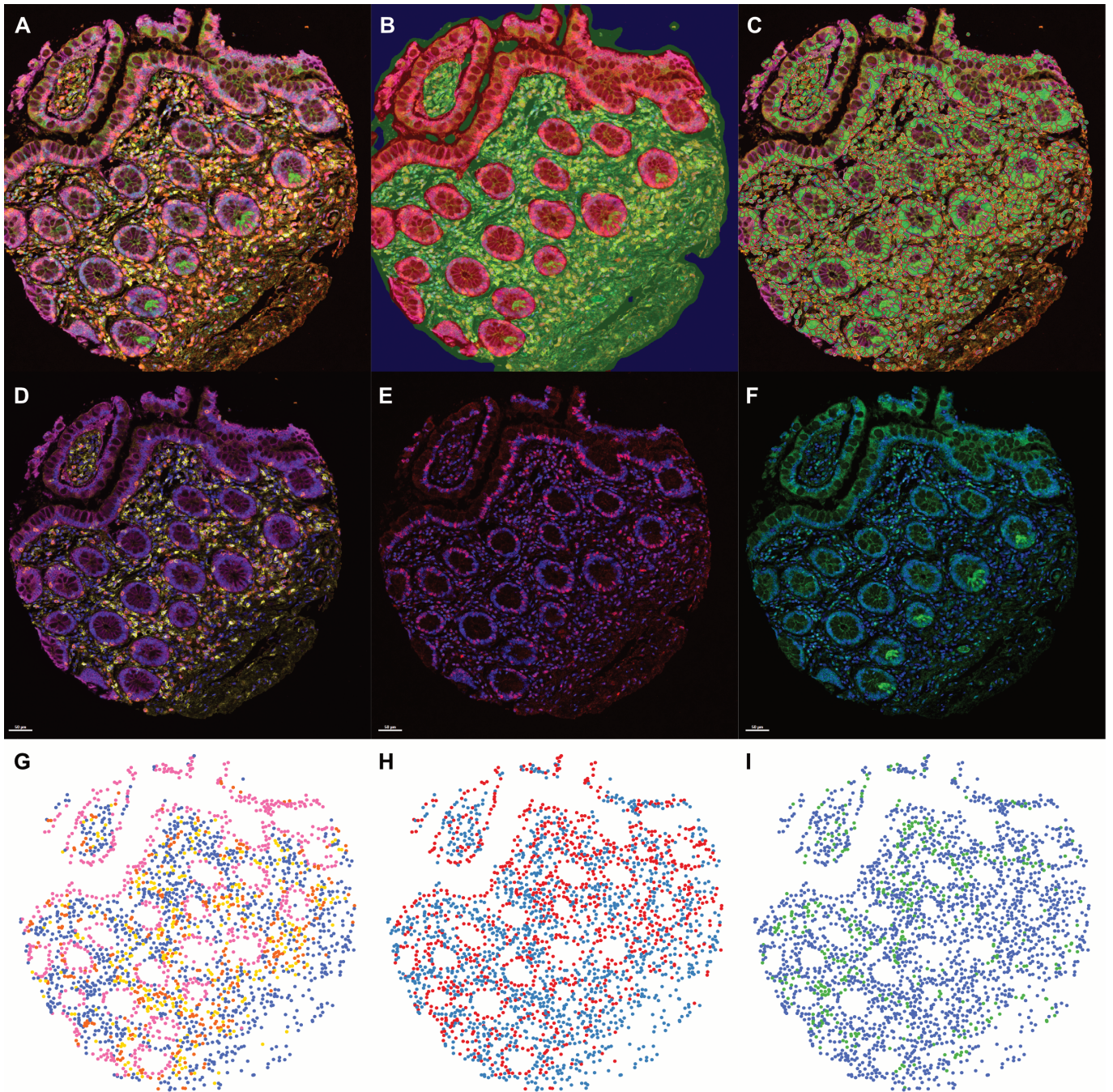
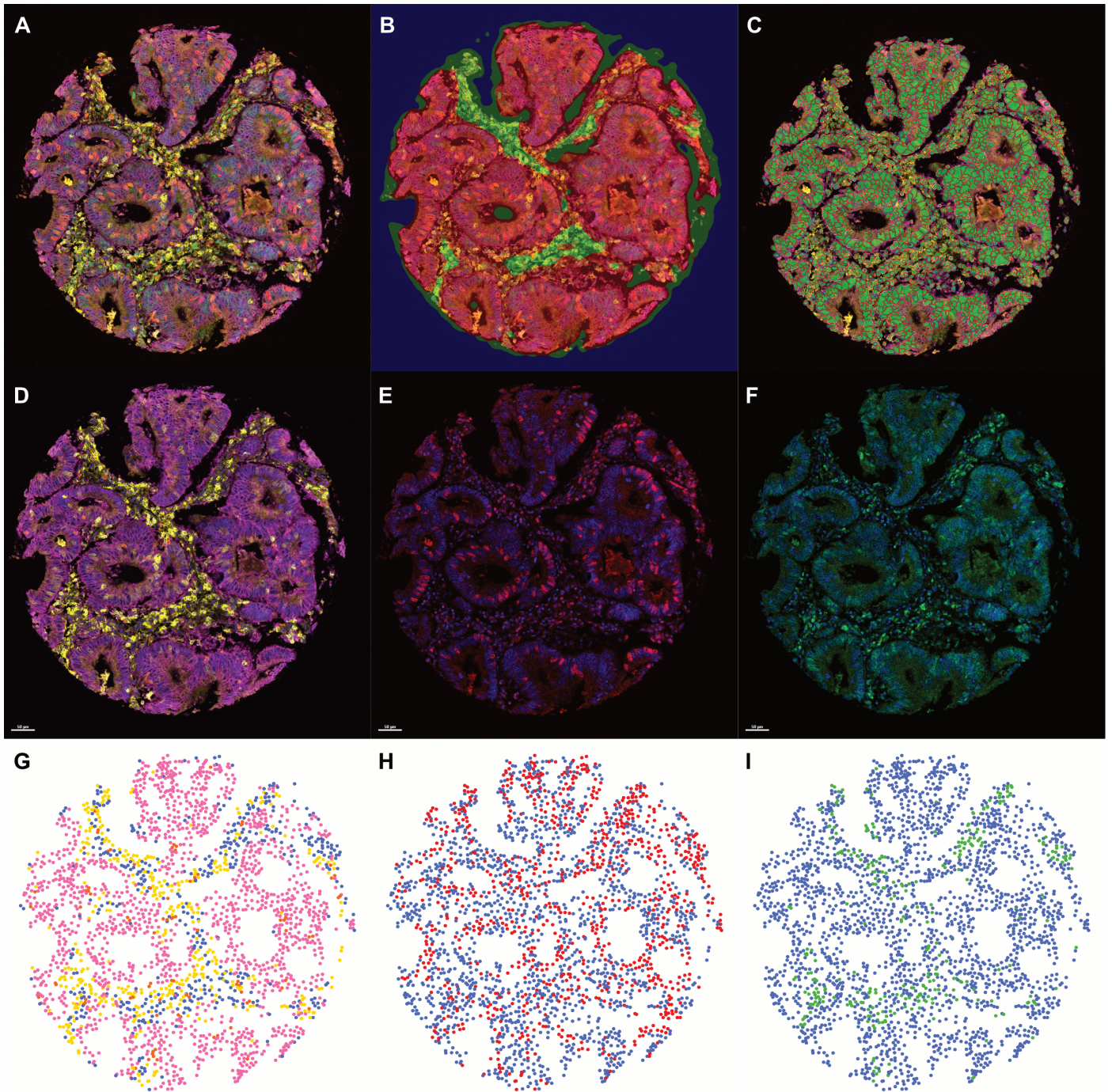




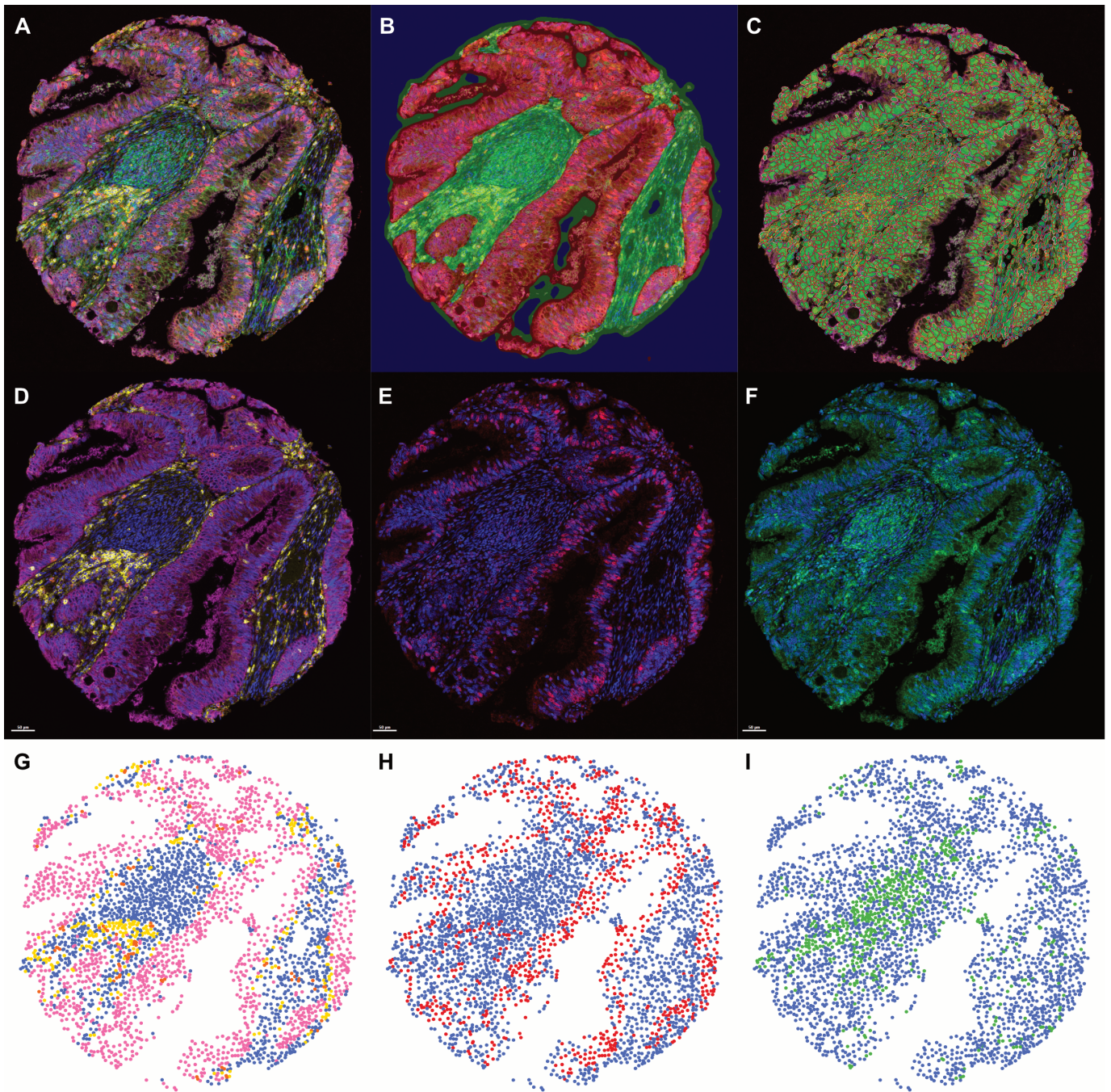
Supplementary Figure 1: Inform Training patient core 1- Tissue segmentation, cell segmentation and cell phenotyping

(A) Unmixed Image of one adjacent normal core fluorescence multiplex imaging of 6 markers CD4 (yellow); CD8 (orange); IL10 (green); Foxp3 (red); EpCAM (purple) nucleus (DAPI blue) (B) Tissue segmentation trained by EpCAM expression and selecting examples of each region: epithelium (red), stroma (green) and blank (Blue). (C) Cell segmentation - Cells were identified and separated based on nuclear biomarkers (DAPI, Foxp3) and membrane biomarkers (CD4, CD8, EpCAM). For the phenotype identification, three separated schemes were used to assure accuracy. (D, E, F) show training images and (G,H,I) show the trained phenotypes with each dot representing a cell with blue dots representing other cells. (D&G) images depicting CD4+ (yellow), CD8+(orange), EpCAM+(Purple). (E&H) images depict Foxp3+ (red) (F&I) IL-10+ (green). All phenotypes were merged into the final phenotypes, which were used for all analyses.



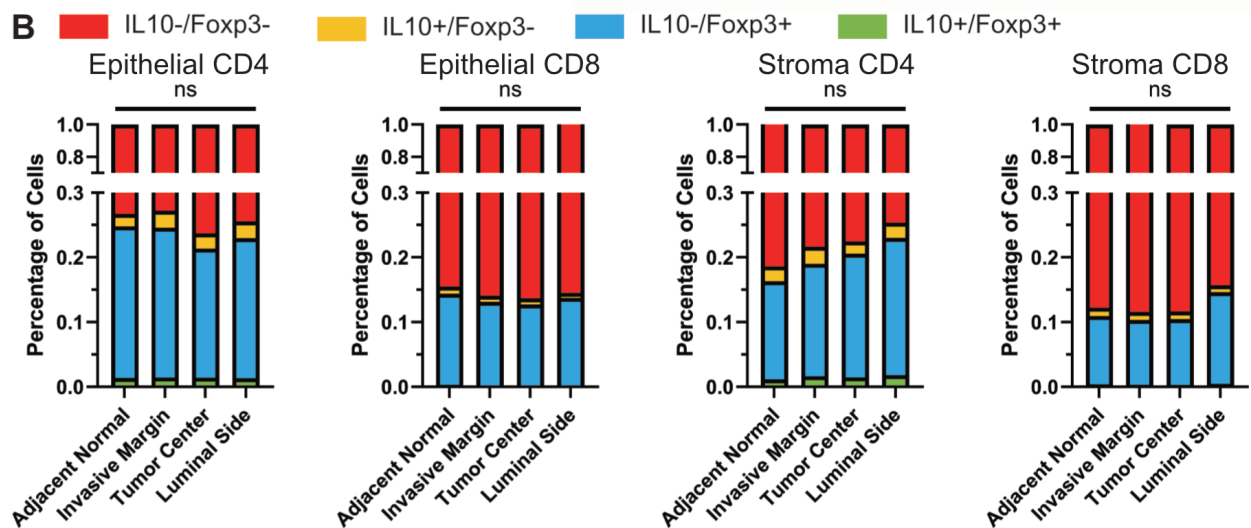
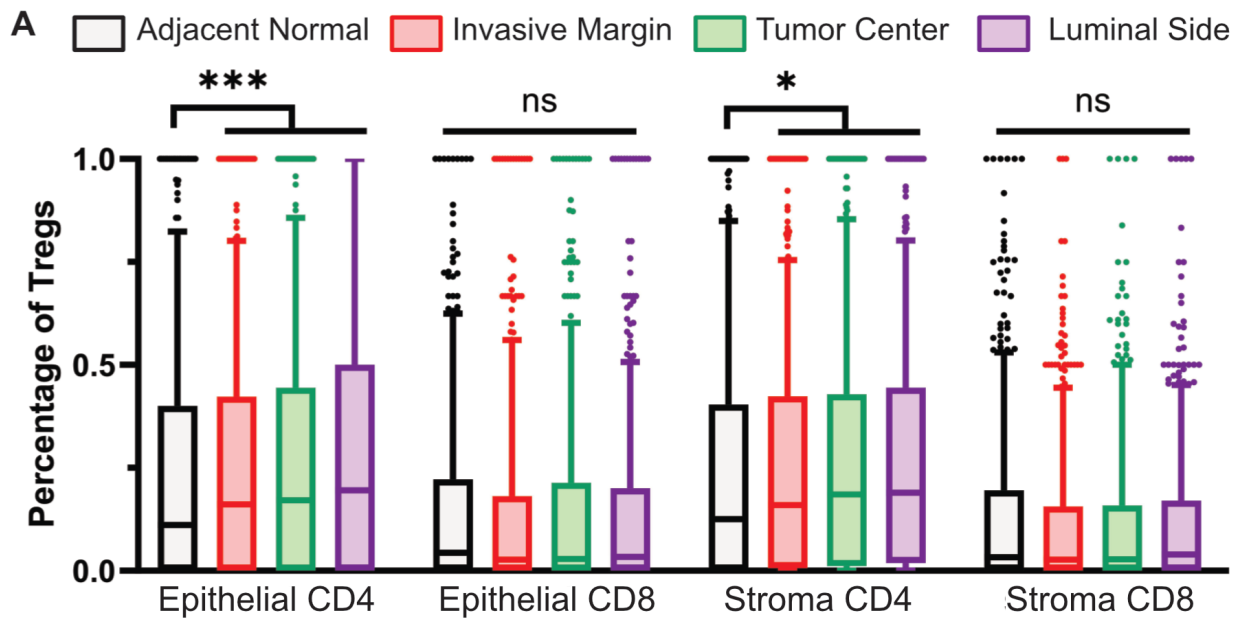
Supplementary Figure 2: Inform Training patient core 2- Tissue segmentation, cell segmentation and cell phenotyping

(A) Unmixed Image of one tumour core fluorescence multiplex imaging of 6 markers CD4 (yellow); CD8 (orange); IL10 (green); Foxp3 (red); EpCAM (purple) nucleus (DAPI blue) (B) Tissue segmentation trained by EpCAM expression and selecting examples of each region: tumour epithelium (red), stroma (green) and blank (Blue). (C) Cell segmentation - Cells were identified and separated based on nuclear biomarkers (DAPI, Foxp3) and membrane biomarkers (CD4, CD8, EpCAM). For the phenotype identification, three separated schemes were used to assure accuracy. (D, E, F) show training images and (G,H,I) show the trained phenotypes with each dot representing a cell with blue dots representing other cells. (D&G) images depicting CD4+ (yellow), CD8+(orange), EpCAM+(Purple). (E&H) images depict Foxp3+ (red) (F&I) IL-10+ (green). All phenotypes were merged into the final phenotypes, which were used for all analyses.



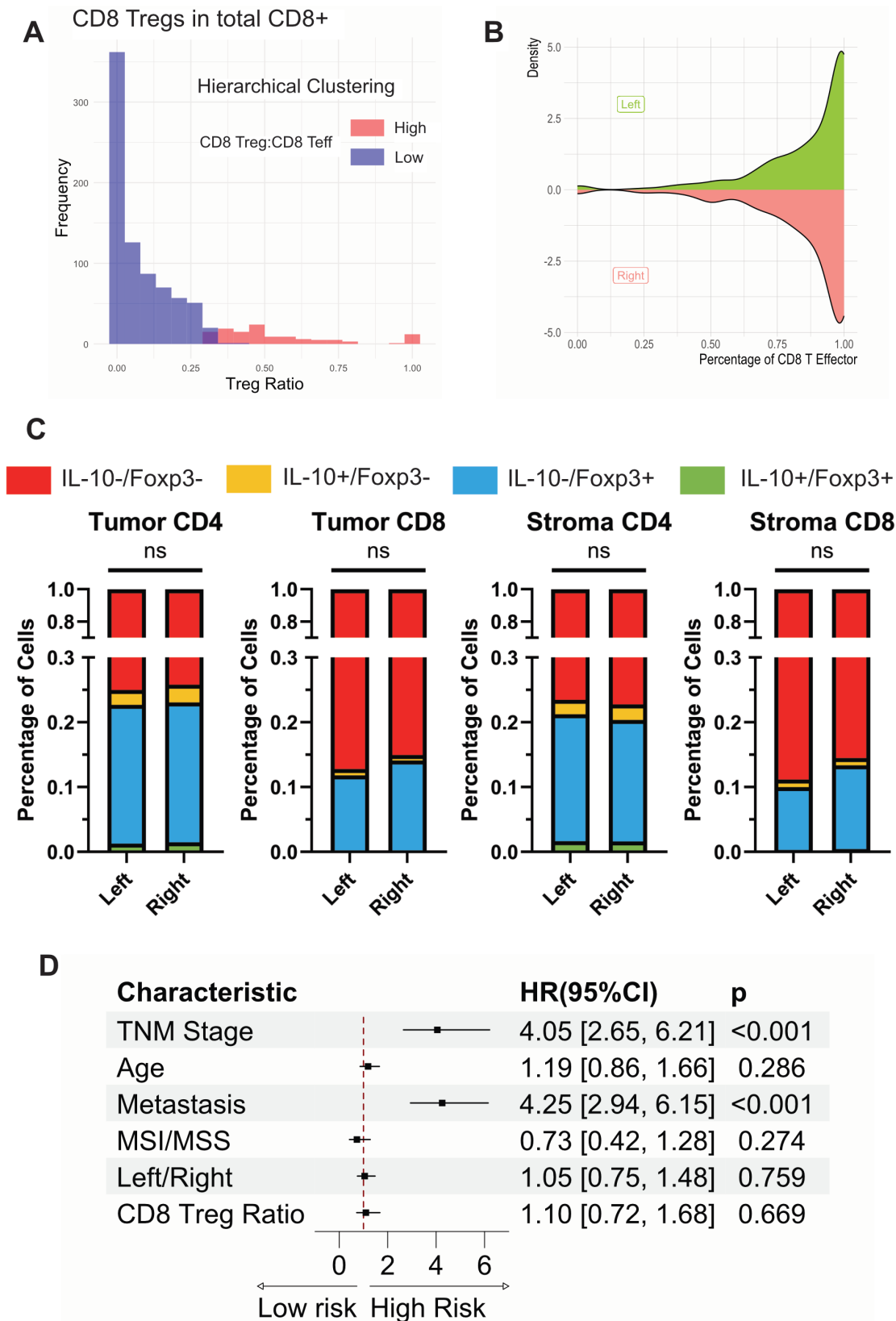
Supplementary Figure 3: Inform Training patient core 3- Tissue segmentation, cell segmentation and cell phenotyping

(A) Unmixed Image of one tumour core fluorescence multiplex imaging of 6 markers CD4 (yellow); CD8 (orange); IL10 (green); Foxp3 (red); EpCAM (purple) nucleus (DAPI blue) (B) Tissue segmentation trained by EpCAM expression and selecting examples of each region: tumour epithelium (red), stroma (green) and blank (Blue). (C) Cell segmentation - Cells were identified and separated based on nuclear biomarkers (DAPI, Foxp3) and membrane biomarkers (CD4, CD8, EpCAM). For the phenotype identification, three separated schemes were used to assure accuracy. (D, E, F) show training images and (G,H,I) show the trained phenotypes with each dot representing a cell with blue dots representing other cells. (D&G) images depicting CD4+ (yellow), CD8+(orange), EpCAM+(Purple). (E&H) images depict Foxp3+ (red) (F&I) IL-10+ (green). All phenotypes were merged into the final phenotypes, which were used for all analyses.

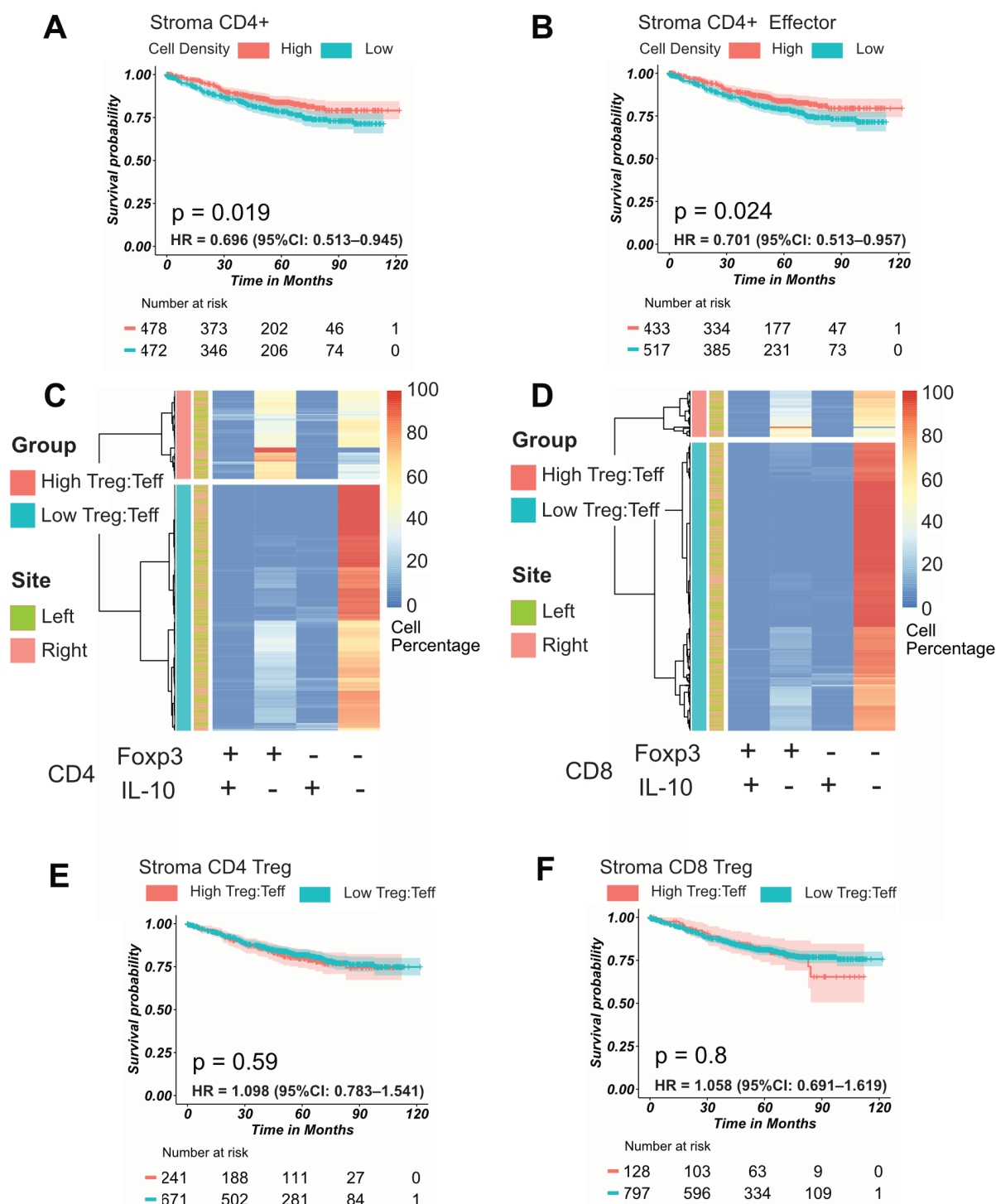


Supplementary Figure 4: Percentage of Treg cells in total CD4 and CD8 population.

(A) Box and whiskers showing percentage of Tregs (Foxp3⁺ and/or IL10⁺) of total epithelial CD4⁺ cells, epithelial CD8⁺ cells, stroma CD4⁺ cells and stroma CD8⁺ cells in the adjacent normal, invasive margins, tumor center and luminal side. **(B)** Bar graphs depicting comparison of the percentage of different Treg phenotypes of total CD4⁺ and CD8⁺ cells in the tumor epithelium and stroma areas of the adjacent normal, invasive margin, tumor center and luminal side. IL10⁻Foxp3⁻ (Red), IL10⁺Foxp3⁻ (yellow), IL10⁻Foxp3⁺ (blue) and IL10⁺Foxp3⁺ (green).

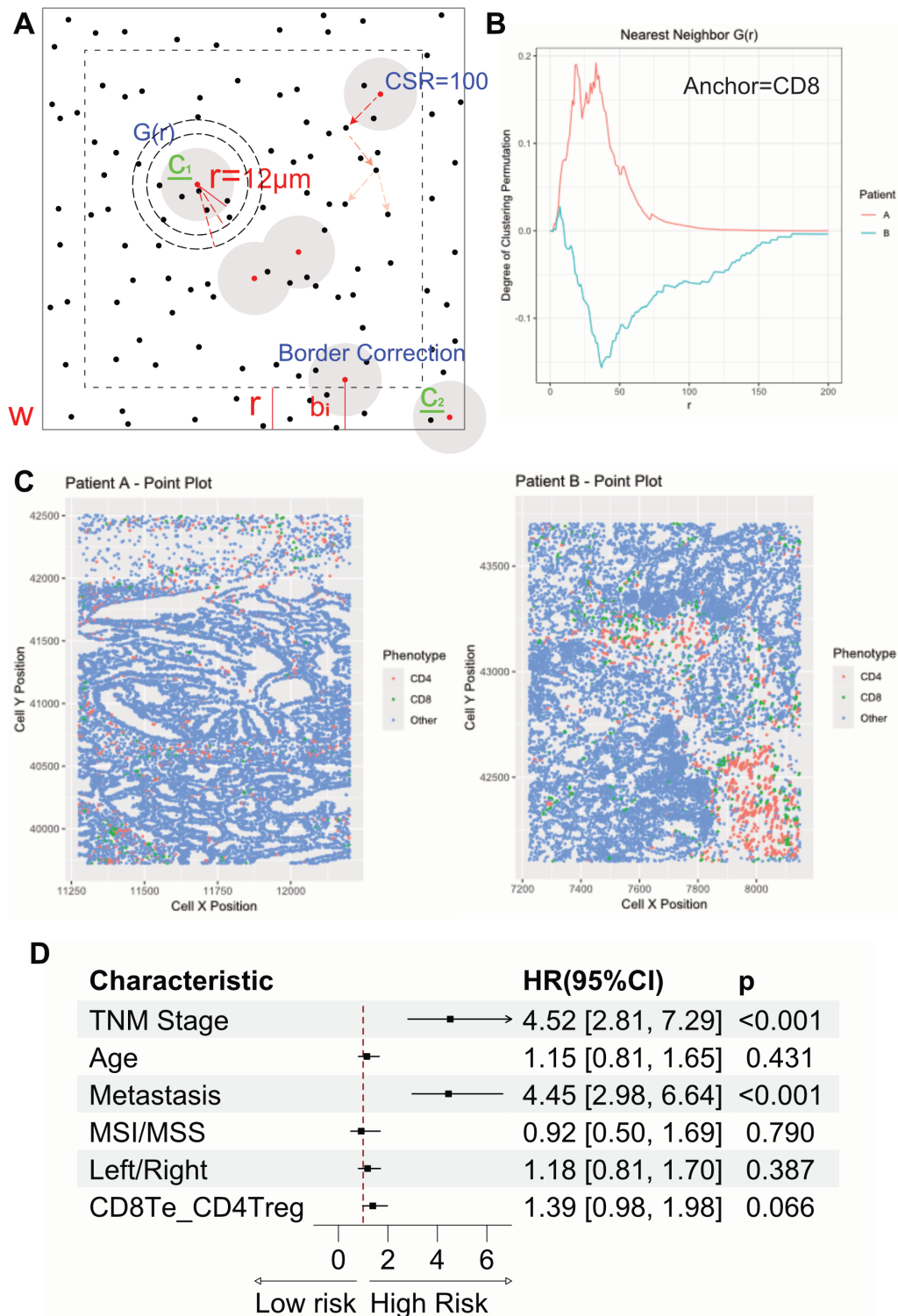


Supplementary Figure 5: Hierarchical clustering of CD8 Treg and effector T cells Hierarchical clustering was used to divide patients into those with high and low percentages of CD8 Treg to CD8 Teff cells as shown in Figure 4. **(A)** Histogram of frequency of different percentages of CD8+Treg of total CD8+ cell in patient tumour cores **(B)**Percentage of CD8+ Teff in tumor cores from both right and left sided CRC patients **(C)** Percentage of different Treg subtypes as a percentage of total CD4 and CD8 cells in the tumor and stroma areas of left and right-sided patients tumour cores



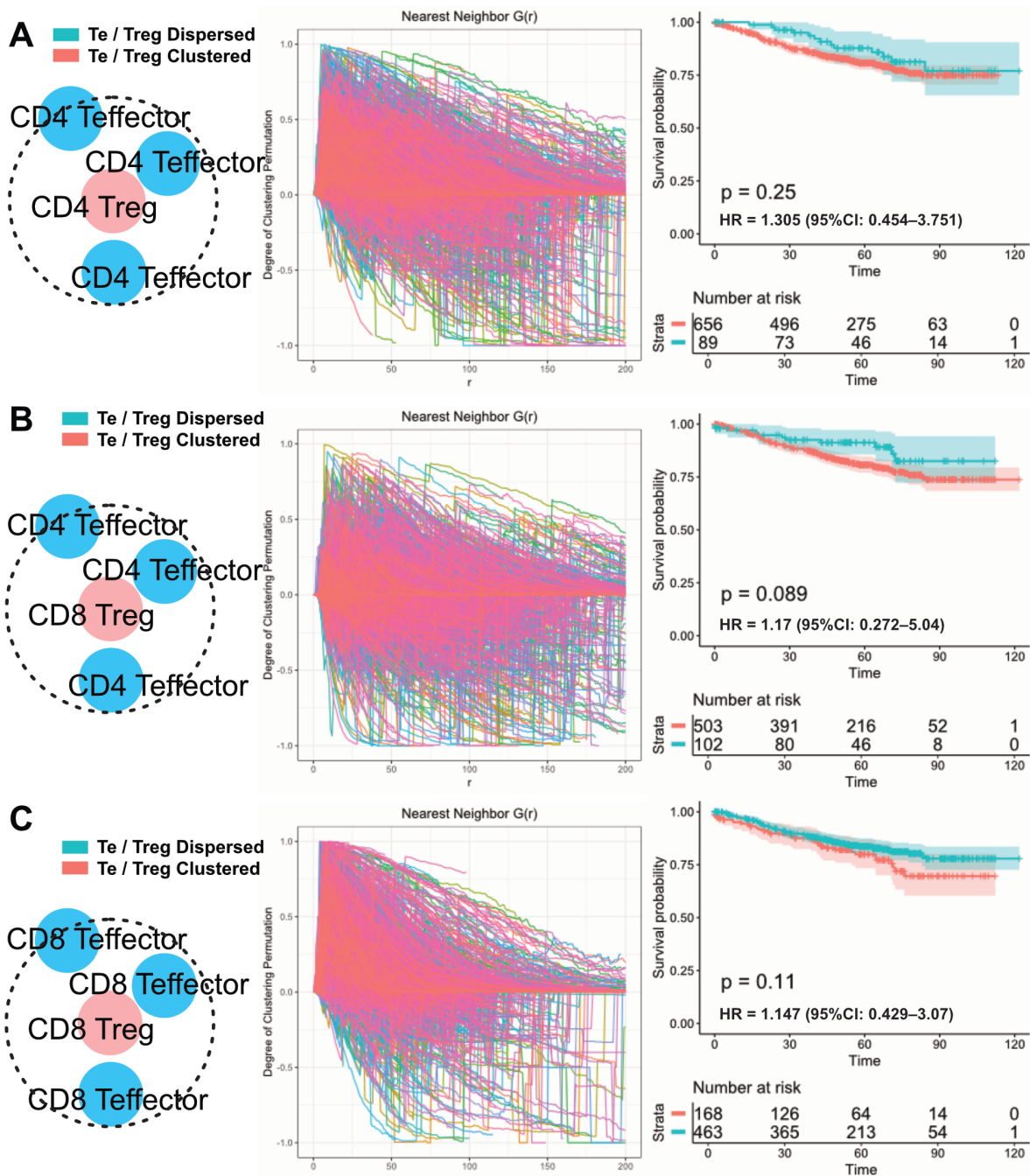
Supplementary Figure 6: Stromal CD4⁺ T effector cells associated with better survival but no effect of the ratio of stromal CD4 or CD8 Tregs on survival

Kaplan Meier survival curves for the density of **(A)** total CD4⁺ T cells and **(B)** Teff CD4⁺ T cells (CD4⁺Foxp3⁻IL10⁻) cells in the stroma with 95% confidence intervals shown in shaded red (high) and green (low). Hazard ratios calculated by univariant Cox regression. **(C)** Patients' tumours were divided into high and low ratios of CD4⁺ Treg to CD4⁺ Teff cells in stroma by hierarchical clustering. **(D)** Patients were divided into high and low ratios of CD8 Treg cells to Teff cells the in stroma by hierarchical cluster. Kaplan-Meier survival curves for the density of **(E)** high and low percentages of stromal CD4⁺ Treg to CD4⁺ Teff cells and **(F)** high and low percentages of stromal CD8⁺ Treg to CD8⁺ Teff cells with 95% confidence intervals shown in shaded red (high) and green (low). Hazard ratios calculated by univariant Cox regression.



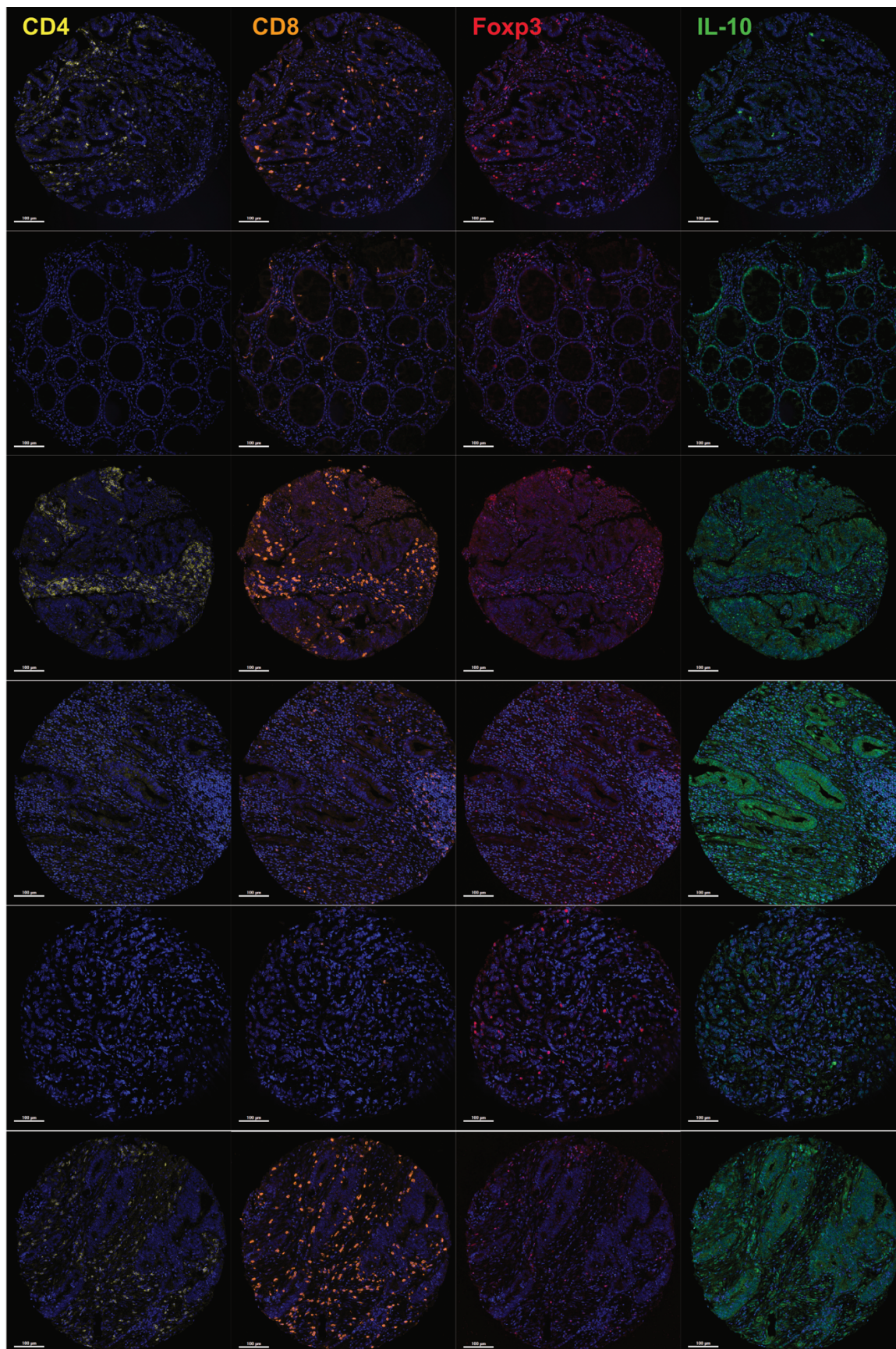
Supplementary Figure 7: The mechanism and example of $G(r)$ function and Multivariate Cox analysis of CD8 Teff to CD4Treg

For the distribution of the nearest neighbour distances, $G(r)$. **(A)** The percentage of counted cell within a radius (e.g. 12 μm) of anchor cells with border correction was calculated, then the observed results were deducted by 100 times random permutation to get the final result. **(B-C)** The positive result implies two types of cells (counted cell and anchor cell) are clustered to each other compared to random distribution, for example, the CD4 and CD8 in patient A; While negative result imply two cells are dispersed compared to random distribution, for example, the CD4 and CD8 in patient B. **(D)** Multi-variant Cox regression showing clustered and dispersed CD8Teff (CD8Te) to CD4 Treg is not independent of TMN stage, patient age, metastasis, MSI status or tumour location (left versus right sided CRC) with hazard ratios and 95% confidence intervals.



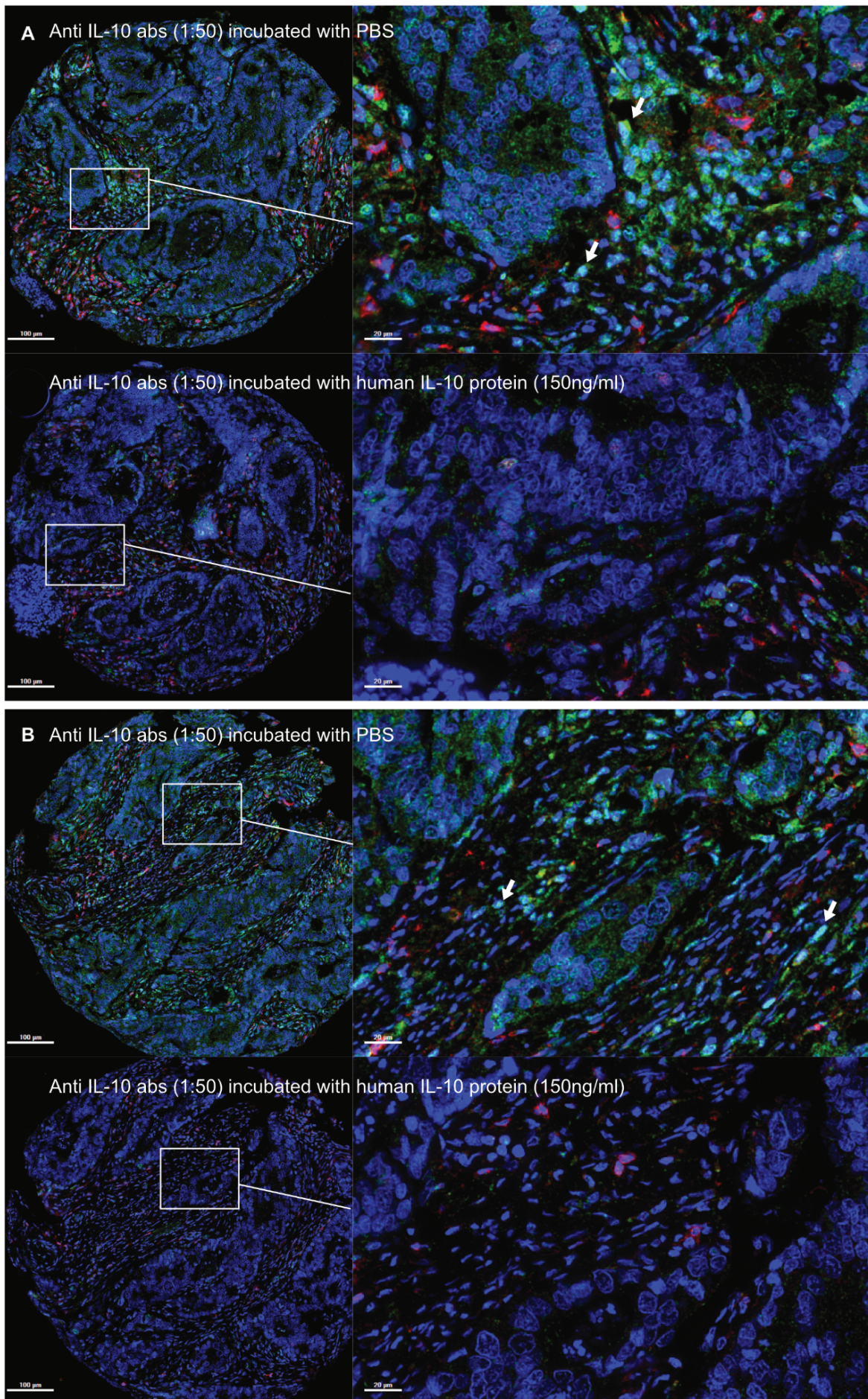
Supplementary Figure 8: No effect of cell-cell contact between CD4⁺ Teff-CD4⁺ Treg, CD4⁺ Teff-CD8⁺ Treg, or CD8⁺ Teff-CD8⁺ Treg and patients survival

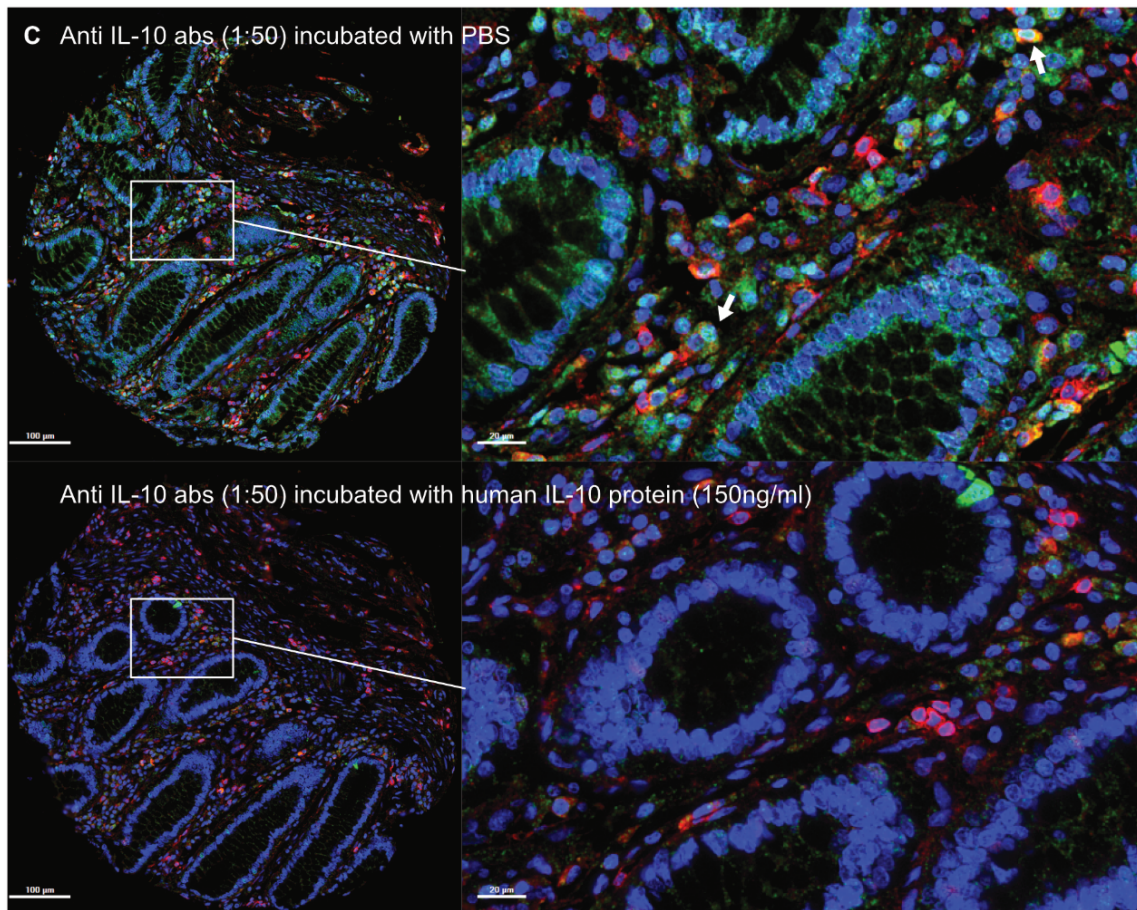
(A) G-cross function was calculated between CD4⁺ Teff cells and CD4⁺ Treg cells, and the prognostic value were tested by Kaplan-Meier survival analysis. **(B)** G-cross function was calculated between CD4⁺ Teff cells and CD8⁺ Treg cells, and the prognostic value was tested by Kaplan-Meier survival analysis with 95% confidence intervals shown in shaded red (high) and green (low). Hazard ratios calculated by univariant Cox regression. **(C)** G-cross function was calculated between CD8⁺ Teff to CD8⁺ Treg cells, and the prognostic value were tested by Kaplan-Meier survival analysis.



Supplementary Figure 9: Phenotype of Cells showing individual marker staining

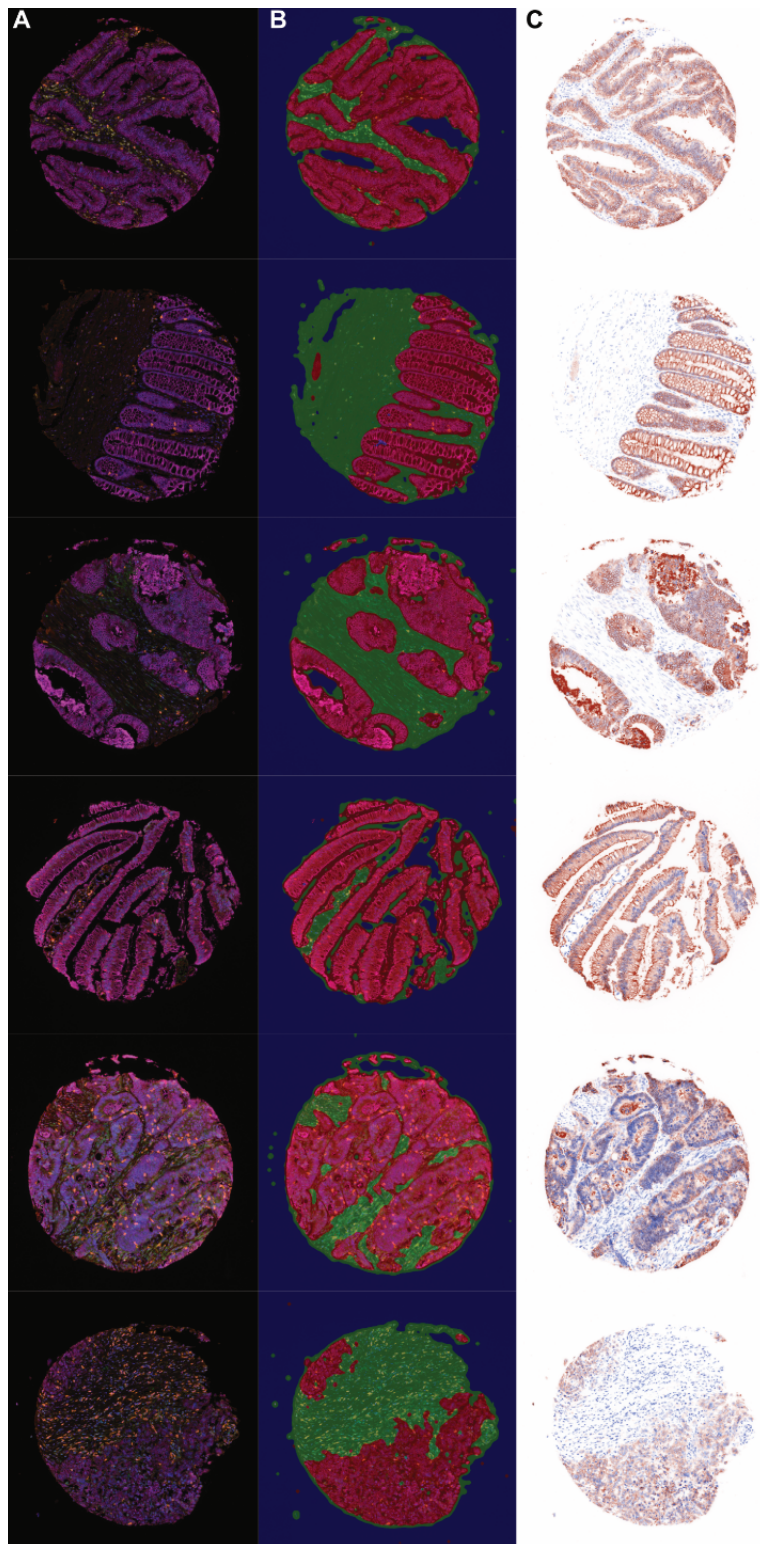
Representative TMA core showing multiplex IHC with all markers (antibody-opal combinations). Individual images of the 6 patient cores showing CD4⁺ cells (yellow), CD8⁺ cells (orange), IL10⁺ (green) Foxp3⁺ (Red) and EpCAM (magenta). Different levels of positive cells and differences in fluorescence intensity can be seen between cores. For Foxp3 staining examples of dual staining with CD4 and CD8. Staining of epithelial cells is evident in some cores but not all cores.





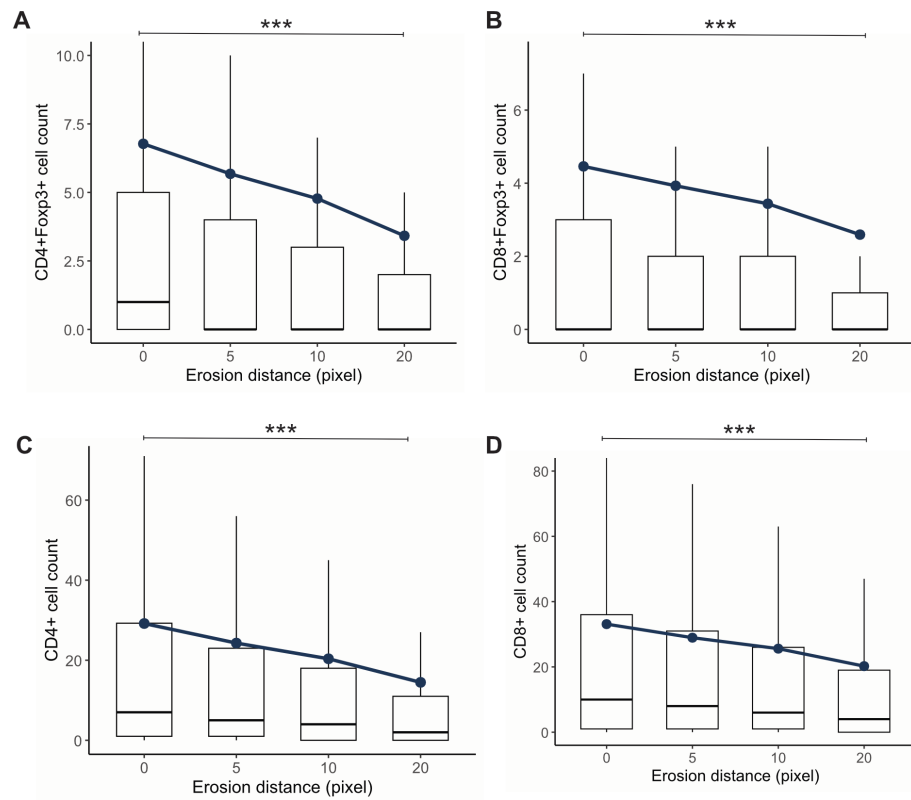
Supplementary Figure 10: Multiplex IHC immunoprobing with anti- IL10 antibody and with anti-IL-10 and IL10 protein block.

Anti- IL-10 antibody (1:50) was incubated with or without 150ng/ml human IL-10 protein at 4°C overnight (pH6). This was then used to immunoprobe control CRC slides. IL10+ cells are shown in **green**. CD4 was chosen as control. Anti-CD4 antibody immunoprobing is depicted i **(red)**. **(A) (B) (C)** represent three patients without human IL-10 protein (top panel) or with IL-10 protein block (bottom panel).



Supplementary Figure 11: Image Training by InForm® - examples of tissue segmentation

(A) The Acquired Image was unmixed **(B)** Tumour epithelium (red), Stroma (green) and Blank (blue) area was trained by manual annotations based on EpCAM expression depicting tumour epithelium. **(C)** Pseudo-pathological view based on EpCAM stain



Supplementary Figure 12: Erosion of the tumour mask by 5, 10 and 20 pixels shows similar decline in CD4+Foxp3+, CD8+Foxp3-, as total CD4 and CD8 T cell count.

The tumour mask was erased by 5, 10 and 20 pixels and the number of **A** CD4+Foxp3+ **B** CD8+Foxp3+ **C** CD4 **D** CD8 T cell count

Supplementary Table 1: Clinical Characteristics of CD8 Treg: CD8 effector T Ratio and CD4 T Effector Group in the tumour epithelium. Chi-squared-test analysis of clinical characteristic with CD8⁺Treg:CD8⁺ T cell ratio and with CD4⁺Teff density in the tumour epithelium.

Clinical Characteristic	CD8 Treg: CD8 effector T cells percentage		P value	CD4 T Effector density Tumour epithelium		P value
	High n (%)	Low n (%)		High n (%)	Low n (%)	
Gender						
Female	50 (40.7)	339 (43.7)	0.520	209 (43.7)	198 (41.9)	0.580
Male	73 (59.3)	436 (56.3)		269 (56.3)	274 (58.1)	
Age						
≤69	77 (62.6)	377 (48.6)	0.004	234 (49)	250 (53)	0.216
>69	46 (37.4)	398 (51.4)		244 (51)	222 (47)	
Site						
Left	62 (50.4)	414 (53.4)	0.534	260 (54.4)	236 (50)	0.175
Right	61 (49.6)	361 (46.6)		218 (45.6)	236 (50)	
TNM stage						
I	15 (12.2)	133 (17.2)	0.050	83 (17.4)	69 (14.6)	0.125
II	42 (34.1)	324 (41.8)		204 (42.7)	181 (38.3)	
III	47 (38.2)	274 (25.4)		145 (30.3)	160 (33.9)	
IV	19 (15.4)	77 (9.9)		46 (9.6)	62 (13.1)	
MSS/MSI						
MSS	101 (85.6)	631 (82.8)	0.452	395 (83.9)	377 (82.5)	0.577
MSI	17 (14.4)	131 (17.2)		76 (16.1)	80 (17.5)	

Raw data used for analysis available in following pages

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855	856	857	858	859	860	861	862	863	864	865	866	867	868	869	870	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885	886	887	888	889	890	891	892	893	894	895	896	897	898	899	900	901	902	903	904	905	906	907	908	909	910	911	912	913	914	915	916	917	918	919	920	921	922	923	924	925	926	927	928	929	930	931	932	933	934	935	936	937	938	939	940	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956	957	958	959	960	961	962	963	964	965	966	967	968	969	970	971	972	973	974	975	976	977	978	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000
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