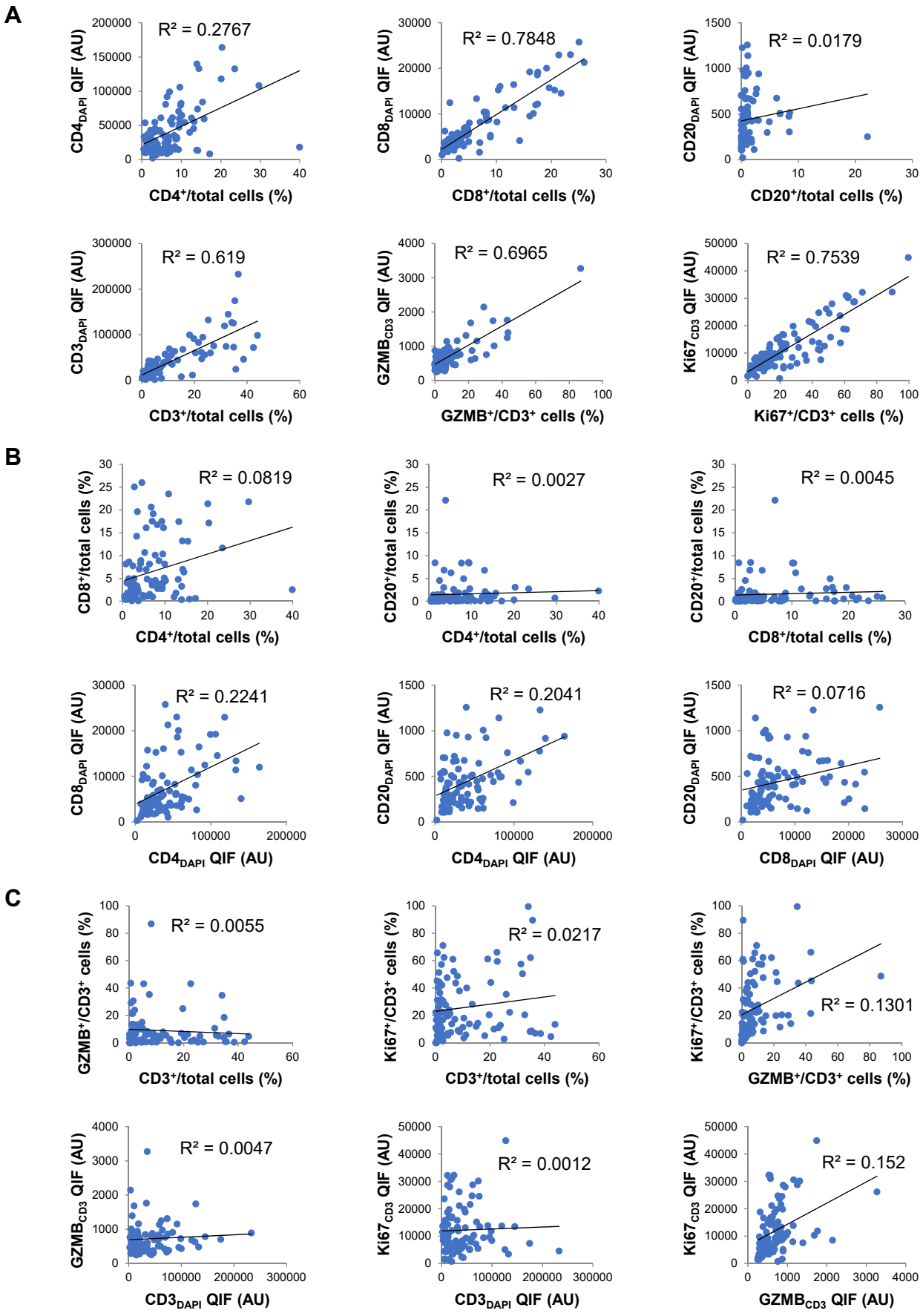
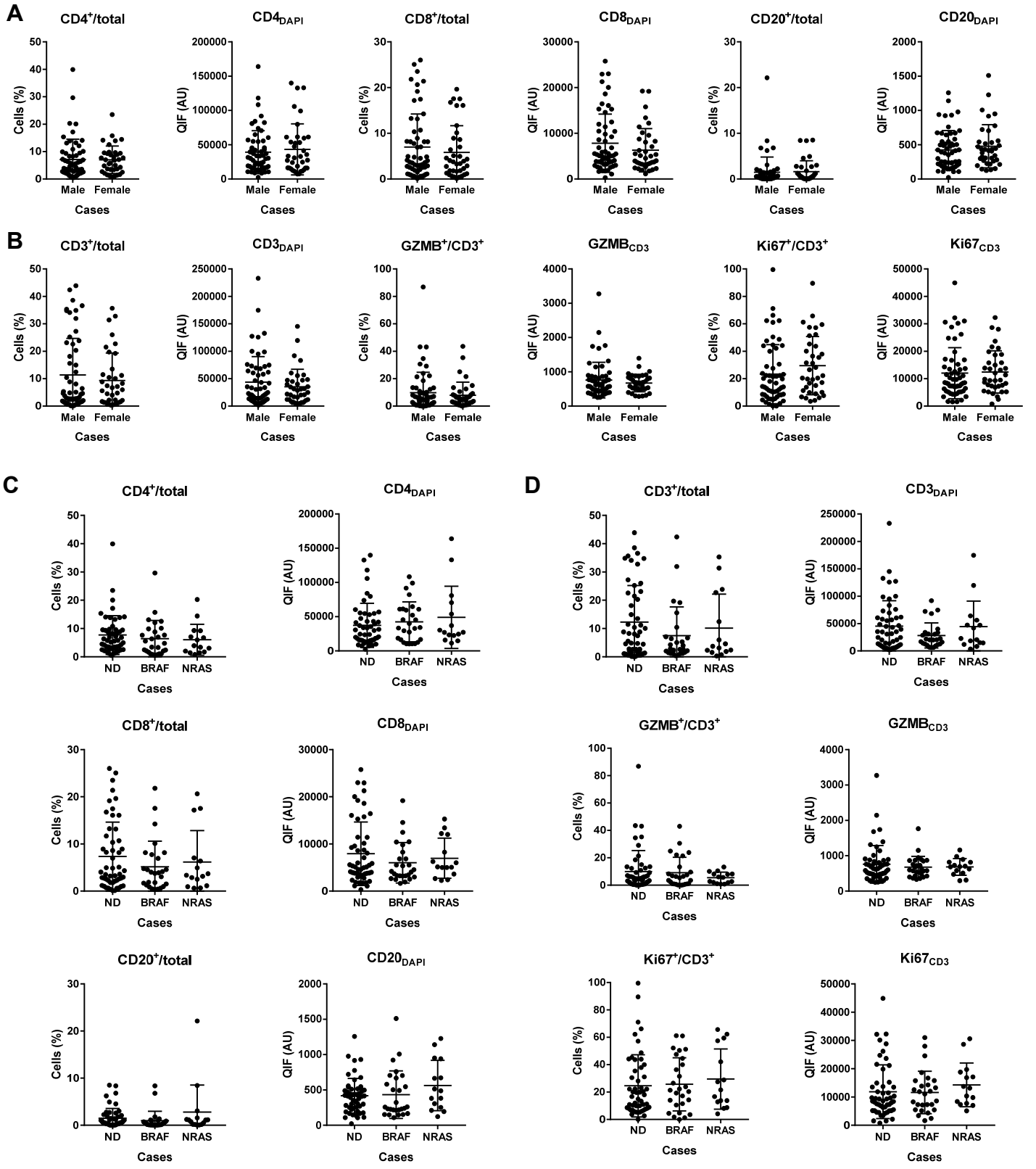
Supplementary Methods

Multiplex immunofluorescence TIL quantification and activation panels

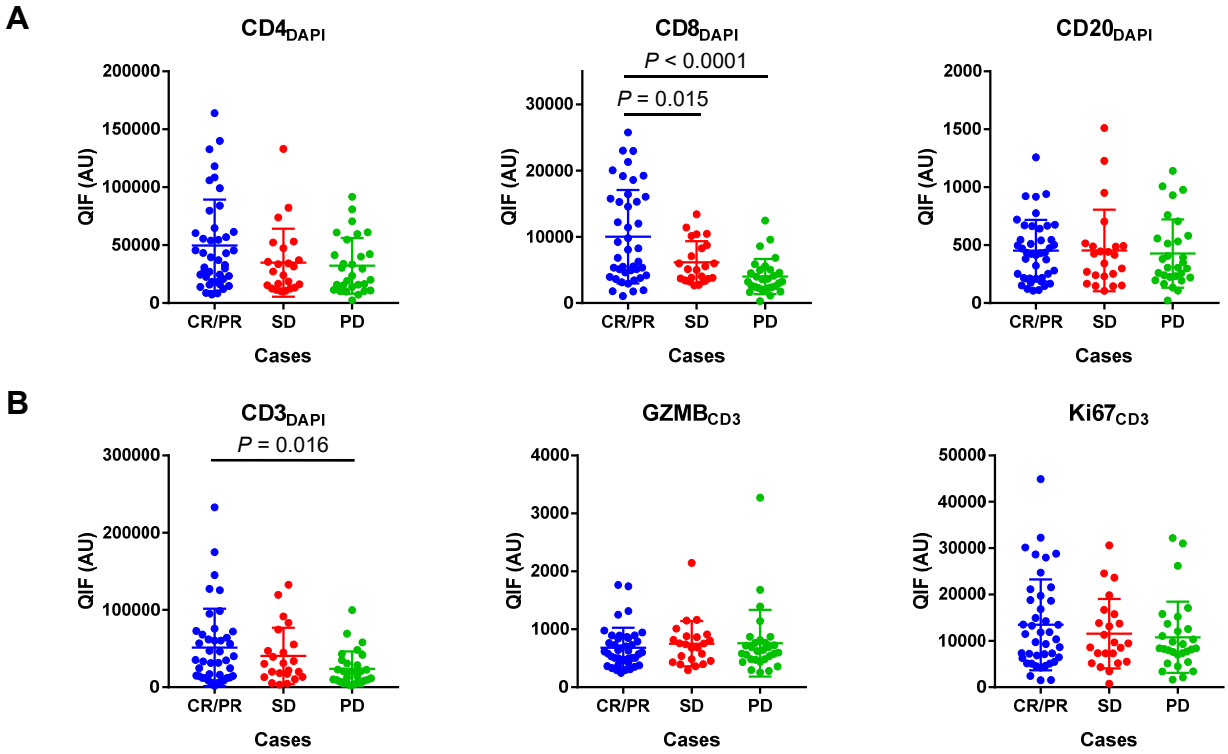
Sections underwent deparaffinization at 60 °C for 30 min followed by xylene washes, then rehydration in an ethanol series. Antigen retrieval was performed in 1 mM EDTA (pH 8) at 97 °C for 20 min in a Lab Vision PT Module (Thermo Scientific, Waltham, MA, USA). Endogenous peroxidases were blocked with 2.5% hydrogen peroxide in methanol for 30 min. The following steps employed a Lab Vision Autostainer 720 (Thermo Scientific). Non-specific antigens were blocked with 0.3% BSA in TBST for 30 min. Primary monoclonal antibodies against CD4 (1:100; SP35; Spring Bioscience, Pleasanton, CA, USA), CD8 (1:250; C8/144B; Dako, Carpinteria, CA, USA), and CD20 (1:150; L26; Dako) for TIL quantification, or CD3 (1:100; SP7; Novus Biologicals, Littleton, CO, USA), GZMB (1:2000; 4E6; Abcam, Cambridge, MA, USA), and Ki67 (1:100; MIB-1; Dako) for TIL activation were co-incubated at room temperature for 1 h. Sections were incubated sequentially with three horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h before tyramide-based labelling for 10 min, followed by 1 mM benzoic hydrazide with 0.15% hydrogen peroxide for 10 min twice to quench HRP activity. The secondary antibodies were anti-rabbit EnVision reagent (Dako), anti-mouse IgG1 (1:100; eBioscience, San Diego, CA, USA), and anti-mouse IgG2a (1:200; Abcam), and the substrates were biotin tyramide (1:50; PerkinElmer, Waltham, MA, USA), TSA Plus Cy3 tyramide (1:100; PerkinElmer), and Cy5 tyramide (1:50; PerkinElmer), respectively. Sections were then treated with streptavidin–Alexa Fluor 750 conjugate (1:100; Invitrogen, Carlsbad, CA, USA) for 1 h. Finally, sections were incubated with mouse anti-S100 (1:100; 15E2E2; BioGenex, Fremont, CA, USA) and HMB45 (1:100; BioGenex) then goat anti-mouse Alexa Fluor 488 (1:100; Invitrogen) for 1 h to identify melanoma cells, counterstained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei, and mounted with ProLong Gold Antifade (Invitrogen).


A
R² = 0.2767
CD4DAPI QIF (AU)
CD4+/total cells (%)
R² = 0.7848
CD8DAPI QIF (AU)
CD8+/total cells (%)
R² = 0.0179
CD20DAPI QIF (AU)
CD20+/total cells (%)
R² = 0.619
CD3DAPI QIF (AU)
CD3+/total cells (%)
R² = 0.6965
GZMBCD3 QIF (AU)
GZMB+/CD3+ cells (%)
R² = 0.7539
Ki67CD3 QIF (AU)
Ki67+/CD3+ cells (%)
B
R² = 0.0819
CD8+/total cells (%)
CD4+/total cells (%)
R² = 0.0027
CD20+/total cells (%)
CD4+/total cells (%)
R² = 0.0045
CD20+/total cells (%)
CD8+/total cells (%)
R² = 0.2241
CD8DAPI QIF (AU)
CD4DAPI QIF (AU)
R² = 0.2041
CD20DAPI QIF (AU)
CD4DAPI QIF (AU)
R² = 0.0716
CD20DAPI QIF (AU)
CD8DAPI QIF (AU)
C
R² = 0.0055
GZMB+/CD3+ cells (%)
CD3+/total cells (%)
R² = 0.0217
Ki67+/CD3+ cells (%)
CD3+/total cells (%)
R² = 0.1301
Ki67+/CD3+ cells (%)
GZMB+/CD3+ cells (%)
R² = 0.0047
GZMBCD3 QIF (AU)
CD3DAPI QIF (AU)
R² = 0.0012
Ki67CD3 QIF (AU)
CD3DAPI QIF (AU)
R² = 0.152
Ki67CD3 QIF (AU)
GZMBCD3 QIF (AU)

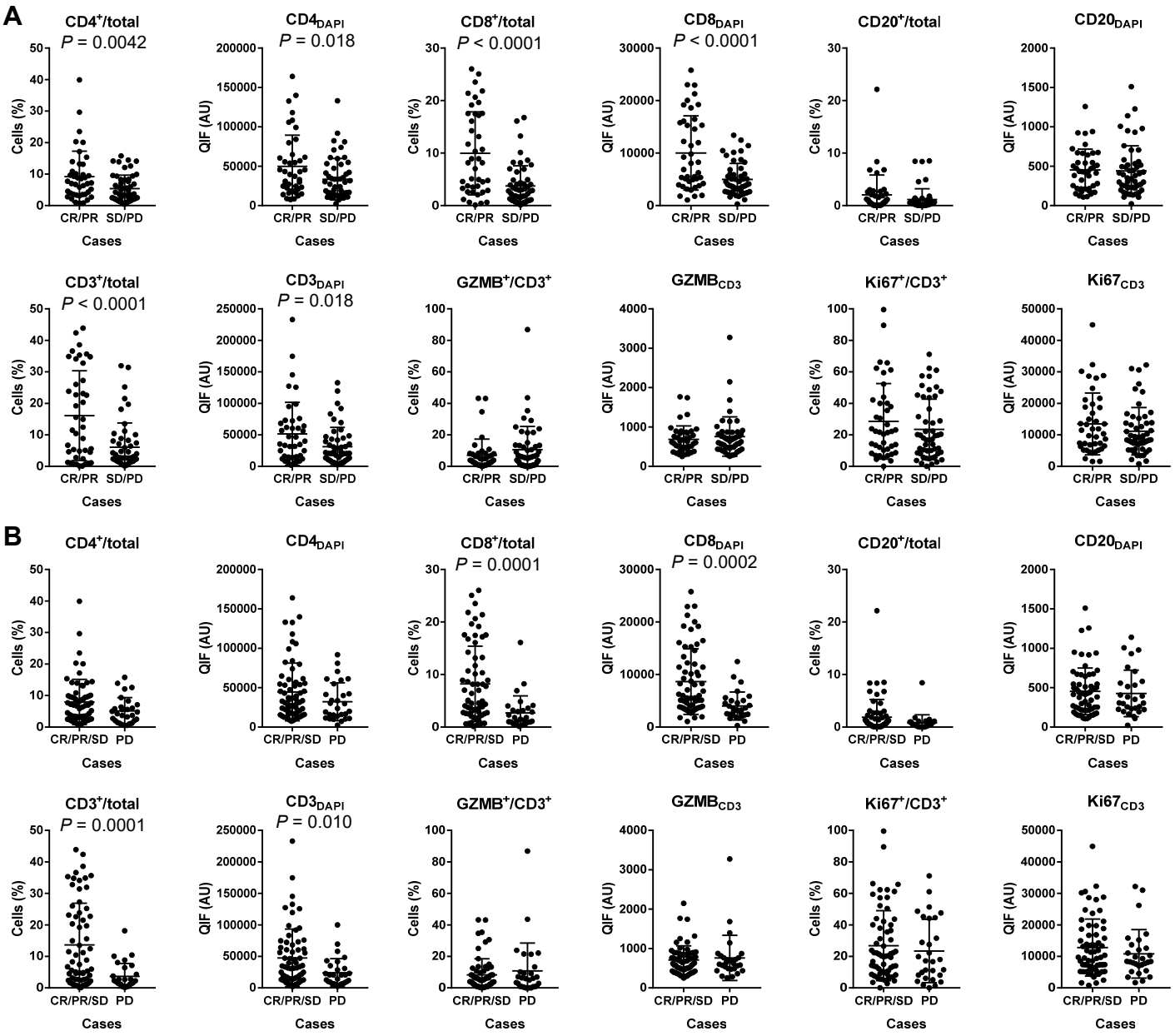
Supplementary Figure 1. Linear regressions of TIL parameters in melanoma by cell counts and quantitative immunofluorescence.
Correlation between cell counts and QIF scores is best for abundant markers and cell types (A). CD4, CD8, and CD20 relationships (B) and CD3, GZMB, and Ki67 relationships (C) by cell counts and QIF scores.
Abbreviations: AU, arbitrary units; QIF, quantitative immunofluorescence; TIL, tumor-infiltrating lymphocyte.



Supplementary Figure 2. Sex and mutation status of melanoma patients and TIL parameters.
TIL quantification (CD4, CD8, CD20) (A, C) and TIL activation (CD3, GZMB, Ki67) (B, D) parameters by cell counts and QIF per sex (A, B) and mutation status (C, D) of melanoma patients. Data are presented as mean with standard deviation (error bars). Abbreviations: AU, arbitrary units; ND, no detection of BRAF or NRAS mutations; QIF, quantitative immunofluorescence; TIL, tumor-infiltrating lymphocyte.

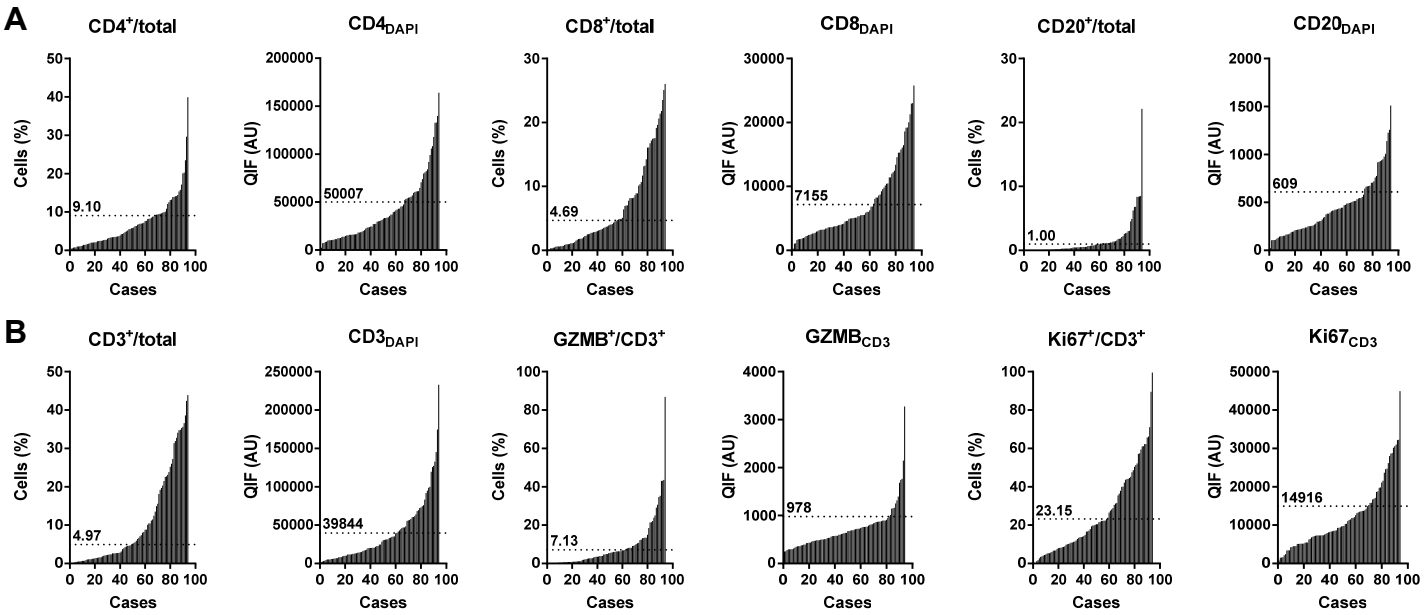


Supplementary Figure 3. RECIST categories of melanoma patients treated with anti-PD-1 therapy and TIL parameters by quantitative immunofluorescence.
TIL quantification (CD4, CD8, CD20) (A) and TIL activation (CD3, GZMB, Ki67) (B) parameters by QIF per RECIST categories of best overall response. Data are presented as mean with standard deviation (error bars). Abbreviations: AU, arbitrary units; CR, complete response; PD, progressive disease; PR, partial response; QIF, quantitative fluorescence; RECIST, Response Evaluation Criteria in Solid Tumors; SD, stable disease; TIL, tumor-infiltrating lymphocyte.

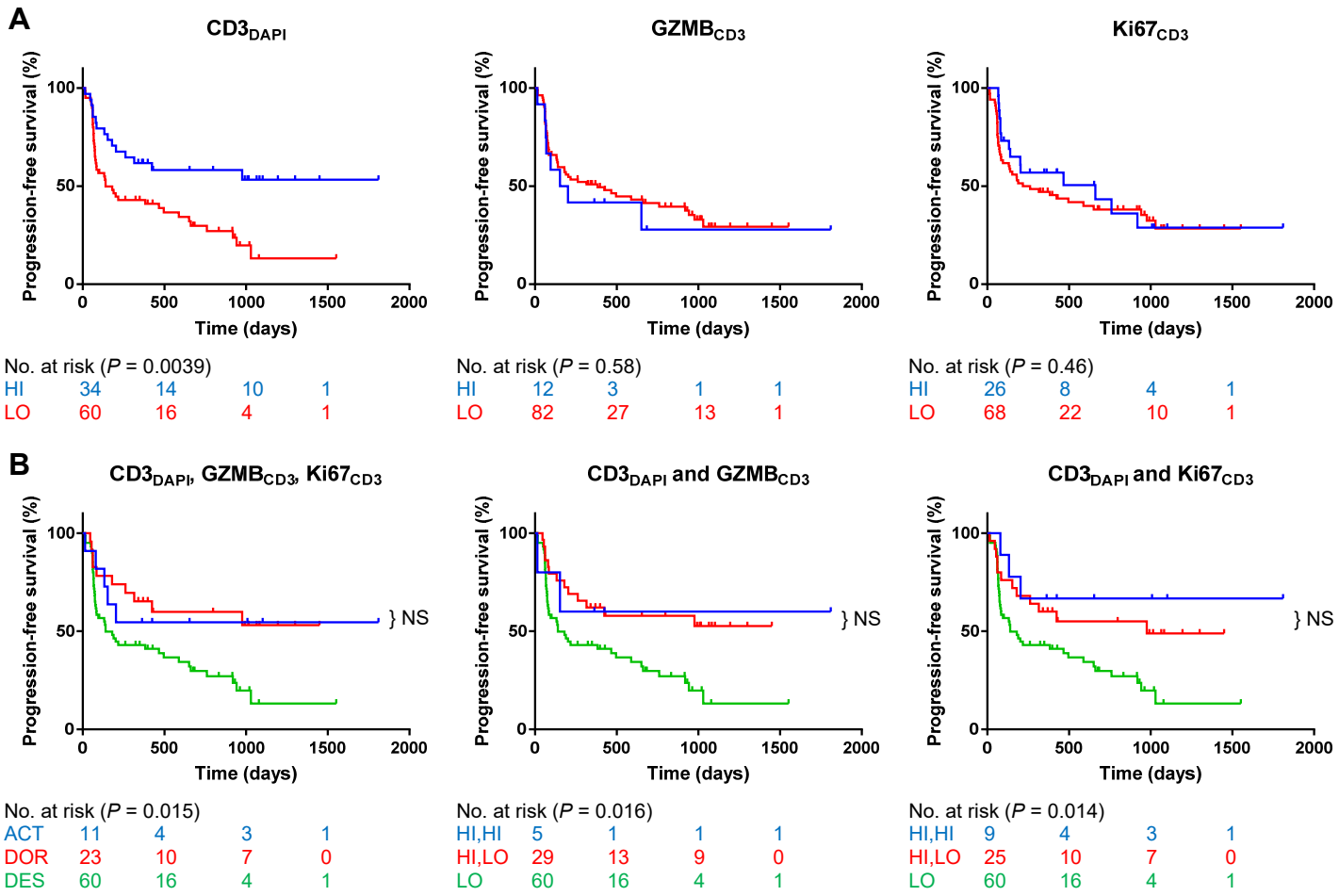


Supplementary Figure 4. Anti-PD-1 objective response rate or disease control rate and TIL parameters in melanoma patients.

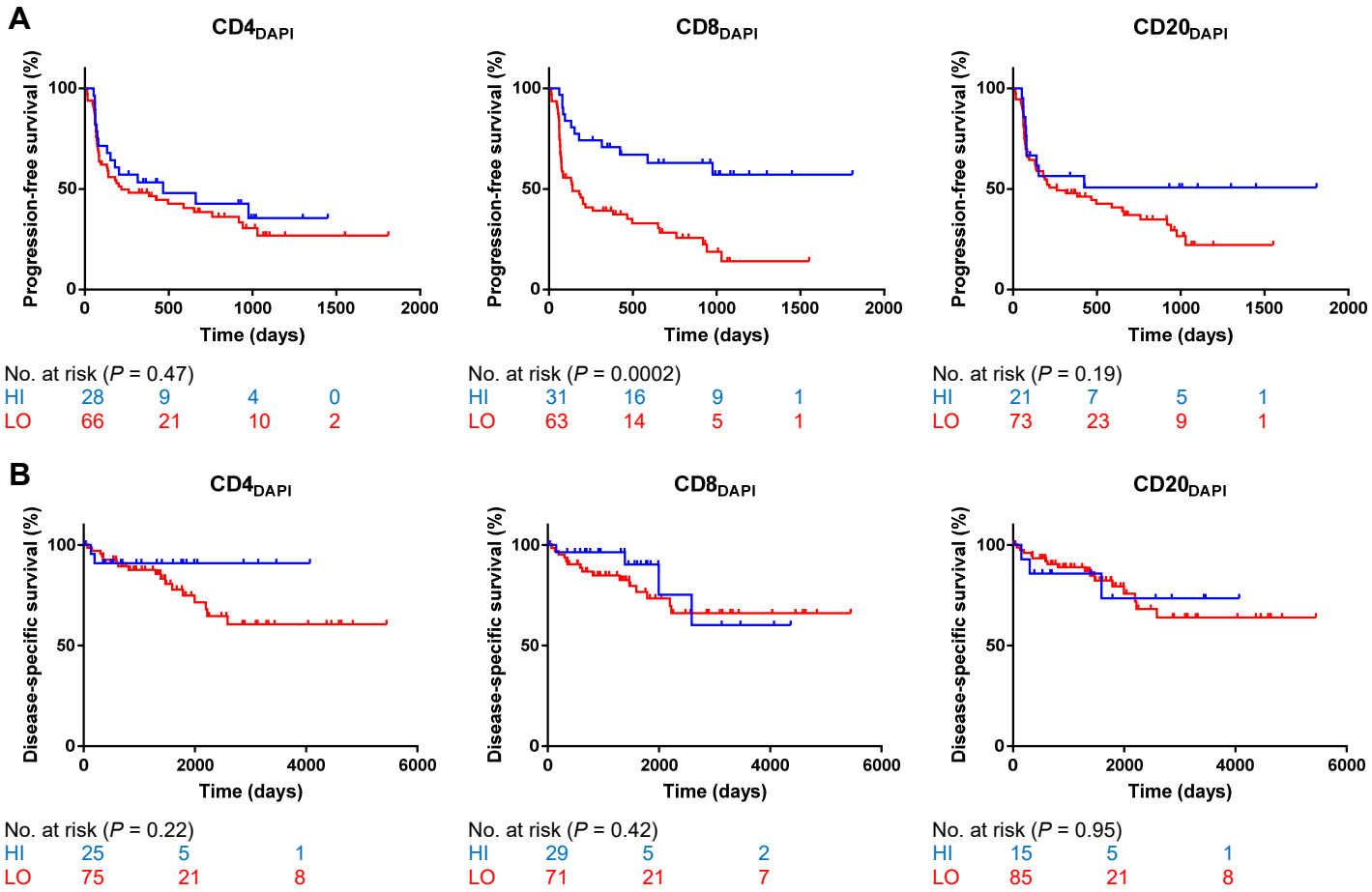
TIL quantification (CD4, CD8, CD20) and TIL activation (CD3, GZMB, Ki67) parameters by cell counts and QIF in relation to anti-PD-1 objective response rate (A) and disease control rate (B) by RECIST. Data are presented as mean with standard deviation (error bars). Abbreviations: AU, arbitrary units; CR, complete response; PD, progressive disease; PR, partial response; QIF, quantitative fluorescence; RECIST, Response Evaluation Criteria in Solid Tumors; SD, stable disease; TIL, tumor-infiltrating lymphocyte.



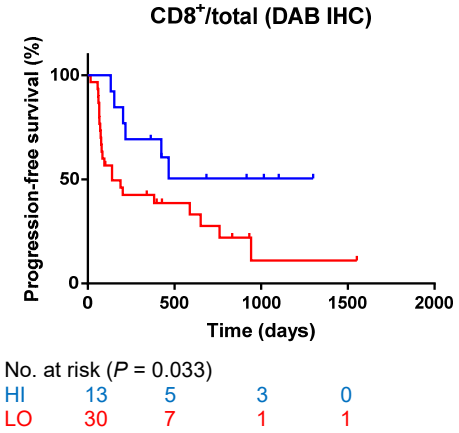
Supplementary Figure 5. Joinpoint thresholds and cohort distributions of TIL parameters.
Cohort distributions of TIL quantification (CD4, CD8, CD20) (A) and TIL activation (CD3, GZMB, Ki67) (B) parameters by cell counts and QIF with Joinpoint thresholds indicated (see Methods). Abbreviations: AU, arbitrary units; QIF, quantitative immunofluorescence; TIL, tumor-infiltrating lymphocyte.



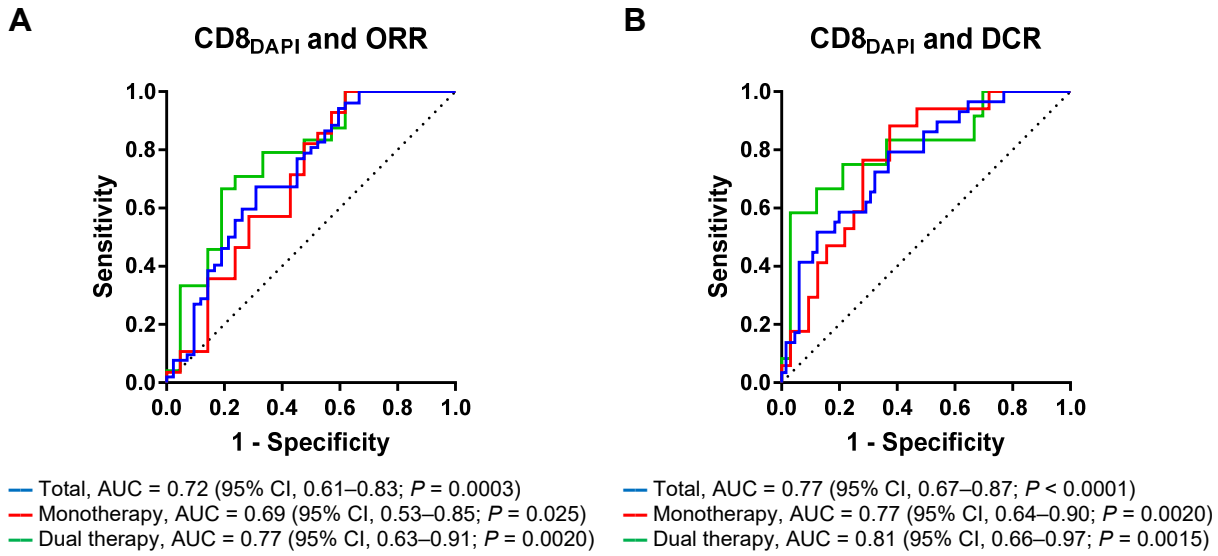
Supplementary Figure 6. TIL activation parameters by quantitative immunofluorescence and survival of melanoma patients treated with anti-PD-1 therapy.
Kaplan–Meier analysis of progression-free survival according to TIL activation (CD3, GZMB, Ki67) (A) parameters by QIF, and the three states of the tumor immune microenvironment (B): immune desert (CD3 low), TIL dormancy (CD3 high, Ki67 and GZMB low), and TIL activation (CD3 high, Ki67 and/or GZMB high). Low and high statuses were objectively defined using thresholds determined by Joinpoint regression (see Methods). Abbreviations: ACT, TIL activation; DES, immune desert; DOR, TIL dormancy; HI, high; LO, low; NS, not significant; QIF, quantitative fluorescence; TIL, tumor-infiltrating lymphocyte.



Supplementary Figure 7. TIL quantification parameters by quantitative immunofluorescence and survival of melanoma patients treated with anti-PD-1 therapy and untreated melanoma patients. Kaplan–Meier analysis of progression-free survival of anti-PD-1 treated melanoma patients (A) and disease-specific survival of untreated melanoma patients (B) according to TIL quantification (CD4, CD8, CD20) parameters by QIF. Low and high statuses were objectively defined using thresholds determined by Joinpoint regression (see Methods). Abbreviations: HI, high; LO, low; QIF, quantitative fluorescence; TIL, tumor-infiltrating lymphocyte.



Supplementary Figure 8. CD8 cell count by chromogenic immunohistochemistry and survival of melanoma patients treated with anti-PD-1 therapy.
Kaplan–Meier analysis of progression-free survival of anti-PD-1 treated melanoma patients according to CD8 cell count by DAB IHC. Low and high statuses were objectively defined using thresholds determined by Joinpoint regression (see Methods). Abbreviations: DAB, 3,3'-diaminobenzidine; HI, high; IHC, immunohistochemistry; LO, low.



Supplementary Figure 9. Receiver operating characteristic (ROC) curve analysis of CD8 by quantitative immunofluorescence for the prediction of anti-PD-1 objective response rate or disease control rate in melanoma.

ROC curves constructed from logistic regression models for the prediction of anti-PD-1 response in terms of ORR (A) and DCR (B) for the total cohort, monotherapy (pembrolizumab or nivolumab), or dual therapy (ipilimumab plus nivolumab). AUC of 0.50 represents performance of random chance (line of identity, dotted); 1.00 represents perfect predictive performance. *P* values indicate probability that the AUC is significantly different from 0.50. Abbreviations: AUC, area under curve; CI, confidence interval; DCR, disease control rate; ORR, objective response rate.

Supplementary Table 1. Univariable and multivariable Cox regression analyses for survival of melanoma patients and TIL parameters by quantitative immunofluorescence.

Variable (LO/HI)	Untreated patients		Anti-PD-1 patients							
	Univariable analysis		Univariable analysis		Trivariable analysis for TIL activation		Multivariable* analysis per variable		Multivariable* analysis for CD3 and CD8	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
$CD4_{DAPI}$	2.44 (0.70–15.4)	0.18	1.23 (0.71–2.26)	0.48			1.23 (0.66–2.41)	0.52		
$CD8_{DAPI}$	1.57 (0.57–5.50)	0.41	**3.12 (1.70–6.18)**	**0.0001**			**2.99 (1.54–6.33)**	**0.0008**	**2.73 (1.29–6.20)**	**0.0081**
$CD20_{DAPI}$	1.04 (0.34–4.47)	0.95	1.56 (0.83–3.28)	0.18			1.43 (0.71–3.14)	0.71		
$CD3_{DAPI}$			**2.33 (1.32–4.37)**	**0.0031**	**2.35 (1.33–4.40)**	**0.0028**	**1.91 (1.04–3.70)**	**0.036**	1.21 (0.60–2.53)	0.60
$GZMB_{CD3}$			0.81 (0.41–1.85)	0.59	0.74 (0.37–1.70)	0.45	0.72 (0.33–1.74)	0.44		
$Ki67_{CD3}$			1.25 (0.71–2.32)	0.46	1.29 (0.73–2.41)	0.39	1.03 (0.57–1.95)	0.91		

Abbreviations: CI, confidence interval; HI, high; HR, hazard ratio; LO, low; TIL, tumor-infiltrating lymphocyte.
*Cox proportional hazards model included age, sex, mutation status, stage, treatment, and prior immune checkpoint blockade as covariates.

Supplementary Table 2. Univariable and multivariable Cox regression analyses for survival of melanoma patients treated with anti-PD-1 therapy and TIL parameters by cell counts per treatment group.

Variable, LO vs. HI	Monotherapy (pembrolizumab or nivolumab) PFS			
	Univariable analysis		Multivariable* analysis per variable	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
$CD4^+$/total	1.36 (0.66–2.99)	0.42	1.03 (0.45–2.54)	0.95
$CD8^+$/total	**3.55 (1.66–8.50)**	**0.0008**	**4.11 (1.76–10.56)**	**0.0008**
$CD20^+$/total	1.65 (0.77–3.93)	0.20	1.17 (0.47–3.21)	0.75
$CD3^+$/total	1.58 (0.80–3.31)	0.19	1.42 (0.67–3.17)	0.36
$GZMB^+/CD3^+$	0.71 (0.35–1.53)	0.36	0.43 (0.18–1.06)	0.067
$Ki67^+/CD3^+$	0.70 (0.35–1.39)	0.31	0.84 (0.39–1.80)	0.65
Variable, LO vs. HI	Dual therapy (ipilimumab plus nivolumab) PFS			
	Univariable analysis		Multivariable* analysis per variable	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
$CD4^+$/total	0.78 (0.33–2.14)	0.60	0.60 (0.22–179)	0.34
$CD8^+$/total	**4.58 (1.85–13.14)**	**0.0007**	**8.02 (2.43–34.31)**	**0.0003**
$CD20^+$/total	1.44 (0.65–3.41)	0.37	1.25 (0.51–3.27)	0.64
$CD3^+$/total	**5.16 (2.26–12.85)**	**<0.0001**	**5.91 (2.04–19.31)**	**0.0008**
$GZMB^+/CD3^+$	1.01 (0.46–2.29)	0.97	1.23 (0.49–3.21)	0.66
$Ki67^+/CD3^+$	1.63 (0.71–4.20)	0.26	2.18 (0.80–6.80)	0.13

Abbreviations: CI, confidence interval; HI, high; HR, hazard ratio; LO, low; PFS, progression-free survival; TIL, tumor-infiltrating lymphocyte.

*Cox proportional hazards model included age, sex, mutation status, stage, and prior immune checkpoint blockade as covariates.

Supplementary Table 3. Univariable and multivariable Cox regression analyses for survival of melanoma patients treated with anti-PD-1 therapy and TIL parameters by quantitative immunofluorescence per treatment group.

Variable, LO vs. HI	Monotherapy (pembrolizumab or nivolumab) PFS			
	Univariable analysis		Multivariable* analysis per variable	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
$CD4_{DAPI}$	1.17 (0.55–2.77)	0.70	0.99 (0.41–2.60)	0.98
$CD8_{DAPI}$	**3.28 (1.50–8.20)**	**0.0022**	**3.40 (1.45–9.06)**	**0.0043**
$CD20_{DAPI}$	1.11 (0.46–3.29)	0.84	0.68 (0.24–2.21)	0.49
$CD3_{DAPI}$	1.52 (0.73–3.48)	0.27	1.27 (0.57–3.07)	0.57
$GZMB_{CD3}$	1.14 (0.44–3.87)	0.80	0.94 (0.33–3.32)	0.91
$Ki67_{CD3}$	0.70 (0.35–1.39)	0.31	1.74 (0.72–4.64)	0.23
Variable, LO vs. HI	Dual therapy (ipilimumab plus nivolumab) PFS			
	Univariable analysis		Multivariable* analysis per variable	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
$CD4_{DAPI}$	1.28 (0.57–3.15)	0.56	0.97 (0.34–2.95)	0.96
$CD8_{DAPI}$	**3.09 (1.27–8.67)**	**0.012**	**5.88 (1.90–22.47)**	**0.0014**
$CD20_{DAPI}$	2.43 (0.92–8.34)	0.074	2.08 (0.67–7.99)	0.22
$CD3_{DAPI}$	**3.56 (1.48–9.89)**	**0.0038**	**2.84 (1.14–8.12)**	**0.024**
$GZMB_{CD3}$	1.39 (0.41–8.69)	0.64	1.26 (0.31–8.48)	0.77
$Ki67_{CD3}$	**5.23 (1.10–93.5)**	**0.035**	**8.45 (1.58–157.78)**	**0.0084**

Abbreviations: CI, confidence interval; HI, high; HR, hazard ratio; LO, low; PFS, progression-free survival; TIL, tumor-infiltrating lymphocyte.

*Cox proportional hazards model included age, sex, mutation status, stage, and prior immune checkpoint blockade as covariates.