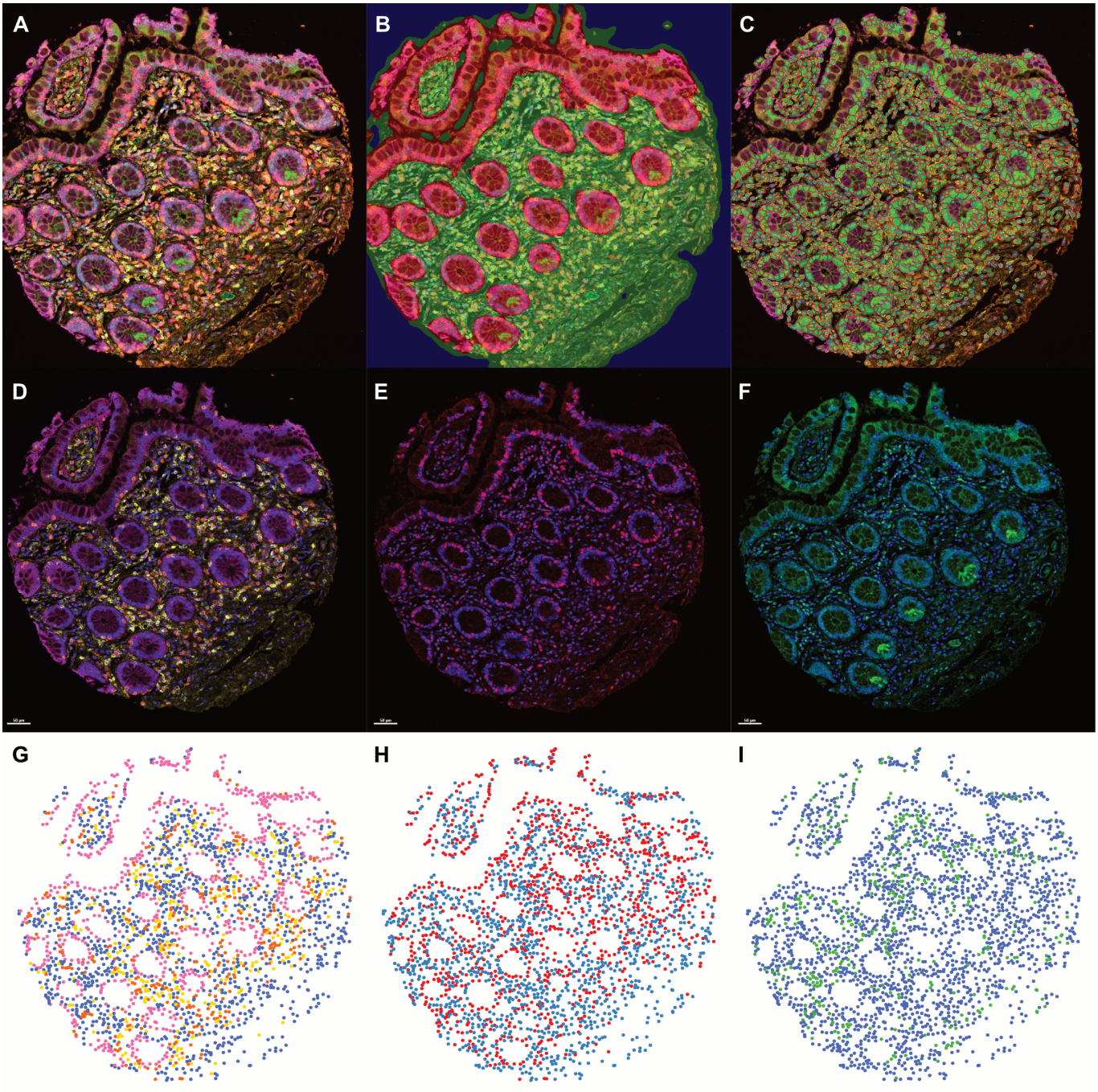
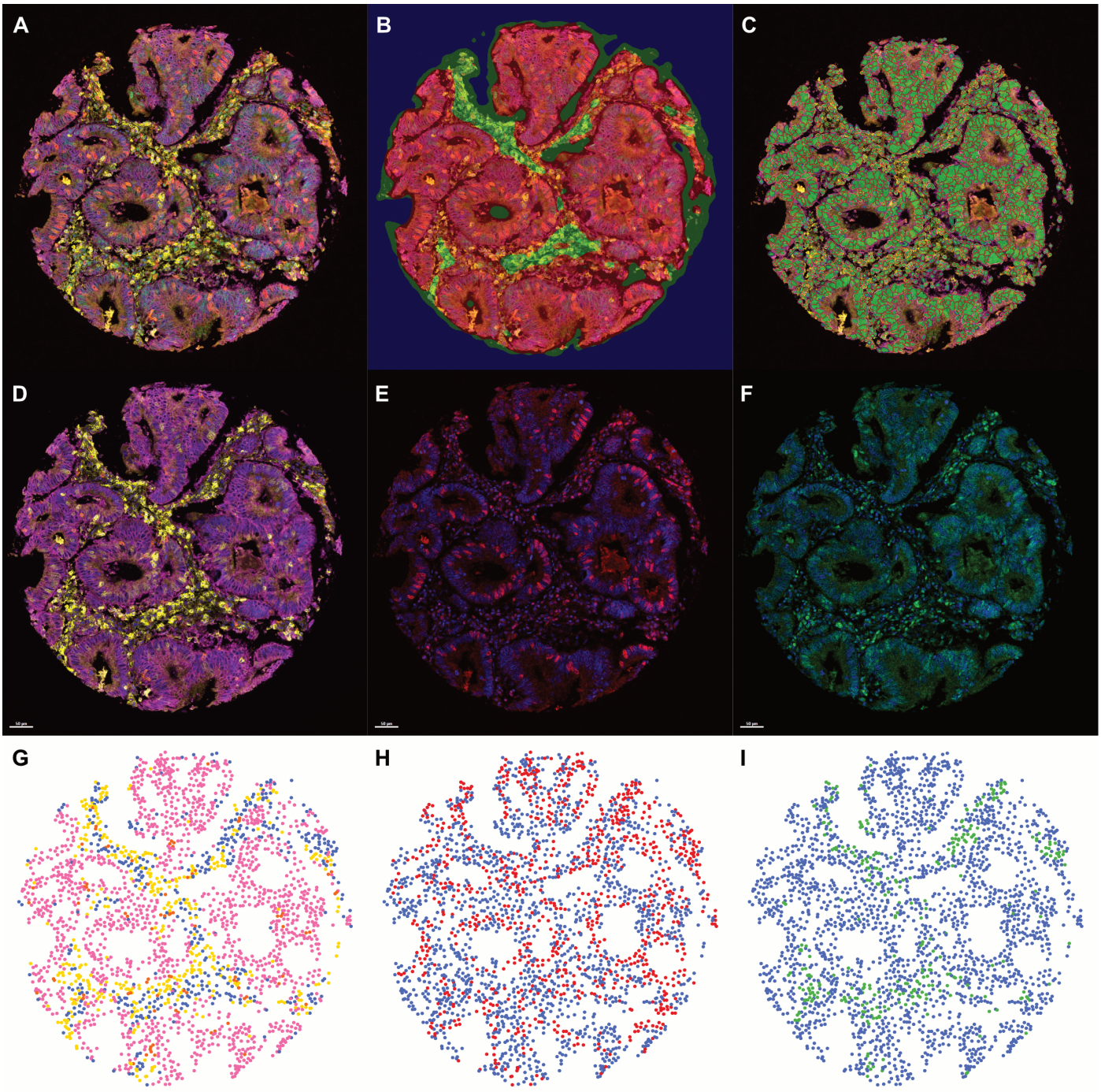


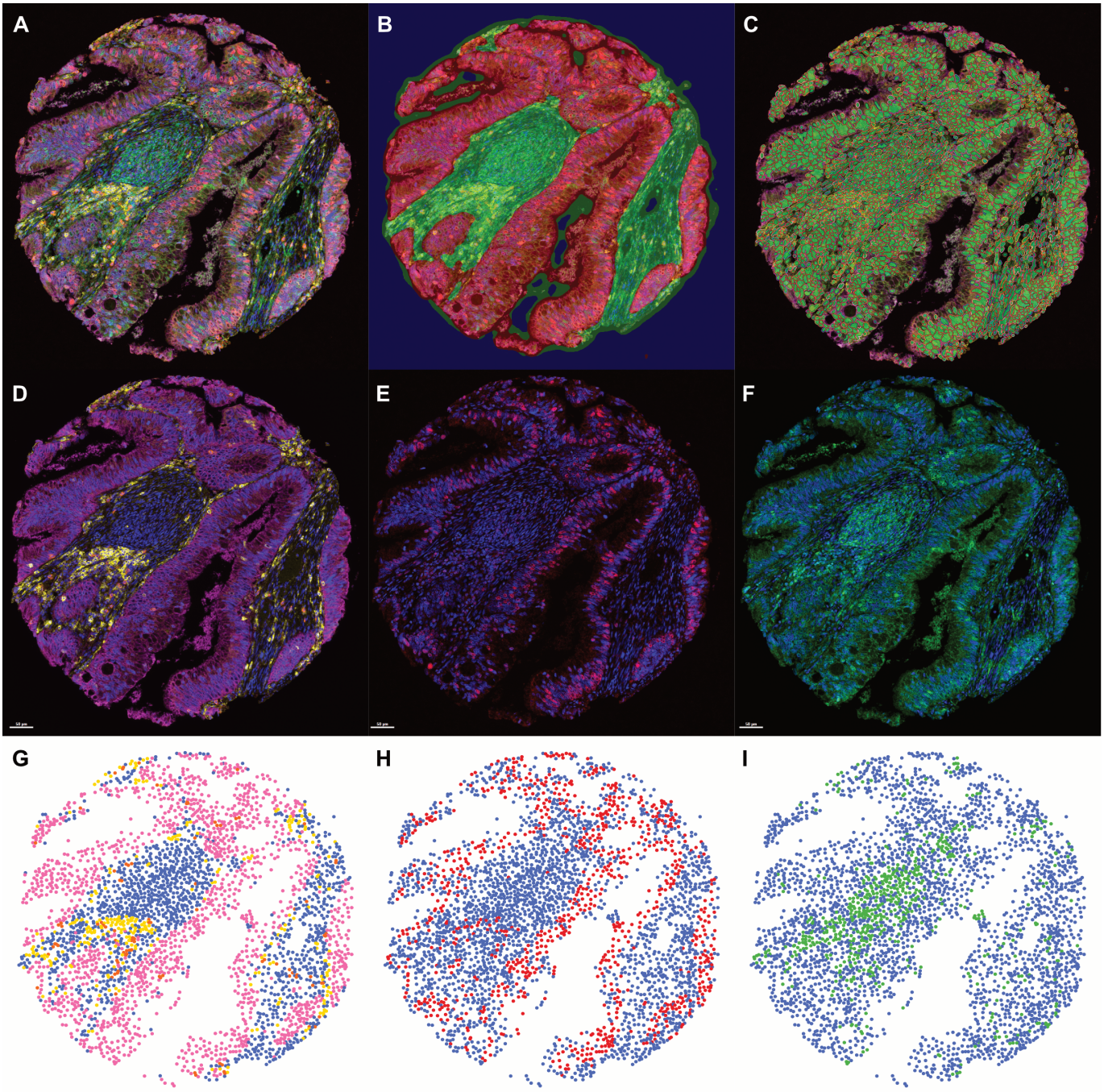
Supplementary Figure 1: Inform Training patient core 1- Tissue segmentation, cell segmentation and cell phenotyping

(**A)** Unmixed Image of one 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 biomakers (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.



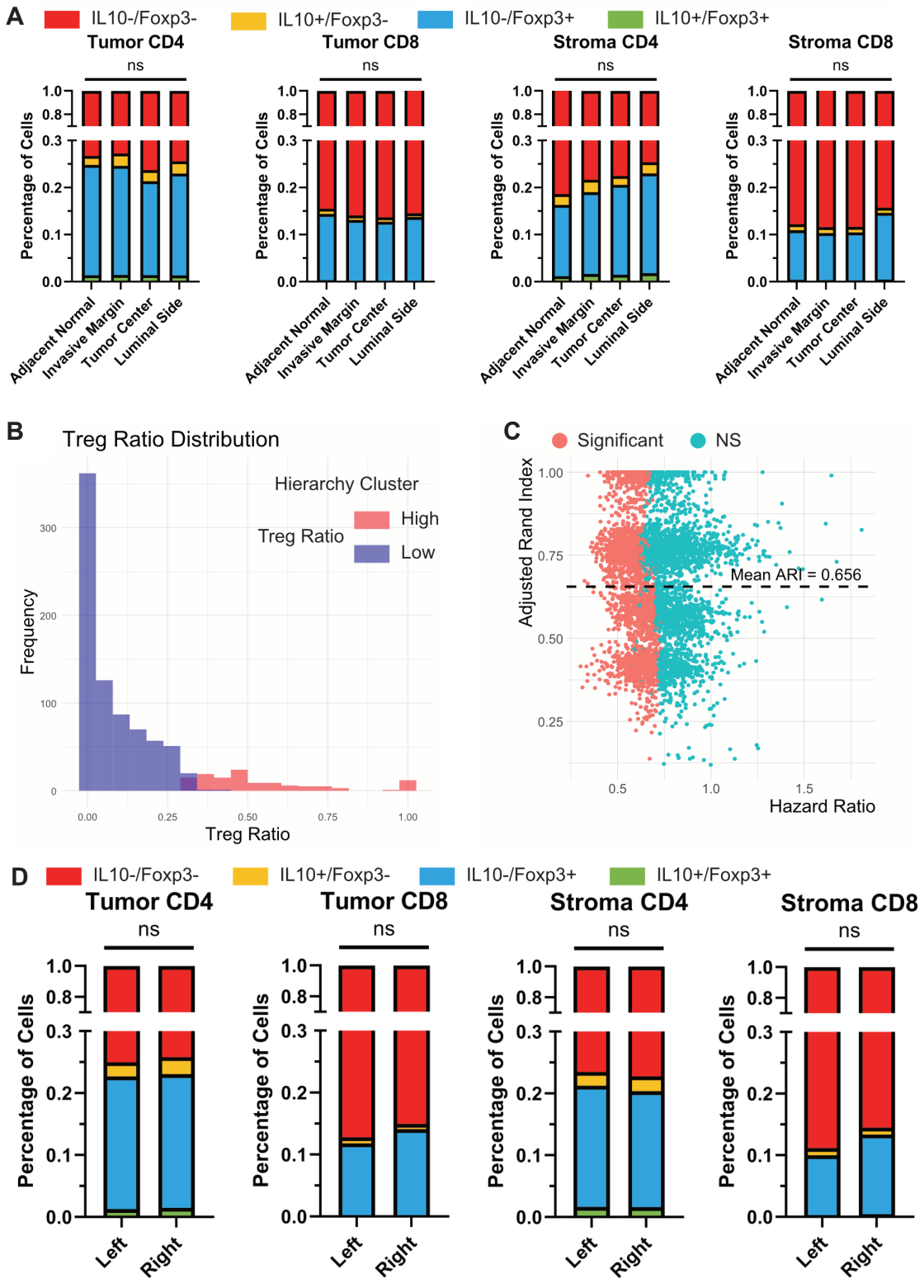
Supplementary Figure 2: Inform Training patient core 2- Tissue segmentation, cell segmentation and cell phenotyping

(**A)** Unmixed Image of one 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 biomakers (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.



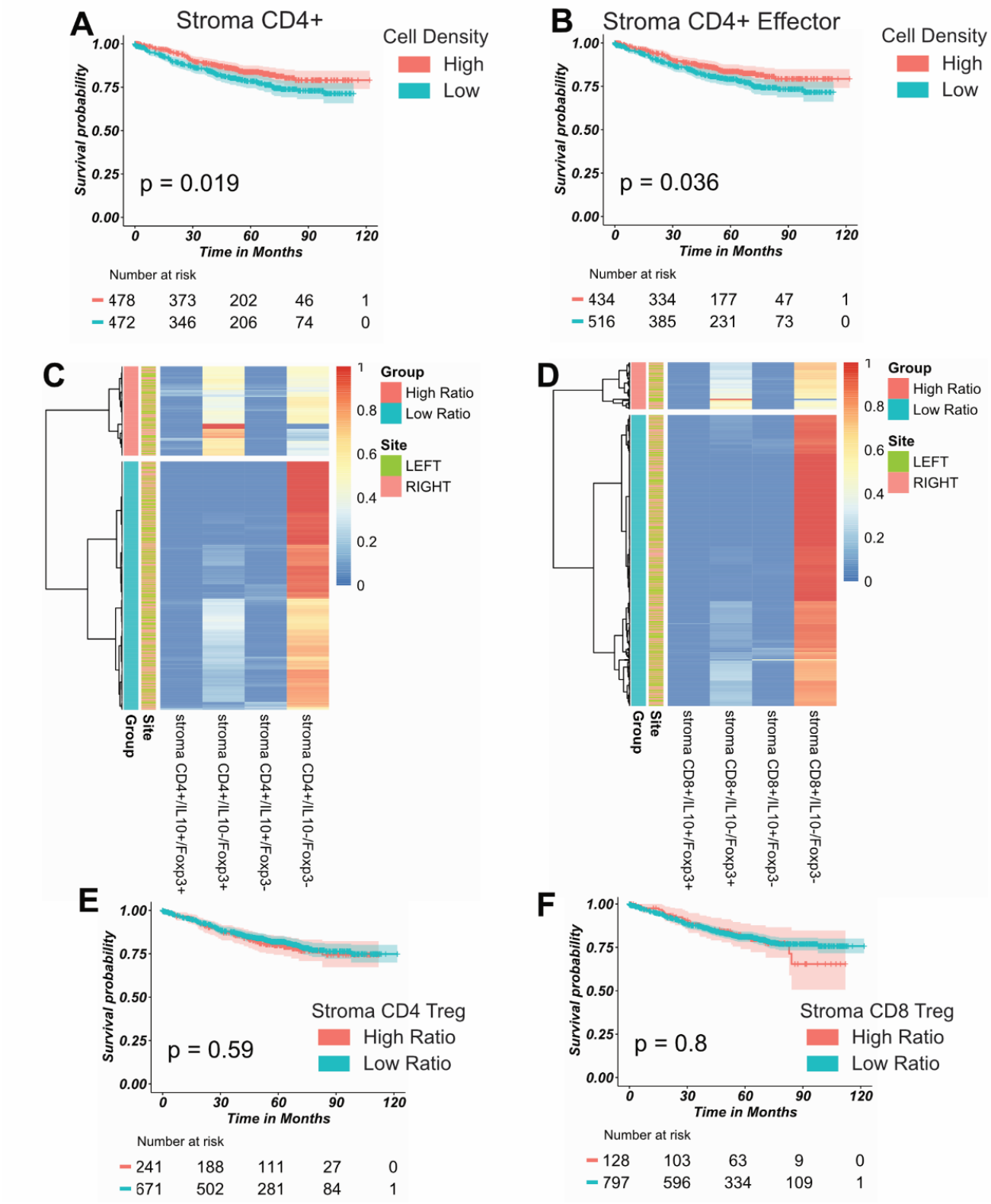
Supplementary Figure 3: Inform Training patient core 3- Tissue segmentation, cell segmentation and cell phenotyping

(**A)** Unmixed Image of one 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 biomakers (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.



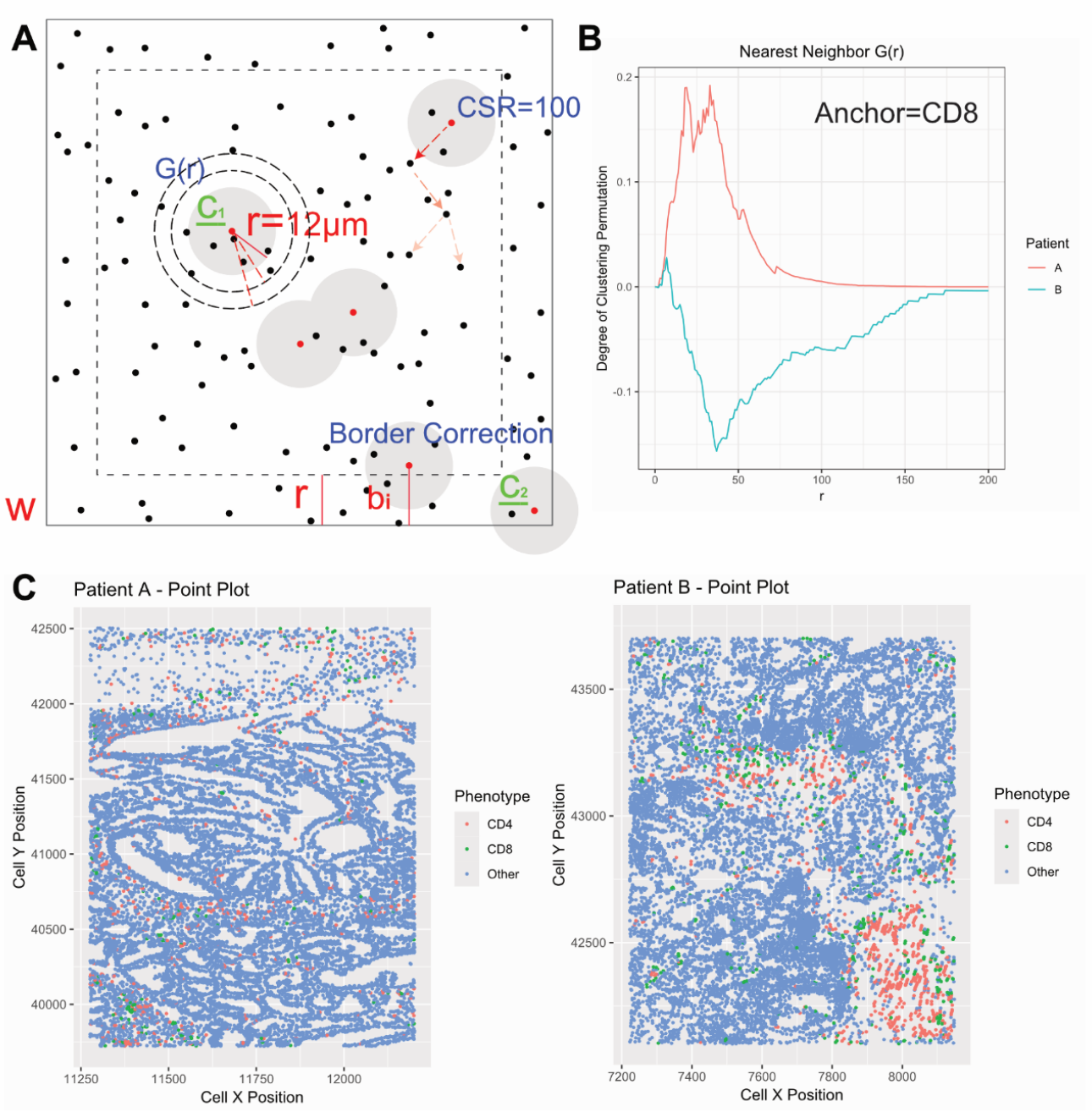
Supplementary Figure 4: Percentage of Treg cells in total CD4 and CD8 population.

(A) Bar graphs depicting comparison of the percentage of different Treg phenotypes of total $CD4^+$ and $CD8^+$cells in the tumour epithelium and stroma areas of the adjacent normal, invasive margin, tumour centre and luminal side. $IL10^-Foxp3^-$ (Red), $IL10^+Foxp3^-$ (yellow), $IL10^-Foxp3^+$ (blue) and $IL10^+Foxp3^+$ (green). **(B)** Treg Ratio distribution **(C)** Bootstrapping was applied 5000 times (N= per analysis) (with replacement, no control on clinical characteristics). **(D)** Bar graphs depicting comparison of the percentage of different Treg phenotypes of total $CD4^+$ and $CD8^+$ cells in the tumour epithelium and stroma areas of the left and right-sided CRC. $IL10^-Foxp3^-$ (Red), $IL10^+Foxp3^-$ (yellow), $IL10^-Foxp3^+$ (blue) and $IL10^+Foxp3^+$ (green).



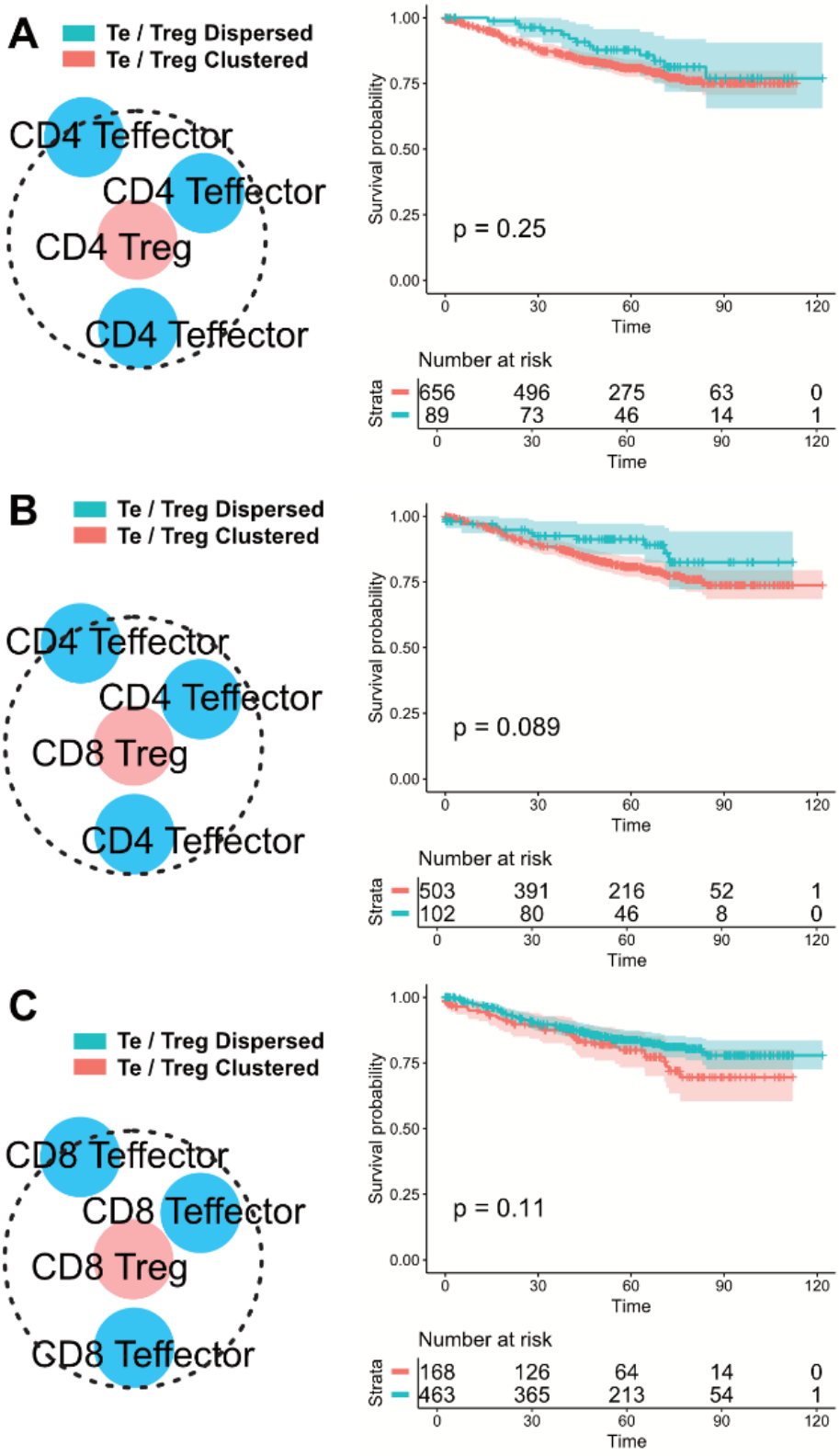
Supplementary Figure 5: Stromal CD4⁺ T effector cells associated with better survival but no effect of the ratio of stromal CD4 or CD8 Tregs on survival

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 **(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-Meir survival curves for the density of **(E)** high and low ratios of stromal $CD4^+$ Treg to $CD4^+$ Teff cells and **(F)** high and low ratio of stromal $CD8^+$ Treg to $CD8^+$ Teff cells.



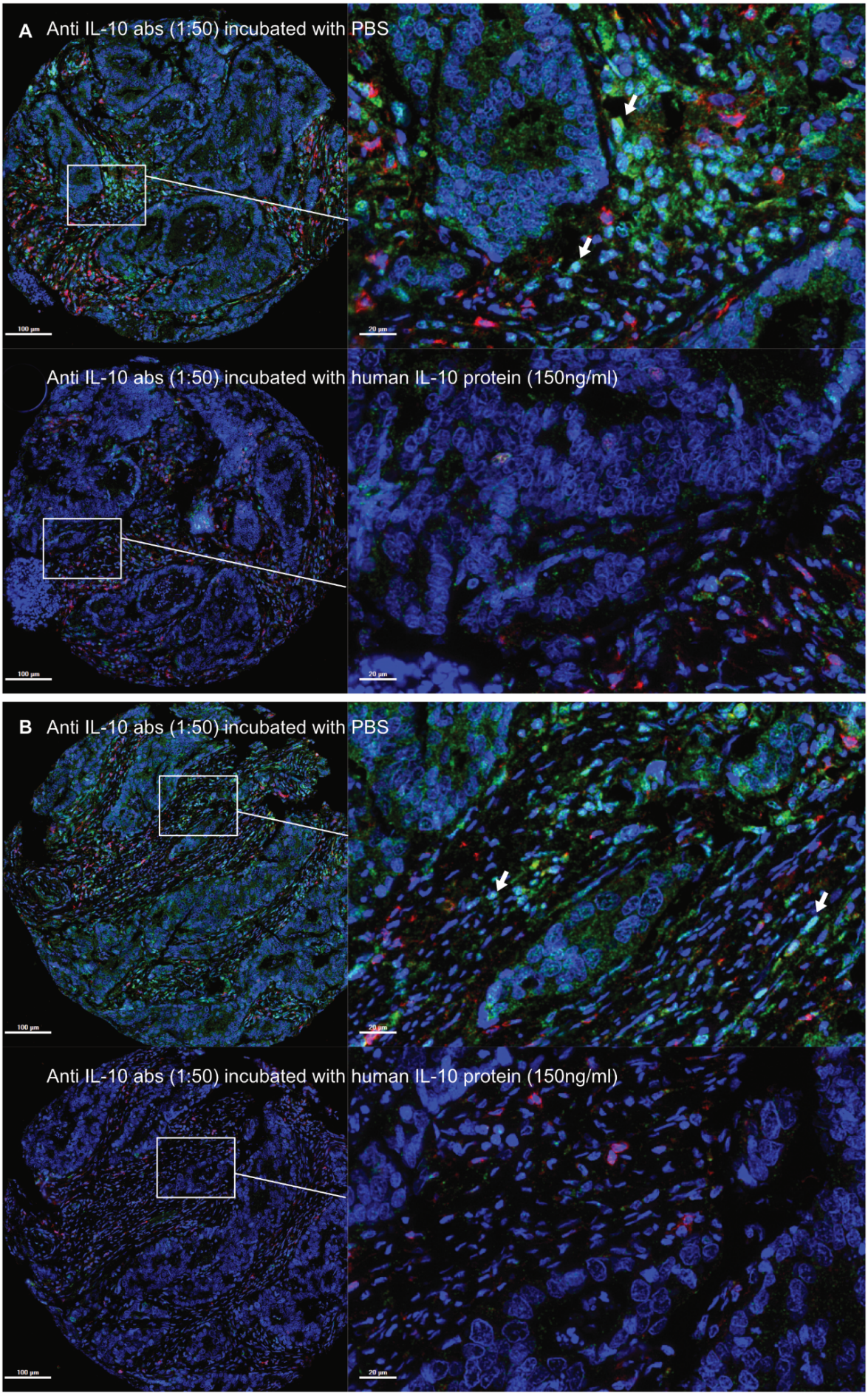
Supplementary Figure 6: No effect of cell-cell contact between CD4⁺ Teff-CD4⁺ Treg, CD4⁺ Teff-CD8⁺ Treg, or CD8⁺ Teff-CD8⁺ Treg and patients survival

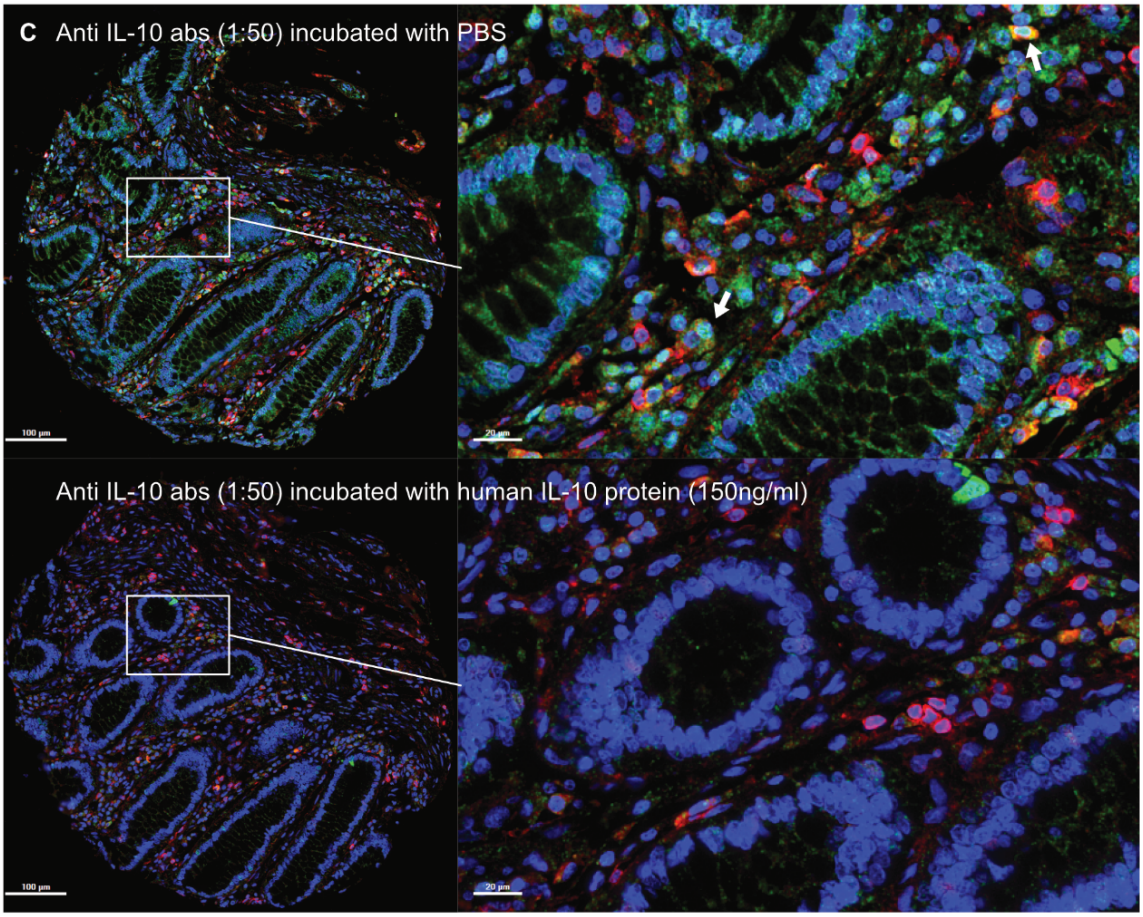
(A) G-cross function was calculated between CD4⁺ Teff cells and CD4⁺ Treg cells, and the prognostic value were tested by Kaplan-Meier survival analysis. **(B)** G-cross function was calculated between CD4⁺ Teff cells and CD8⁺ Treg cells, and the prognostic value was tested by Kaplan-Meier survival analysis. **(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 7: No effect of cell-cell contact between CD4⁺ Teff-CD4⁺ Treg, CD4⁺ Teff-CD8⁺ Treg, or CD8⁺ Teff-CD8⁺ Treg and patients survival

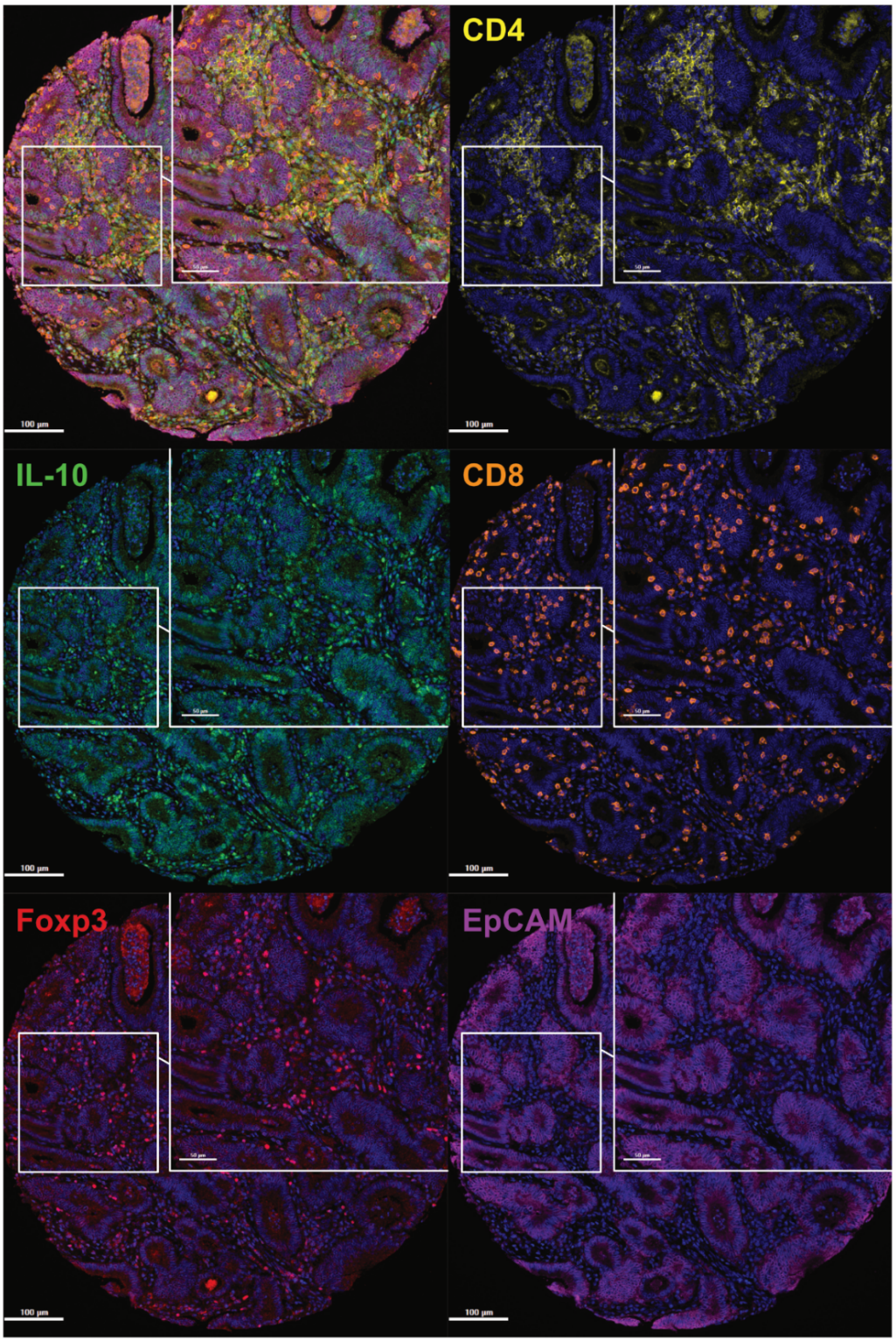
(A) G-cross function was calculated between CD4⁺ Teff cells and CD4⁺ Treg cells, and the prognostic value were tested by Kaplan-Meier survival analysis. **(B)** G-cross function was calculated between CD4⁺ Teff cells and CD8⁺ Treg cells, and the prognostic value was tested by Kaplan-Meier survival analysis. **(C)** G-cross function was calculated between CD8⁺ Teff to CD8⁺ Treg cells, and the prognostic value were tested by Kaplan-Meier survival analysis.


A
Anti IL-10 abs (1:50) incubated with PBS
100 µm
20 µm
Anti IL-10 abs (1:50) incubated with human IL-10 protein (150ng/ml)
100 µm
20 µm
B
Anti IL-10 abs (1:50) incubated with PBS
100 µm
20 µm
Anti IL-10 abs (1:50) incubated with human IL-10 protein (150ng/ml)
100 µm
20 µm

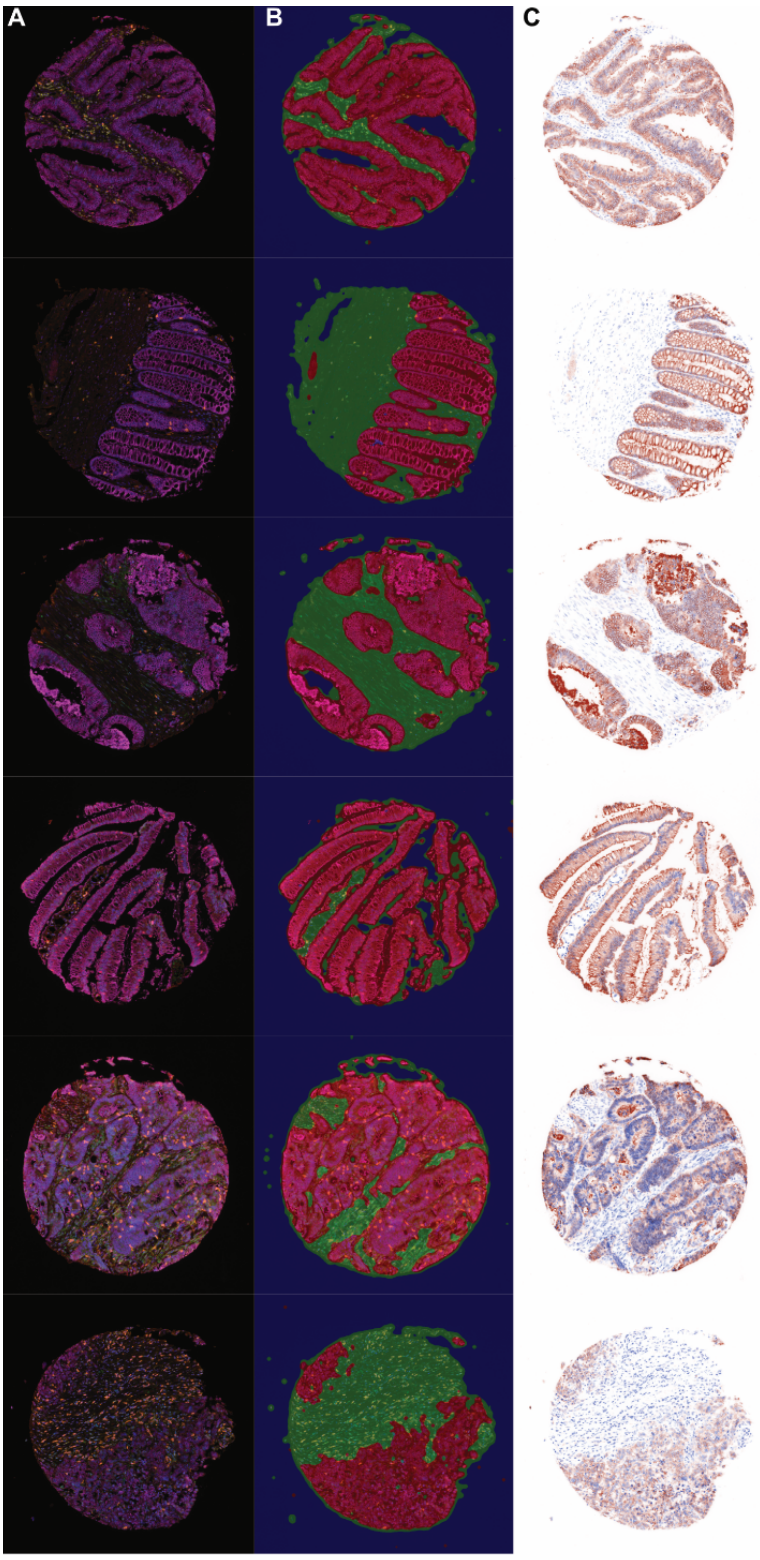


Supplementary Figure 8: Multiplex IHC immunoprobing with anti- IL10 antibody and with anti-IL-10 and IL10 protein block.

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 9: Phenotype of Cells showing individual marker staining
Representative TMA core showing multiplex IHC with all markers (antibody-opal combinations). Individual images of the same core showing CD4+ cells (yellow), $CD8^+$ cells (orange), $IL10^+$ (green) $Foxp3^+$ (Red) and EpCAM (magenta)



Supplementary Figure 10: 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 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 Ratio		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）	