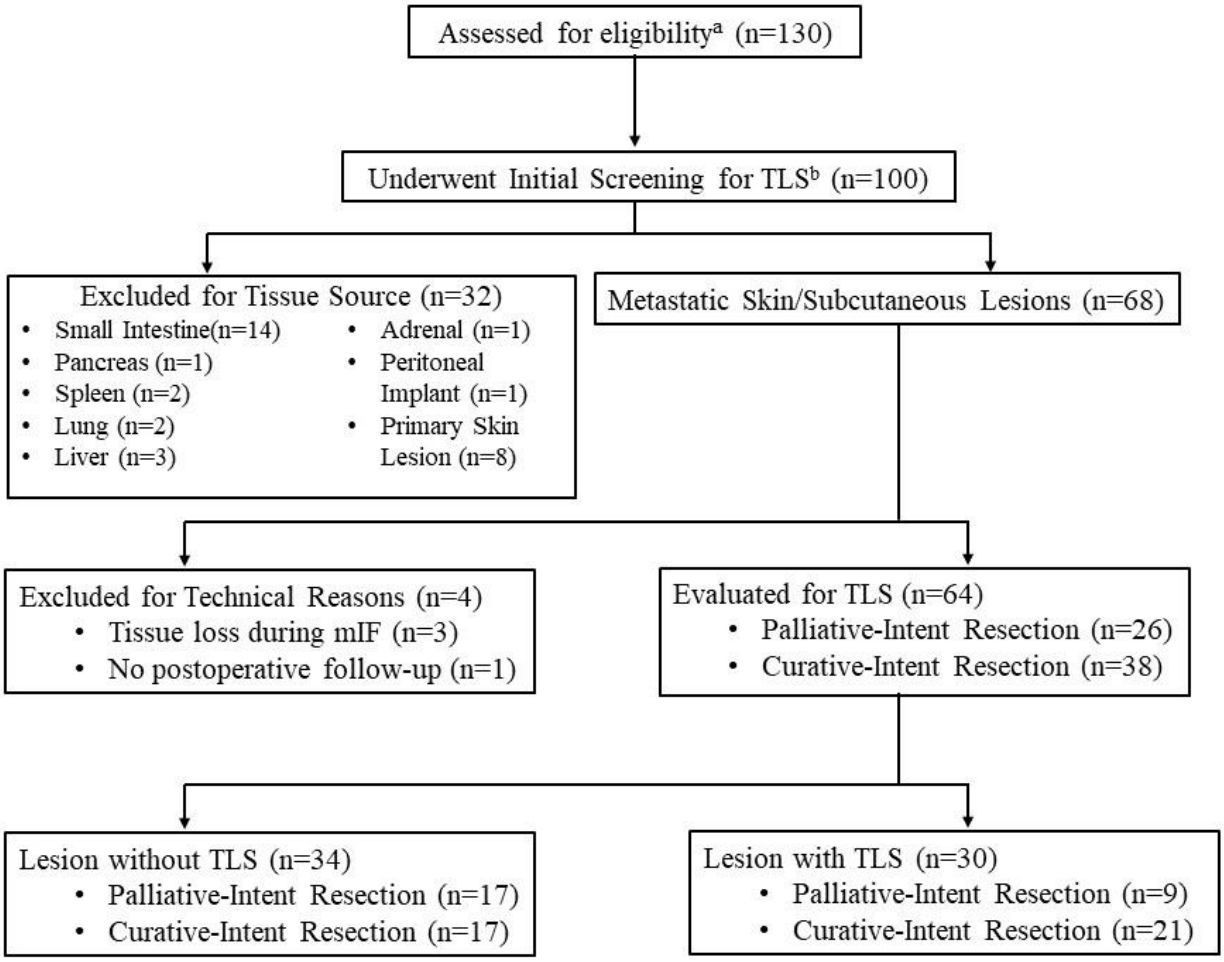
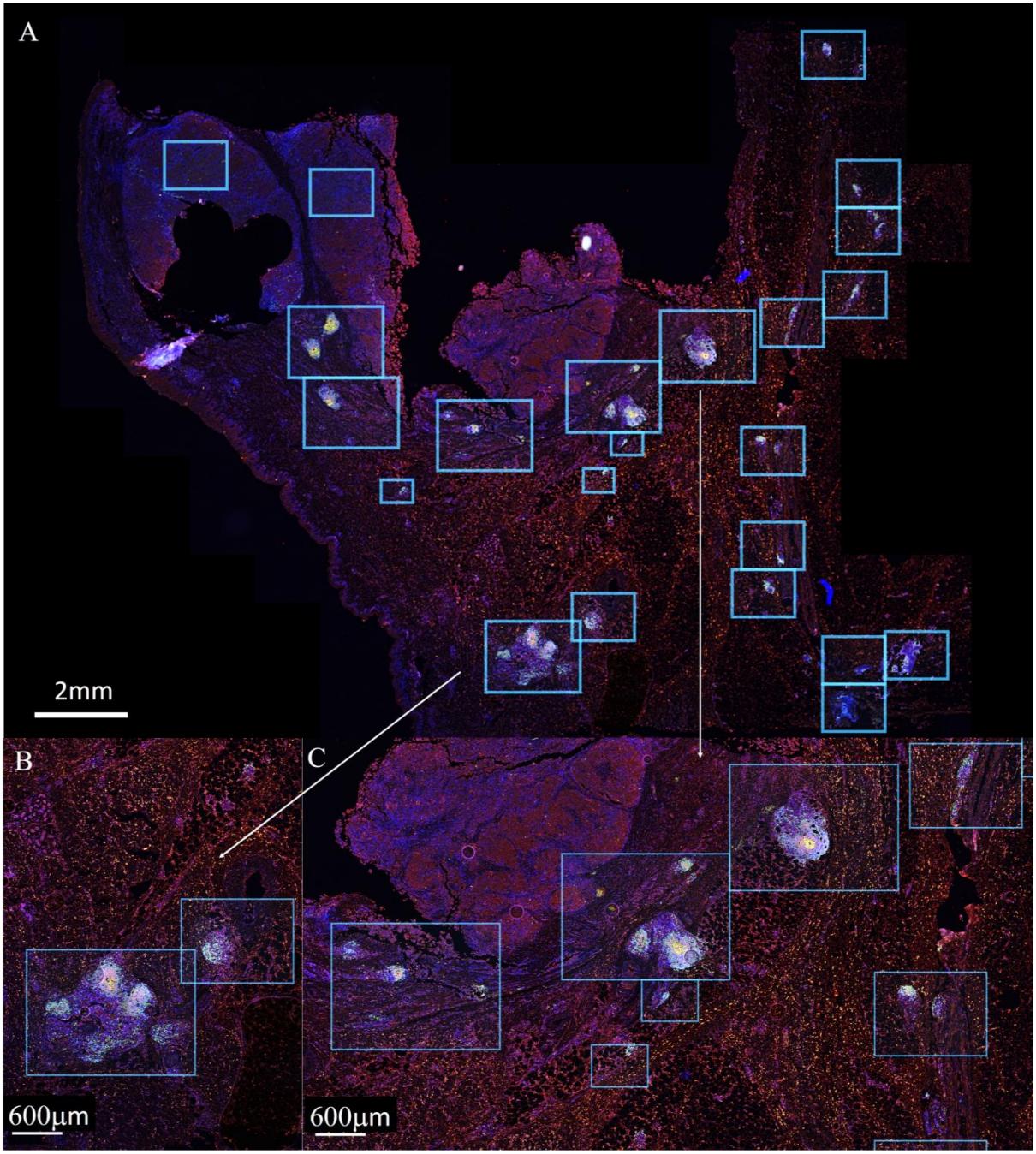
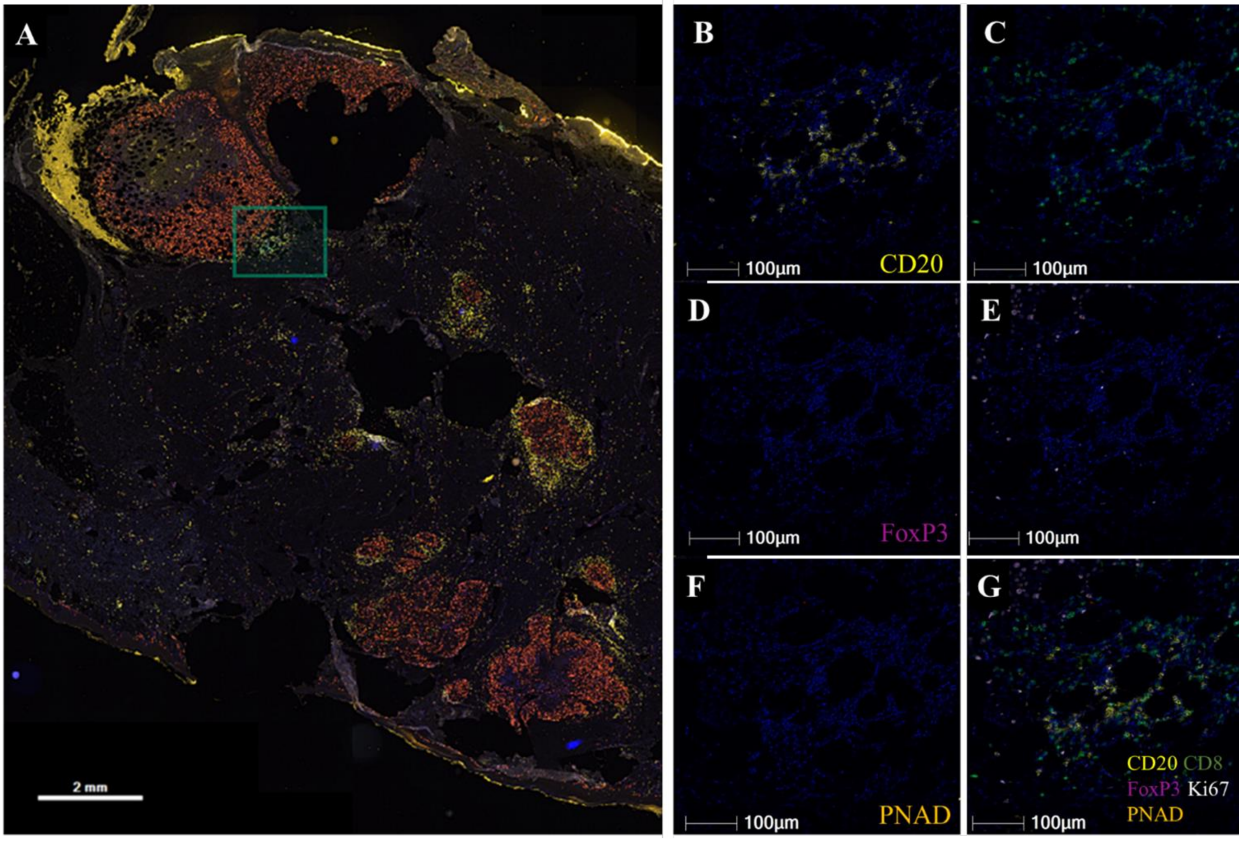


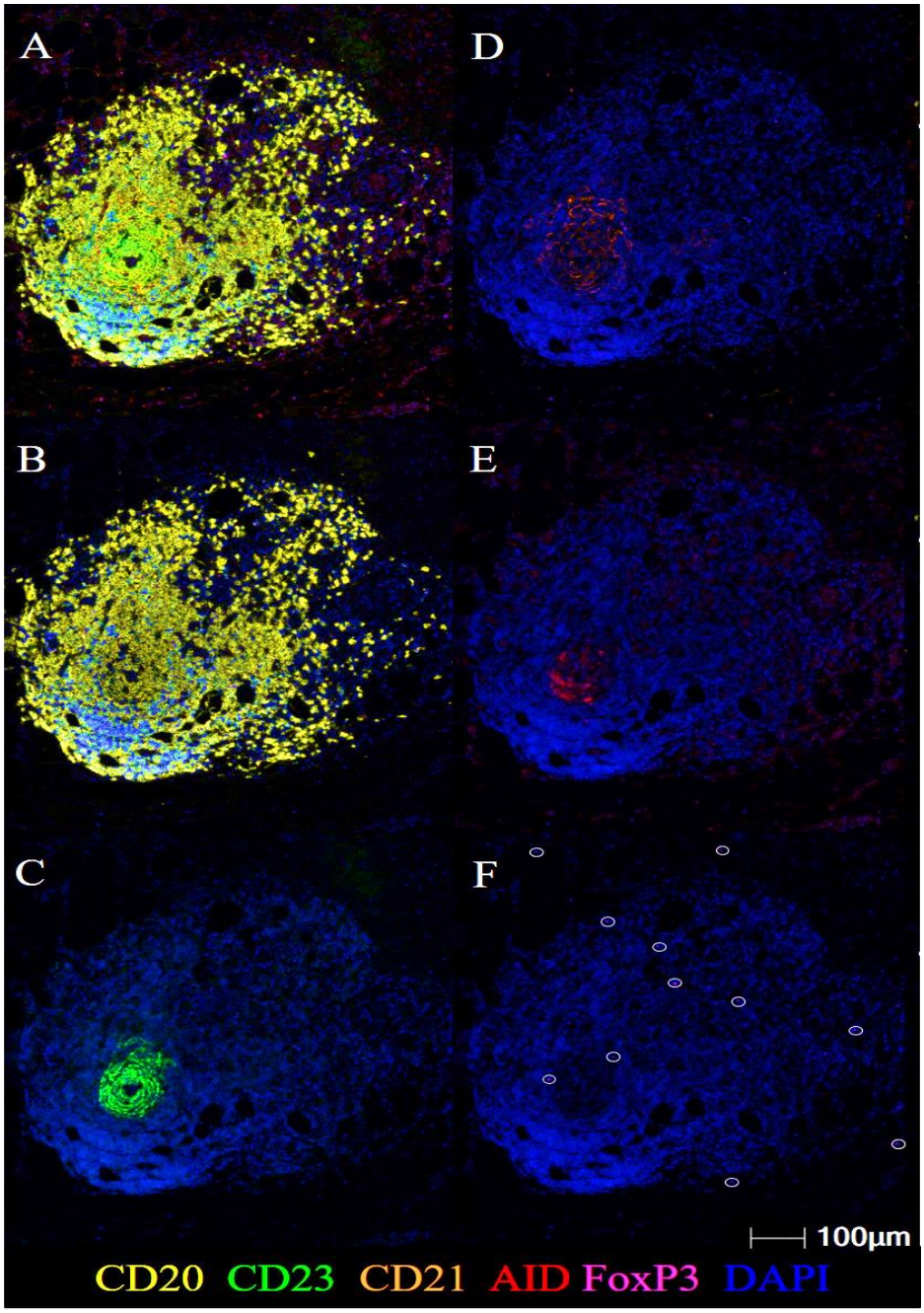
Supplemental Figure 1: Participant Flow Diagram. A) Patients assessed for eligibility included those who previously underwent TMA creation of melanoma tumor cores. B) Screened for presence of TLS Excluded lymph node metastases and tumors without sufficient FFPE material for mIFH.



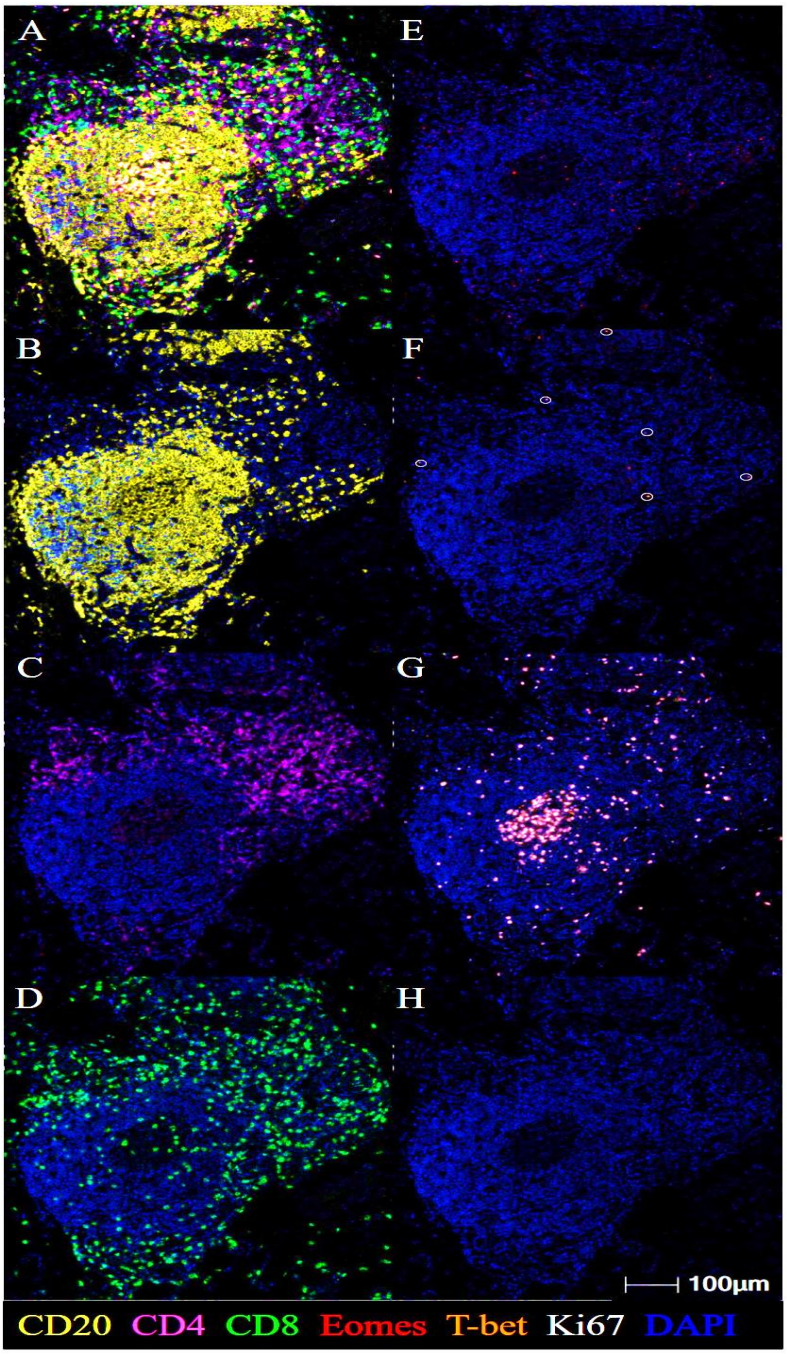
Supplemental Figure 2. Image of TLS Regions of Interest Selection in Melanoma. Images are of a melanoma tumor specimen stained with the TLS identification panel. Regions of interest for analysis were selected on multiplex stained samples **(A-C)**. Image magnifications are indicted, and higher magnification images are shown in **(B-C)** corresponding to the indicated regions of interest selected in **A**. Holes in the melanoma tumor specimen are of punch biopsies which were used in generating melanoma TMAs.



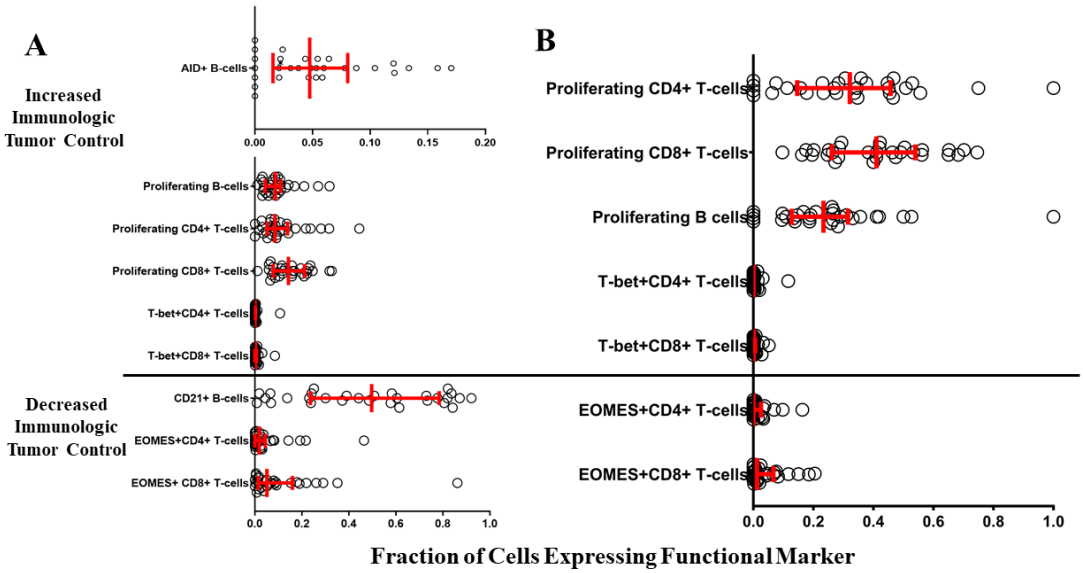
Supplemental Figure 3: Image of TLS^{neg} melanoma metastasis. Images are of a melanoma tumor specimen stained with the TLS identification panel (**A**). **Panels B-G** demonstrate intact staining in one region of interest for CD20^{+} B cells (**B**) CD8^{+} T-cells (**C**) FoxP3 (**D**) Ki67 (**E**) but absence of PNAD staining (**F**). All 5 markers are aggregated in panel **G**.



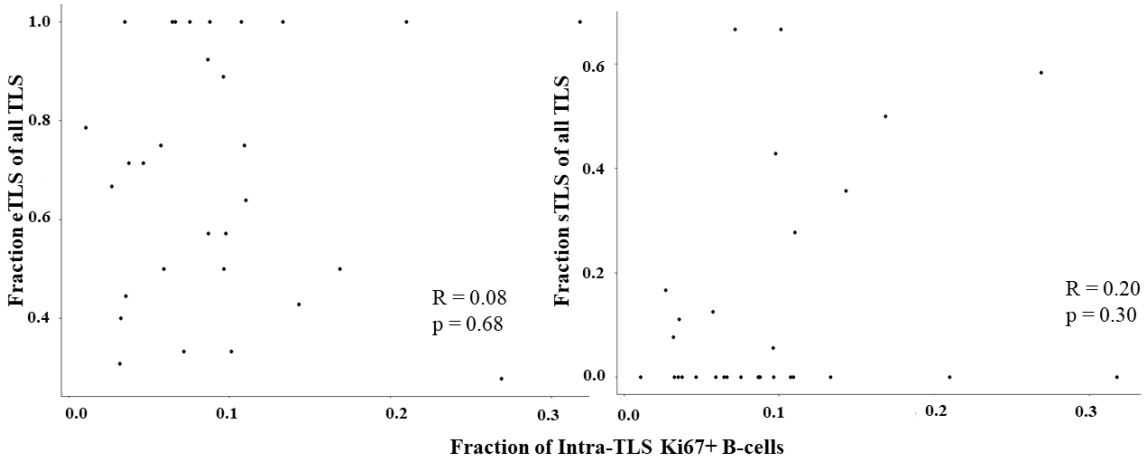
Supplemental Figure 4. **Multiplex and Single Marker Images of a mature secondary follicle-like (SFL) TLS in Melanoma**. Images are of one TLS, stained with the TLS maturation multiplex panel. Images are of the 6-color multiplex stain (A), and single marker images (B-F) are of CD20 (B), CD23 (C), CD21 (D), AID (E), FoxP3 (F) in combination with DAPI shown in blue. Circles denote FoxP3_ells. Marker colors and image magnifications are indicated.



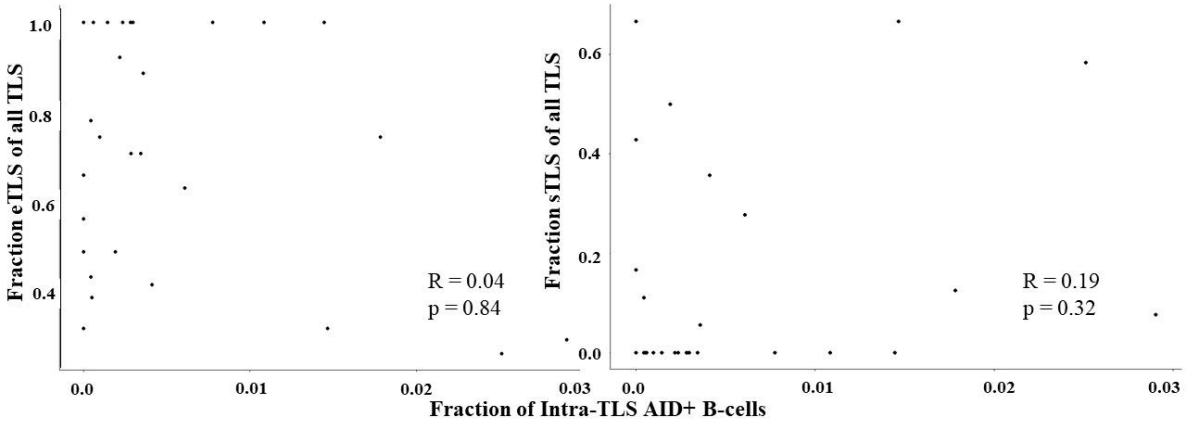
Supplemental Figure 5. **Multiplex and Single Marker Images of a TLS with EOMES^{+} cells**. Images are of one TLS, and were stained with the TLS T-helper cell lineage panel. Images are of the 6-color multiplex stain (A), and single marker images (B-F) are of CD20 (B), CD4 (C), CD8 (D), EOMES (E), T-bet (F), Ki67 (G) in combination with DAPI shown in blue and alone in (H). Marker colors and image magnifications are indicated.



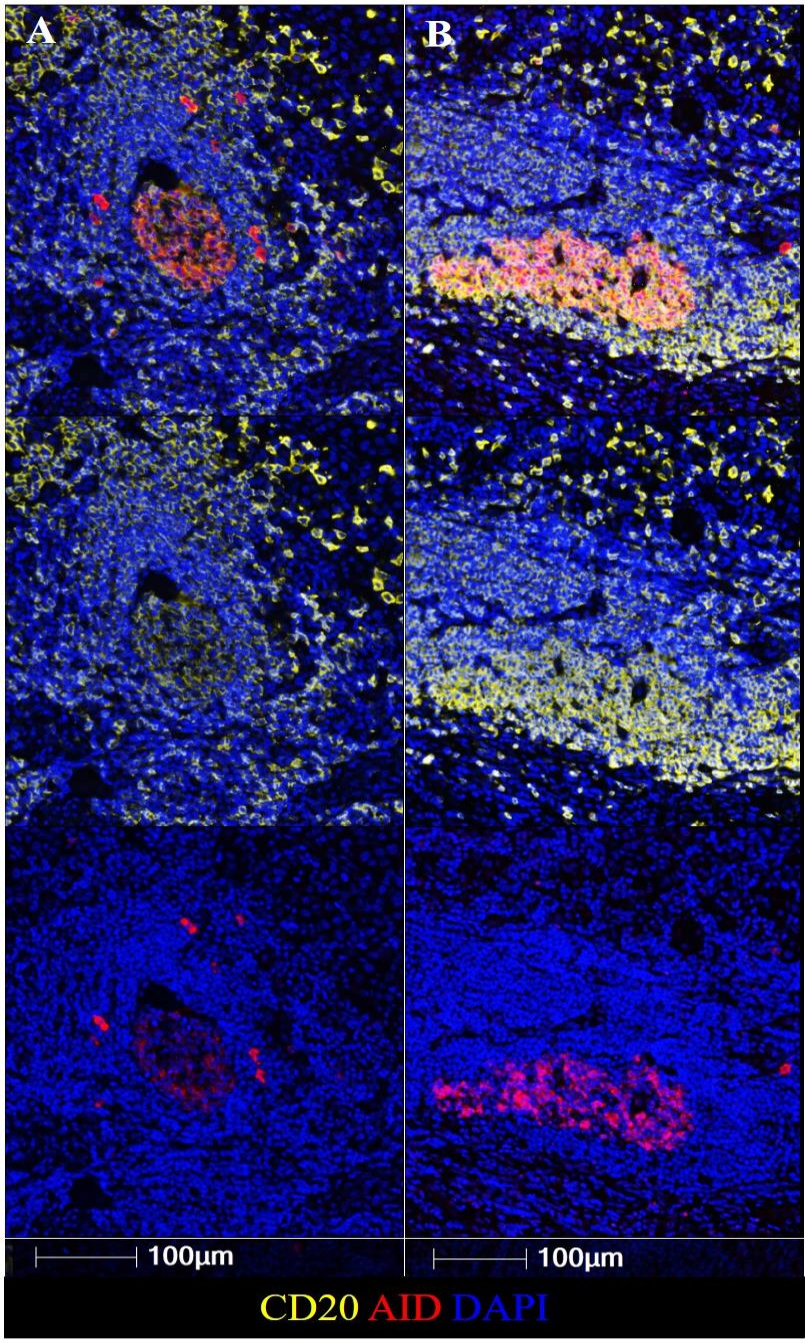
Supplemental Figure 6. Distribution of Intra-TLS and Intra-Tumoral Lymphocyte Expression of Activation/Differentiation Markers Grouped by Perceived Impact on Tumor Control. Fraction of B-cells or T-cells expressing a given marker of proliferation ($Ki67^{+}$), Th1/Tc1 differentiation ($T\text{-}bet^{+)}$ or exhaustion ($EOMES^{+}$) within TLS **(A)** and tumor **(B)**. Bars indicate median and IQR. *Square-root transformed data to stabilize variance, shown on smaller scale as all fractions less than 0.20.



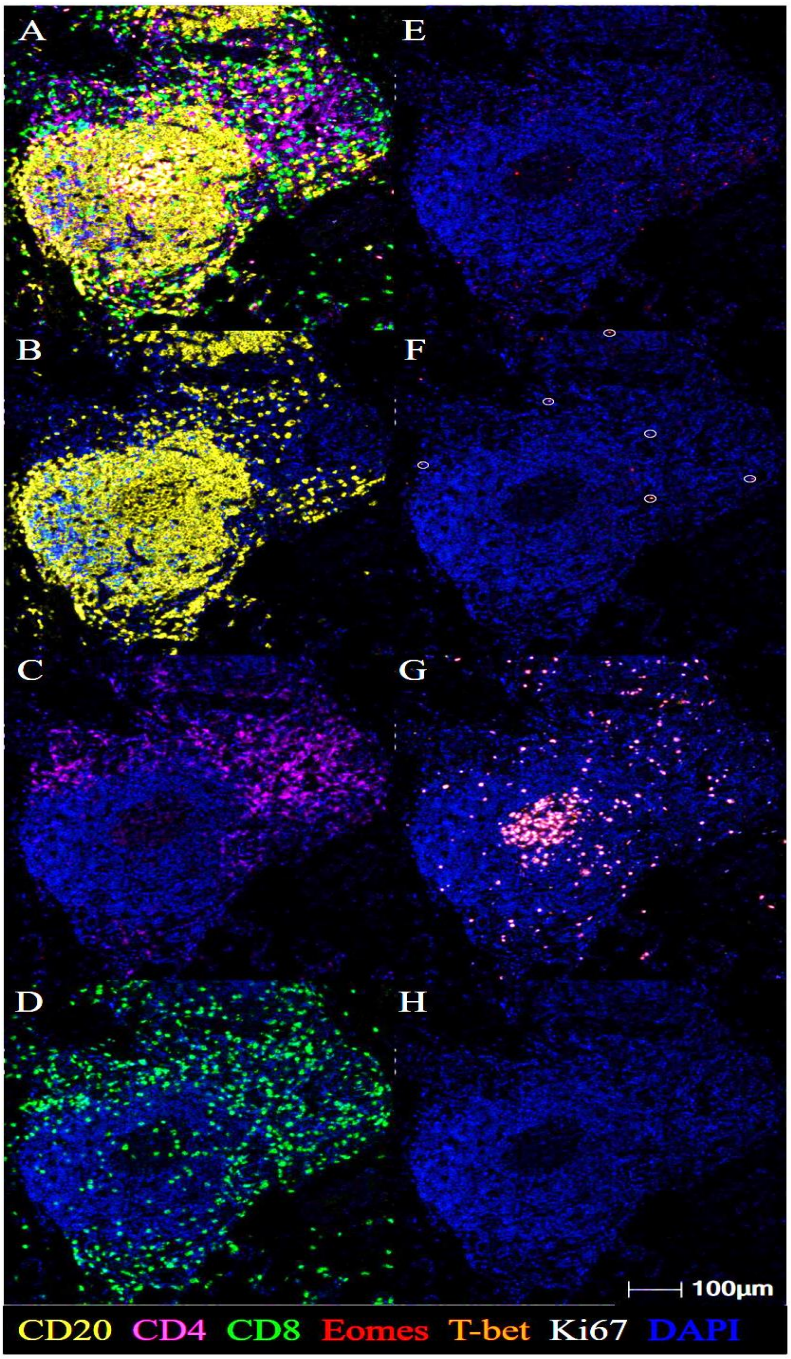
Supplemental Figure 7: TLS Maturation Fraction Does Not Correlate with Fraction of Intra-TLS B-cells Expressing ki67. Correlation between fraction of eTLS and sTLS with fraction of intra-TLS B-cells expressing ki67. Testing for trend with Spearman's rank correlation.



Supplemental Figure 8: TLS Maturation Fraction Does Not Correlate with Fraction of Intra-TLS B-cells Expressing AID. Correlation between fraction of eTLS and sTLS with fraction of intra-TLS B-cells expressing AID. Testing for trend with Spearman's rank correlation.



Supplemental Figure 9: Multiplex and Single Marker Images of AID$^+$ TLS in Melanoma. Images are representative of two TLS (A and B), stained with the TLS maturation multiplex panel. The top panel shows CD20, AID and DAPI cells together, middle panels are of CD20 and DAPI, and bottom panel show AID cells and DAPI. Marker colors and image magnifications are indicated.



Supplemental Figure 10. **Multiplex and Single Marker Images of a TLS with EOMES^{+} cells**. Images are of one TLS, and were stained with the TLS Thelper cell lineage panel. Images are of the 6-color multiplex stain (A), and single marker images (B-F) are of CD20 (B), CD4 (C), CD8 (D), EOMES (E), T-bet (F), Ki67 (G) in combination with DAPI shown in blue and alone in (H). Marker colors and image magnifications are indicated.

Supplemental Tables

Supplemental Table 1: REMARK Analysis Report

a) Patients treatment and variables				
Study and marker	**Remarks**			
Markers	M1 =CD20$^+$, M2 =CD4$^+$, M3 =CD8$^+$, M4 =%CD8$^+$EOMES$^+$, M5 =%CD4$^+$EOMES$^+$, M6 =%CD20$^+$AID$^+$, M7 =%CD20$^+$Ki67$^+$, M8 =%CD4$^+$Ki67$^+$, M9 =CD8$^+$Ki67$^+$, M10 =%CD20$^+$CD21$^+$, M11 =CD4$^+$Tbet$^+$, M12 =CD8$^+$Tbet$^+$, M13 =CD4$^+$FoxP3$^+$ M14 =CD21$^+$, M15 =CD23$^+$, M16 =PNAd$^+$,			
How was marker analyzed?	M1-13: Density of cells expressing marker were quantified using Halo digital software (cells/mm^2). M14-16: Determined as categorically positive or negative by microscopic evaluation.			
If categorical, how were cutpoints determined?	Categorical high vs low cut points were determined for each evaluated immunological marker using the Contal O'Quigley method.			
Further variables	v1 =Age, v2 =Disease Stage (Stage 3/Stage 4), v3 =Patient Sex (female/male), v5 =Intra-tumoral CD8 T-cell density$^+$ V6 =Surgical Intent (Curative-Intent Resection/Palliative-Intent Resection) (yes/no)			
Outcomes	OS, melanoma recurrence			
Patients		**n**	**Remarks**	
Assessed for eligibility		130	Disease: Stage IIIb-IV Metastatic Melanoma previously undergoing TMA creation	
Met Inclusion Criteria		68	Patient Source: University of Virginia, meeting general inclusion criteria[a]	
Excluded		4	General exclusion criteria[b]	
Included		64	Included in analysis: A1-2	
TLS^{pos}		30	Included in analyses	
TLS^{neg}		34	Excluded from subgroup analysis: A3-A16[c]	
b) Statistical analyses of survival outcomes				
Analysis	**Patients**	**Events**	**Variables**	**Results/Remarks**
A1: Univariate	64	52	M16	Lesions associated with TLS (TLS+) are correlated with improved OS.
A2: Multivariable	64	52	M16,v1-v6	TLS+ lesions are correlated improved OS after controlling for patient age, sex, disease stage, intra-tumoral CD8 T-cell density and surgical intent. Model stratified by sex to correct for Schoenfeld residuals <0.01.
A3: Univariate	30	22	M14-16	TLS maturation status was not associated with OS.
A4: Univariate	30	22	M7	Fraction of proliferating B-cells in TLS was not correlated with OS.
A5: Univariate	30	22	M8	Fraction of proliferating CD4$^+$ T-cells in TLS was not correlated with OS.
A6: Univariate	30	22	M9	Fraction of proliferating CD8$^+$ T-cells in TLS was not correlated with OS.
A7: Univariate	30	22	M11	Fraction of Tbet$^+$CD4$^+$ T-cells in TLS was not correlated with OS.
A8: Univariate	30	22	M12	Fraction of CD8$^+$Tbet$^+$ T-cells in TLS was not correlated with OS.
A9: Univariate	30	22	M6	Fraction of AID$^+$ B-cells in TLS was associated with OS.
A10: Multivariate	30	22	M6, v2-v3	Fraction of AID$^+$ B-cells in TLS was associated with OS after controlling for surgical intent and patient sex. Model stratified by sex to correct for Schoenfeld residuals <0.01.
A11: Univariate	30	22	M13	Density of regulatory T-cells in TLS was not correlated with OS.
A12: Univariate	30	22	M5	Fraction of EOMES$^+$CD4$^+$ T-cells in TLS was not correlated with OS.
A13: Univariate	30	22	M4	Fraction of EOMES$^+$CD8$^+$ T-cells in TLS was associated with OS.
A14: Multivariate	30	22	M4, v2-v3	Fraction of EOMES$^+$CD8$^+$ T-cells in TLS not significantly associated after controlling for surgical intent and patient sex.
A15: Univariate	30	22	M10	Fraction of CD21$^+$ B-cells in TLS was associated with OS.
A16: Multivariate	30	22	M10, v2-v2	Fraction of CD21$^+$ B-cells in TLS was associated with OS after controlling for surgical intent and patient sex.

[a] Inclusion criteria: Melanoma skin metastases from unique patients with FFPE specimens available.
[b] Technical failure of mIF (n=3), no post-operative follow up (n=1) [c] Exclusion from analysis A4-16: No TLS identified in lesion

Supplemental Table 2: Dichotomization Points Used in Survival Analyses.

Variable (ratio)	Dichotomization Point*
eTLS/TLS	0.64
pTLS/TLS	0.43
sTLS/TLS	0.08
CD20 Ki67+/CD20	0.11
CD20 AID+/CD20	0.001
CD4 Ki67+/CD4	0.119
CD8 Ki67+/CD8	0.22
CD4 Tbet+/CD4	0.002
CD8 Tbet+/CD8	0.006
CD4 FoxP3+/CD4	0.002
CD4 EOMES+/CD4	0.002
CD8 EOMES+/CD8	0.002
CD20 CD21+/CD20	0.39

* Calculated using the Contal-O'Quigley[4] method.

Supplemental Table 3: Intra-TLS Lymphocyte Activation Markers Not Significantly Associated with Overall Survival.

Lymphocyte Marker	Median OS (high vs. low, months)	Log-rank p-value
CD8Ki67/CD8	29.5 vs. 19.6	0.40
CD20Ki67/CD20	19.6 vs 21.2	0.90
CD4Ki67/CD4	30.9 vs 19.0	0.20
CD8Tbet/CD8	17.0 vs. 29.5	0.20
CD4Tbet/CD4	17.8 vs. 26.4	0.10
FoxP3+	29.8 vs. 18.8	0.20

Supplemental Table 4: Inter-TLS T-cell EOMES Expression Does Not Correlate with OS in a Multivariate Cox Hazard Model.

Variable	HR	p-value
Low Fraction of $CD8^+$ T-cells expressing EOMES	0.62	0.38
Male Sex	3.79	0.02
Palliative-Intent Resection	6.03	<0.01

Includes only patients with at least one TLS (n=30). Model assumptions verified with Schoenfeld residuals with a significance level of <0.01.