boxes. *In some images, the contrast was enhanced by turning down DAPI (gray) intensity. White and yellow scale bars: 100 μm and 10 μm, respectively. (**B**) $CXCL13^+$ (red), $CD20^+$ (green) and $Ki67^+$ (blue) cells identify germinal centers (GC) containing proliferating ($Ki67^+$) $CD20^+$ B- tumor-infiltrating lymphocytes (TIL) in the 3 tertiary lymphoid structures (TLS) shown in Figure 1B. (**C**) Single channel images for the complete TLS shown in Figure 1C. (**D**) $CXCL13^+$ (red) and $CD21^{hi}$ (blue) cells in a tonsil GC. (**E**) Gating strategies for flow cytometric analysis ($CD4^+$ BC TIL in red) and the control images for CXCL13 staining shown in Figure 1D using an isotype control Ab. Low levels of nonspecific CXCL13 control staining are detectable in the viable TIL gate on cells negative for TIL subpopulation markers (ex: $CD4^-$). For this reason, samples with <1% $CXCL13^+$ cells in the viable TIL gate are considered to be $CXCL13^{neg}$. This background was higher in the viable non-TIL gate and markedly increased without dead cell exclusion even in the TIL gate. (**F**) Staining of CXCL13 in conjunction with the pan T cell marker CD3 in the TIL gate. (**G**) Correlation plot showing % $CXCL13^+$ in $CD4^+$ and $CD8^+$ TIL. (**H**) IF staining of $CXCL13^+$ (red) and $CD3^+$ (green) TIL in BC tissues. The yellow arrow indicates infrequently detected $CXCL13^+CD3^-$ TIL.


A
in CD4+ T-cells
%PD-1hiICOSint
%PD-1intCOShi
D PB
P PB
MR
BT
NT
BC
Ctrl tissues
B
CD4+ TIL
PD-1loICOSlo
PD-1hiICOSint
PD-1intICOShi
CD25
FoxP3
C
in CD4+ TIL subpopulations
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N=20
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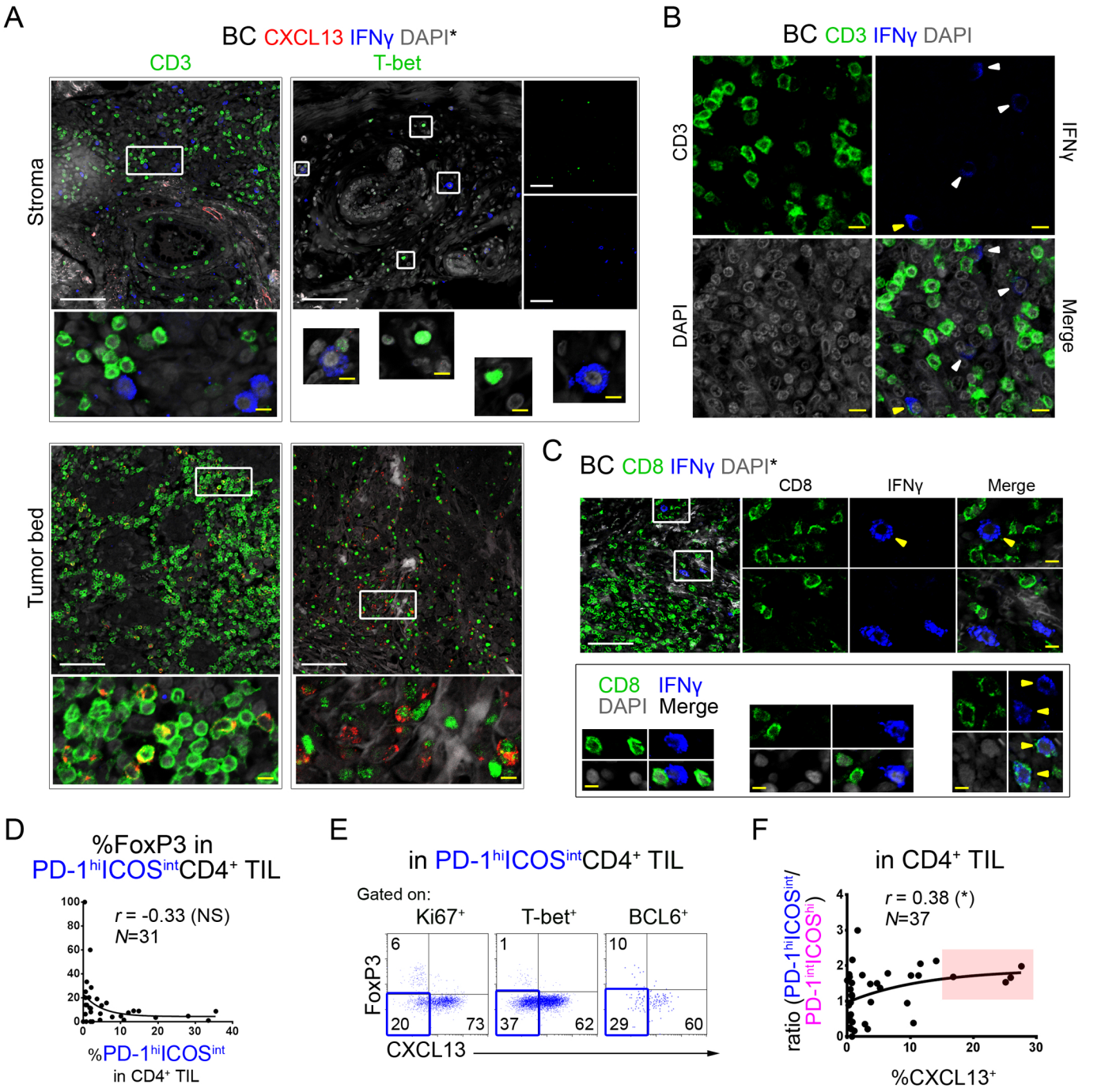
hi/int
int/hi
PD-1/ICOS
D
in CD4+ TIL
%TFHX13 cells
All
Lum A
Lum B
HER2+
TN
N=38
n= 10 7 11 5
E
in CD4+ TIL
%TFHX13 cells
%FoxP3+
Histological grade
n = 3 12 18
n = 4 12 14
F
BC: overall survival
CXCL13 gene expression
Histological grade 1
Histological grade 3
P = 0.37
P = 0.02
High 3
Int 3
Low 3
High 15
Int 16
Low 16
Time (months)

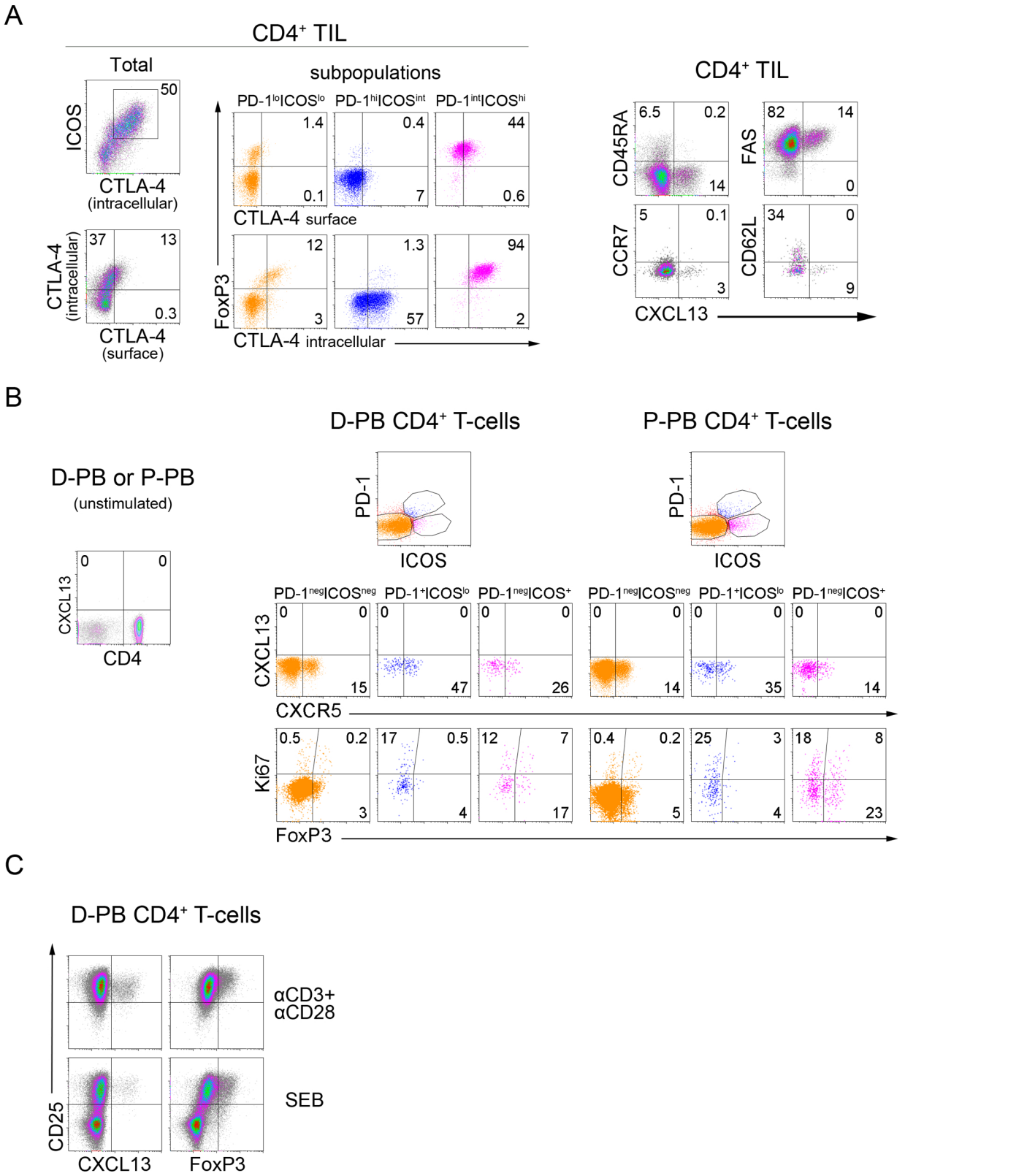
Supplemental Figure 2 (Supporting data for Figure 2).

(**A**) Percentage of PD-1^{hi}ICOS^{int} (left) and PD-1^{int}ICOS^{hi} (right) subpopulations in CD4^{+} T cells from healthy donor peripheral blood (D-PB; *n* = 5), breast cancer (BC) patient PB (P-PB; *n* = 9), mammary reduction tissues (MR; *n* = 5), benign breast tumors (BT; *n* = 5), nonadjacent nontumor breast tissues from BC patients (NT; *n* = 10) and BC (*n* = 40). (**B**) Flow cytometry dot plots showing CD25 and FOXP3 expression patterns for the 3 PD-1/ICOS-defined CD4^{+} tumor-infiltrating lymphocyte (TIL) subpopulations. (**C**) CXCR5 expression in PD-1/ICOS-defined CD4^{+} TIL subpopulations: (left) % CXCR5^{+} in each defined subpopulation; (right) % CXCR5^{+}CD4^{+} TIL that display a PD-1^{lo}ICOS^{lo}, PD-1^{hi}ICOS^{int} or PD-1^{int}ICOS^{hi} phenotype. (**D**) T_{FH}X13 cell abundance (% CXCL13^{+} in CD4^{+} TIL) in BC (all or subtypes). BC (all) are also shown in Figure 2C. (**E**) Percentage of CXCL13^{+} or FOXP3^{+} in CD4^{+} TIL stratified on histological tumor grade. (**F**) Kaplan-Meier analysis of BC overall survival rates for grade 1 and grade 3 tumors stratified on *CXCL13* gene expression. (**C-E**) One-way ANOVA followed by Tukey's test. $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) and $P<0.0001$ (****).


A
BC CXCL13 IFNγ DAPI*
CD3
T-bet
Stroma
Tumor bed
B
BC CD3 IFNγ DAPI
CD3
IFNγ
DAPI
Merge
C
BC CD8 IFNγ DAPI*
CD8
IFNγ
Merge
CD8 IFNγ
DAPI Merge
D
%FoxP3 in
PD-1hiICOSintCD4+ TIL
r = -0.33 (NS)
N=31
%PD-1hiICOSint
in CD4+ TIL
E
in PD-1hiICOSintCD4+ TIL
Gated on:
Ki67+
T-bet+
BCL6+
FoxP3
CXCL13
6
20
73
1
37
62
10
29
60
F
in CD4+ TIL
r = 0.38 (*)
N=37
ratio (PD-1hiICOSint/
PD-1intICOShi)
%CXCL13+

Supplemental Figure 3 (Supporting data for Figure 3).

(**A-C**) Immunofluorescence (IF) staining of breast cancer (BC) tissue sections; the zoomed areas are defined by white boxes. IFNγ^{+} (blue) (and CXCL13^{+} [red] in **A**) with T-bet^{+}, CD3^{+} or CD8^{+} (green) cells. *In some images, the contrast was enhanced by turning down DAPI (gray) intensity. White and yellow scale bars: 100 μm and 10 μm, respectively. (**B** and **C**) White arrows indicate IFNγ^{+}CD3^{-} non-T cells; yellow arrows point to the infrequently detected colocalization of IFNγ with the T cell markers CD3 or CD8. (**D**) Correlation between PD-1^{hi}ICOS^{int} CD4^{+} tumor-infiltrating lymphocytes (TIL; %) and % FOXP3^{+} within this subpopulation. NS = non-significant. (**E**) FOXP3 and CXCL13 expression patterns in Ki67^{+}, T-bet^{+} or BCL6^{+} PD-1^{hi}ICOS^{int}CD4^{+} TIL. Blue frames indicate FOXP3^{-}CXCL13^{-} cells. (**F**) Correlation between cells (%) expressing CXCL13 and the PD-1^{hi}ICOS^{int}/PD-1^{int}ICOS^{hi} ratio within CD4^{+} TIL. Red zone identifies high T_{FH}X13 TIL levels where the PD-1^{hi}ICOS^{int}/PD-1^{int}ICOS^{hi} ratio is stable. (**D** and **F**) $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) and $P<0.0001$ (****).


A
CD4+ TIL
Total
ICOS
CTLA-4 (intracellular)
CTLA-4 (intracellular)
CTLA-4 (surface)
subpopulations
PD-1loICOSlo
PD-1hiICOSint
PD-1intICOShi
CTLA-4 surface
FoxP3
CTLA-4 intracellular
CD4+ TIL
CD45RA
FAS
CCR7
CD62L
CXCL13
B
D-PB or P-PB
(unstimulated)
CXCL13
CD4
D-PB CD4+ T-cells
P-PB CD4+ T-cells
PD-1
ICOS
PD-1negICOSneg
PD-1+ICOSlo
PD-1negICOS+
CXCR5
Ki67
FoxP3
C
D-PB CD4+ T-cells
αCD3+
αCD28
SEB
CD25
CXCL13
FoxP3

Supplemental Figure 4 (Supporting data for Figure 4).

(**A**)(Left) Intracellular and surface CTLA-4 expression in total and PD-1/ICOS-defined $CD4^+$ tumor-infiltrating lymphocyte (TIL) subpopulations. Surface ICOS and intracellular CTLA-4 are coexpressed in the majority of $CD4^+$ TIL; (right) expression of additional (in addition to Figure 4A) surface markers on $CXCL13^+CD4^+$ TIL. (**B**)(Left) CXCL13 expression is not detected in unstimulated healthy donor (D-PB) and patient (P-PB) peripheral blood $CD4^+$ T cells; (right) expression of T_{FH}/Treg markers CXCL13, CXCR5 and FOXP3 together with the proliferation marker Ki67 in PD-1/ICOS-defined D-PB and P-PB $CD4^+$ T cells. (**C**) CD25 expression on activated (αCD3/αCD28 Ab or Staphylococcal enterotoxin B [SEB]) D-PB $CD4^+$ T cells that express either CXCL13 or FOXP3.

Figure S5
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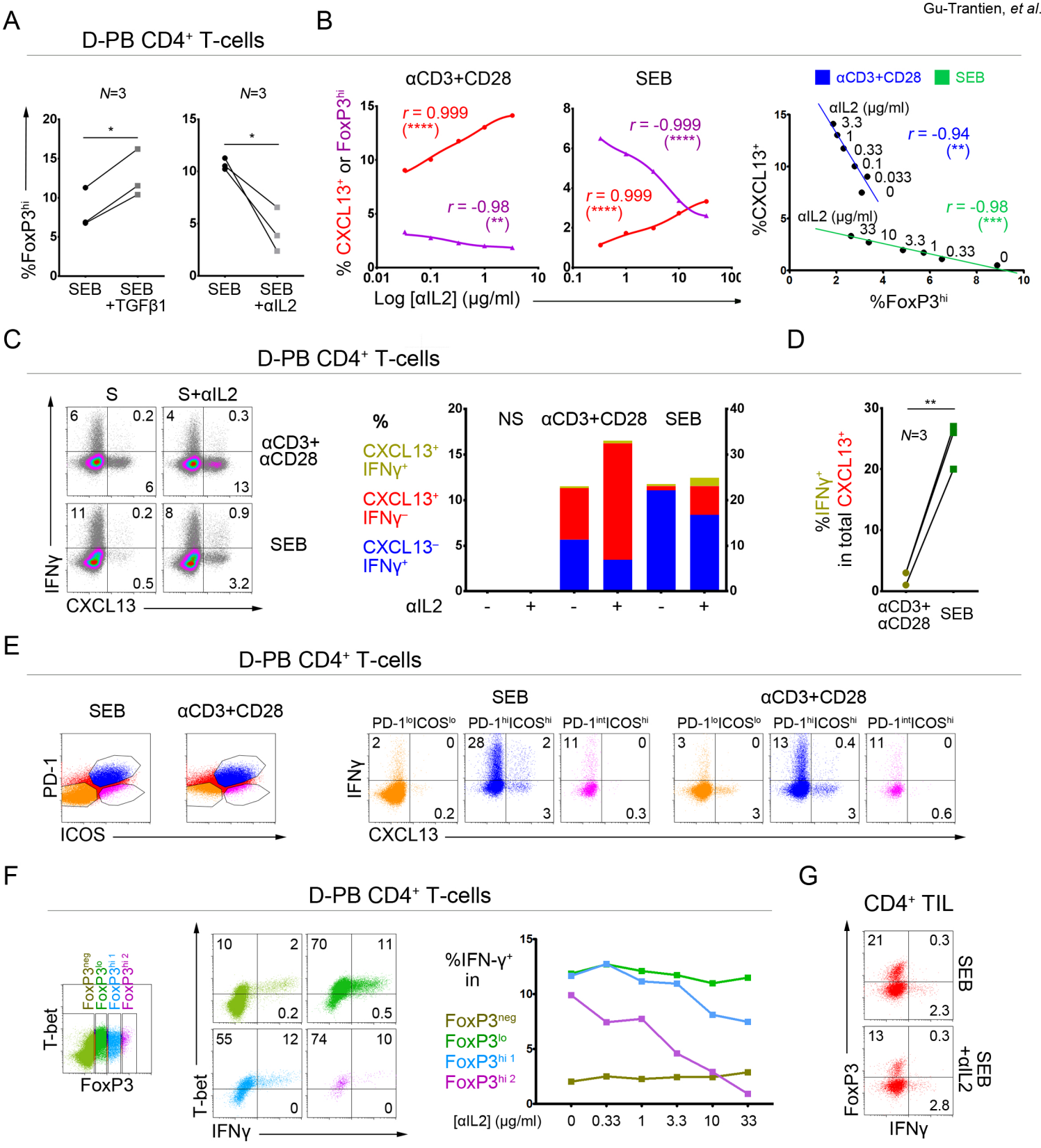
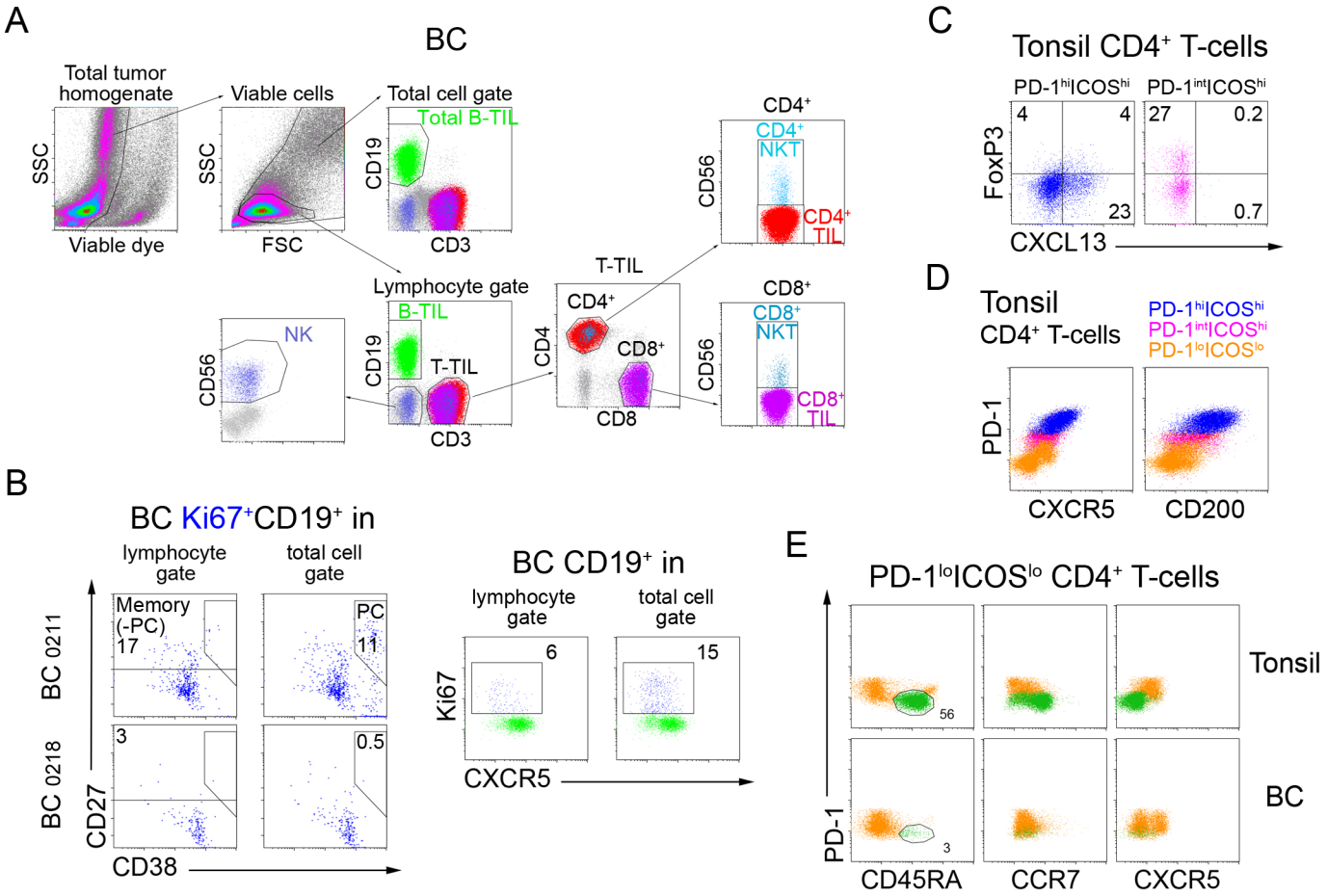


Supplemental Figure 5 (Supporting data for Figure 5).

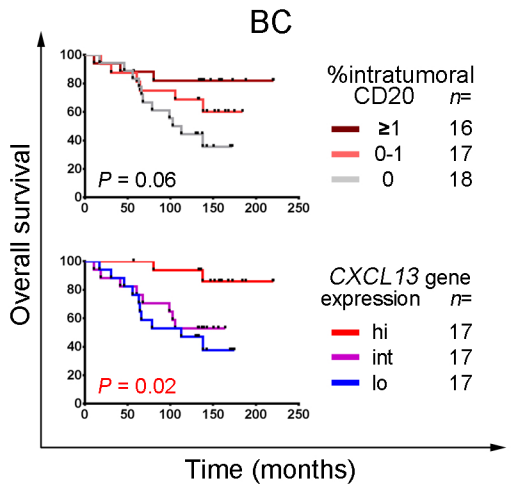
(**A**) % FOXP3^{hi} in Staphylococcal enterotoxin B (SEB)-stimulated healthy donor peripheral blood (D-PB) CD4^{+} T cells: (left) ± TGFβ1; (right) ± αIL2 blocking Ab. Data in the left and right panels are from independent experiments. (**A** and **D**) Paired 2-tailed student *t* test. (**B**) Correlation plots showing D-PB CD4^{+} T cells (%) expressing CXCL13 or FOXP3^{hi} after stimulation (αCD3/αCD28 Ab or SEB) with different concentrations of an αIL2 Ab. (**C**)(dot plots) IFNγ and CXCL13 expression in stimulated (S) D-PB CD4^{+} T cells ± αIL2 Ab; (graph) IFNγ or CXCL13 single or double positive cells (%) in unstimulated (NS) and stimulated cells ± αIL2 Ab (the latter shown in the dot plots). (**A**, **C**, **G**) αIL2 Ab were used at 1 μg/ml and 10 μg/ml, respectively for αCD3/αCD28 Ab and SEB stimulation. (**D**) % IFNγ^{+} in CXCL13^{+}CD4^{+} T cells induced by stimulation with αCD3/αCD28 Ab or SEB. (**E**)(left) PD-1 and ICOS expression on stimulated D-PB CD4^{+} T cells does not define distinct subpopulations corresponding to those in BC TIL; (right) IFNγ and CXCL13 expression are enriched in the gate area of PD-1^{hi}ICOS^{hi} cells, which is most pronounced in the SEB-stimulated cells. (**F**)(dot plots)(left) Gating strategies defining 4 subpopulations with different intensities of FOXP3 expression after SEB stimulation of D-PB CD4^{+} T cells and (middle) IFNγ and T-bet expression in these subpopulations; (graph)(right) IFNγ expression in the 4 FOXP3-defined subpopulations in relation to addition of increasing concentrations of αIL2 Ab during stimulation. (**G**) IFNγ and FOXP3 expression in SEB-stimulated CD4^{+} tumor-infiltrating lymphocytes (TIL) (± αIL2 Ab) from a different BC than that shown in Figure 5F. (**A**, **B**, **D**) $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) and $P<0.0001$ (****).


A
BC
Total tumor homogenate
Viable cells
Total cell gate
Total B-TIL
SSC
Viable dye
FSC
CD19
CD3
CD4+
CD4+ NKT
CD4+ TIL
CD56
Lymphocyte gate
B-TIL
T-TIL
NK
CD4
CD8+
CD8
CD8+ NKT
CD8+ TIL
C
Tonsil CD4+ T-cells
PD-1hiICOShi
PD-1intICOShi
FoxP3
CXCL13
4
4
23
27
0.2
0.7
D
Tonsil
CD4+ T-cells
PD-1hiICOShi
PD-1intICOShi
PD-1loICOSlo
PD-1
CXCR5
CD200
B
BC Ki67+CD19+ in
lymphocyte gate
total cell gate
BC 0211
BC 0218
Memory (-PC)
17
PC
11
3
0.5
CD27
CD38
BC CD19+ in
lymphocyte gate
total cell gate
6
15
Ki67
CXCR5
E
PD-1loICOSlo CD4+ T-cells
Tonsil
BC
56
3
PD-1
CD45RA
CCR7
CXCR5

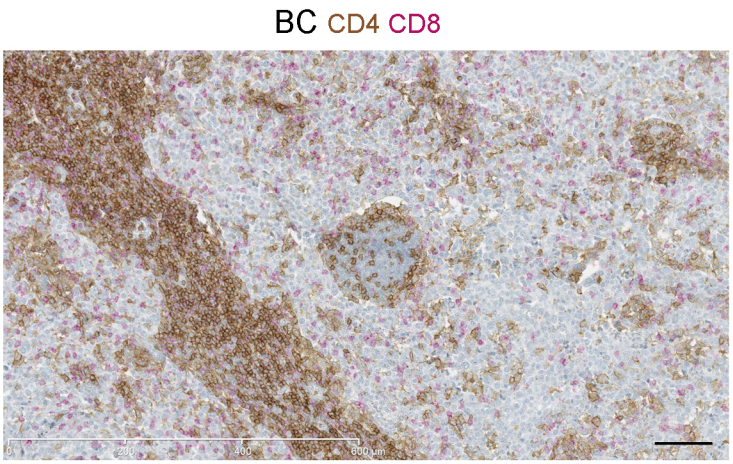
Supplemental Figure 6 (Supporting data for Figure 6).

(**A**) Flow cytometry gating strategies for defining lymphocyte subpopulations in the viable cell gate from breast cancer (BC) tumor homogenates. In some tumors, substantial numbers of $CD19^+$ B cells are detected both in the lymphocyte and the nonlymphocyte gates thus we defined “total B-TIL” (found in the viable total cell gate) and “B-TIL” (viable lymphocyte gate) for analysis of specific B cell subpopulations. TIL = tumor-infiltrating lymphocytes. (**B**)(left) Gating strategies used to define the 2 parameters used for the correlation analysis shown in Figure 6F (images from 2 additional tumors that are not shown in Figure 6D); (right) Ki67 and CXCR5 expression in B-TIL (lymphocyte gate) or total B-TIL (total cell gate). (**C**) FOXP3 and CXCL13 expression in tonsillar $PD\text{-}1^{hi}ICOS^{hi}$ or $PD\text{-}1^{int}ICOS^{hi}$ $CD4^+$ T cell subpopulations. (**D**) Expression patterns for PD-1, CXCR5 and CD200 in tonsillar $CD4^+$ T cell subpopulations. (**E**) $PD\text{-}1^{-}CD45RA^{+}CCR7^{+}CXCR5^{-}$ naïve $CD4^+$ T cells are abundant in tonsils but rare in BC.

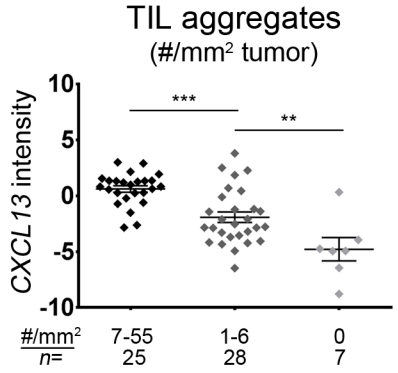
A


BC
Overall survival
Time (months)
%intratumoral CD20 n=
≥1 16
0-1 17
0 18
P = 0.06
CXCL13 gene expression n=
hi 17
int 17
lo 17
P = 0.02

B


BC CD4 CD8

C


TIL aggregates
(#/mm² tumor)
CXCL13 intensity
***
**
#/mm² 7-55 1-6 0
n= 25 28 7

Supplemental Figure 7 (Supporting data for Figure 7).

(**A**) Kaplan-Meier analysis: (top) Breast cancer (BC) overall survival rates stratified on the percentage of intratumoral (apart from the more abundant stromal) $CD20^+$ B-tumor-infiltrating lymphocytes (TIL) (determined by IHC staining); (bottom) BC overall survival rates stratified on *CXCL13* gene expression are shown for comparison. (**B**) $CD4^+$ (brown) and $CD8^+$ (red) IHC staining of the tertiary lymphoid structure (TLS)-containing tumor bed shown in Figure 7F. Black scale bar: 100 µm. (**C**) *CXCL13* gene expression in BC divided into 3 levels of lymphoid aggregate density (TIL aggregate number [#] scored on CD45 IHC-stained sections) per mm^2 tumor surface. One-way ANOVA followed by Sidak's test. $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) and $P<0.0001$ (****).