

Supplementary Materials for
**Macrophages restrict tumor immune infiltration by controlling
collagen topography**

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Supplementary Text
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Other Supplementary Material for this manuscript includes the following:

Data files S1 to S13
MDAR Reproducibility Checklist

Supplementary Material and Methods

In vitro topotactic migration assay

T cell extraction and activation

Splenocytes of tdTomato/GFP male and female mice were harvested and T cells were isolated using dynabeads untouched Pan T cell isolation kit (Dynabeads, 10425073) following manufacturer's instructions and activated with CD3/CD28 beads (Gibco, 10022593, 2 beads for 1 T cell) and 25U/mL of IL-2 (Novartis, Proleukin) in T cell media (RPMI Glutamax, 10% FBS, 1% Penicillin/Streptomycin, 1% HEPES (Gibco, 15630-056), and 1% non-essential amino acids (Gibco, 11140-035). After 3 to 5 days, beads were washed off the cell using a magnet and rinsed twice with T cell media.

Fibrillar network design and preparation

Deformable fiber arrays were fabricated as previously described (40), using TPP with a QSwitch Teem Photonics laser (10 kHz, 5 ns pulses, 532 nm, 10 μ J) and a IX70 Olympus microscope equipped with water (60 $\times$ NA 1.2) and oil (100 $\times$ NA 1.4) objectives, a piezo-z stage, and a Guppy CCD camera.

The system was controlled by Lithos software with autofocus functionality. Fibrous scaffolds were designed using Sympoly V4. Each scaffold consisted of three 21-fiber layers (160 μ m length, 8 μ m z-spacing). Slicing parameters included a lateral voxel size of 0.17 μ m and a vertical voxel size of 0.55 μ m for fibers (overlap: 80%). Two resins were used: Resin 1 (PEGDA575 (Sigma, 26750-48-9)) with 15% PETA (PentaErythritol TetraAcrylate, Sigma, 986-89-4) then 4,54% (w/w) Irgacure 651 (Sigma, 24650-42-8) for anti-adhesive supporting walls, and Resin 2 (PEGDA250 (Sigma, 25852-47-5) with 10% PETA then 4,54% (w/w) Irgacure 651) for fibers. Supporting walls were printed with a 60x objective (laser power: 6.4 mW, exposure: 1000 μ s) and Resin 2 was added after rinsing to print fibers using a 100 $\times$ objective (laser power: 4.9 mW, exposure: 800 μ s). Scaffolds were washed in ethanol and coated with 10 μ g/mL fibronectin (Thermofisher, 33010018) labeled with CF[™]640R (Sigma, SCJ4600044) to mimic the coating of collagen fibers observed *in vivo* (Fig. S2J). Scaffolds were washed in RPMI medium before seeding 100,000 activated T cells (0.5 mL) at a density of 15–30 cells per scaffold. Cells were allowed to settle for 20 minutes at 37°C and 5% CO₂.

Single cell RNA sequencing

MC38 tumors from CD64^{DTR} and CD64^{WT} were harvested at day 14 d.p.i., digested in Liberase TL 0,25mg/ml (Roche, #5401020001) DNase 0,5mg/ml (Sigma, DN25-100MG) in CO₂ independent medium (Gibco, 18045088) using GentleMacs Octo dissociator pre-set program (37C_m_TDK1, 41min at 37° under agitation). Cells were filtered on a 100 μ m cell strainer and red blood cells were lysed using red

blood cells lysis buffer 1X (Biolegend, 420301) diluted in water. After centrifugation (5min, 1500rpm), cells were incubated 10 min with Fcblock solution diluted in FACS buffer (BD, 553142, 1:100) and stained either with (1) DAPI (Invitrogen, 11534886, 1:10000) for the first experiment (batch #1), or (2) with DAPI, anti-CD45 APC-Cy7 (BD, 557659, 1:200) and anti-CD64 APC (Biolegend, 139306, 1:200) (for the second experiment (batch #2) diluted in FACS buffer (PBS 1X, 0.5% BSA, 2mM EDTA). For batch#1, DAPI⁻ cells were sorted while for batch#2, DAPI⁻CD45⁻, DAPI⁻CD45⁺CD64⁺, DAPI⁻CD45⁺CD64⁻ populations were sorted separately. For batch #1, 15 000 cells from CD64^{WT} and CD64^{DTR} tumors were loaded. For Batch #2, 10000 cells from CD64^{WT} and CD64^{DTR} tumors were loaded. Sequencing was performed on a Novaseq 6000, with the Single Cell 3' V3.1 Next-GEM kits. Batch #1 and DAPI⁻CD45⁻ from batch #2 were sequenced at 50,000 reads / cell, DAPI⁻CD45⁺CD64⁺, DAPI⁻CD45⁺CD64⁻ from batch #2 were sequenced at 100,000 reads / cell. ScRNAseq data were analyzed with R using the Seurat suite (v4). Cells were then filtered out when expressing less than 1000 genes, or when expressing more than 10% of mitochondrial genes. Doublets were identified using the DoubletFinder package (78). Clusters containing less than 100 cells were excluded from the analysis. Differential analysis was done using the DESeq2 package (79) and gene set enrichment analysis was performed using the FGSEA package (80). For sub-clustering of non-immune, Mo/Mac/DC and Lymphocyte clusters, batch #1 and batch #2 CD45⁻, CD45⁺CD64⁺ and CD45⁺CD64⁻, or CD45⁺CD64⁻ sorted samples were selected respectively. Cell-Cell interaction analysis was performed using LIANA package (81). Data management, quality control and primary analysis were performed by the Bioinformatics platform of the Institut Curie.

Orion multiplex imaging

Orion Antibody Preparation and Conjugation

All antibodies were monoclonal, rabbit-derived, and carrier-free. The MHCII antibody was directly conjugated to biotin, while other antibodies were conjugated to ArgoFluor dyes using amine conjugation chemistry. The following antibodies (Epitope (Clone, Catalog#, Argo reporter, dilution)) were used: CD3 (SP162, Abcam #ab245731, Argo515, 1:20), Ki67(D3B5, Rarecyte #62-1002-501B, Argo555L, 1:100), PD1 (EPR20665, Abcam #ab228857, Argo535, 1:13), CD4 (EPR19514, Abcam #ab271945, Argo580L, 1:50), Lyve1 (EPR21771, Abcam #ab230377, Argo660L, 1:50), TCF1 (CD63D9, Cell Signaling #85942SF, Argo 572, 1:13), Epcam (65, SinoBiological #50591-R065, Argo 602, 1:50), CD11c (D1V9Y, Rarecyte #63-1006-601B, Argo 624, 1:100), CCR7 (EPR23192-57, Abcam #ab272938, Argo662, 1:50), Ly6G (1A8, Abcam #ab210204, Argo686, 1:20), F4/80 (EPR26545-166, Abcam #ab300422), Argo730, 1:13), CD8a (D4W2Z, Ozyme #60168SF, Argo760, 1:13), FoxP3 (1054C, R&D system #MAB8214, Argo784, 1:50), CD31 (D8V9E, Ozyme #92841SF, Argo810, 1:13), CD45 (D3F8Q, Ozyme #98819SF, Argo845, 1:20), MHCII-biot (M5/114.15.2, Biolegend#107602, Argo890, 1:50). Antibodies were first concentrated by filtering on Amicon filters (Sigma-Aldrich, UFC503096). Following centrifugation at 14,000 × g for 10 minutes, 475 µL of Reaction Buffer (Sigma-Aldrich, 36486) was added. The solution was transferred to a clean Amicon collection tube and centrifuged at 1,000 × g for 2 minutes, yielding 25 µL of concentrated antibody (2-5 mg/mL). To conjugate the antibodies, 5 µL of ArgoFluor dye dissolved in DMSO was added to the concentrated antibody solution and vortexed for 10 seconds and incubated in the dark at room temperature for 1 hour. The reaction was quenched by adding 1.5 µL of 1 M Tris-HCl (Teknova, T1083), followed by vortexing on high for 5 seconds and further incubation in the dark for 20 minutes at room temperature. The conjugate was purified using a clean Zeba column (ThermoFisher, 7766) by centrifugation at 5,000 × g for 5 minutes. The final volume was measured, and labeling efficiency was determined via absorbance spectroscopy. The conjugated antibodies were diluted in PBS Antibody Stabilizer (Candor Bioscience, 131125) to a concentration of 0.1 mg/mL.

Orion Slide Preparation

Slides were immersed in a bleaching buffer (30% H₂O₂, 1 M NaOH in PBS) and exposed to LED light for 1 hour to quench autofluorescence. Subsequently, slides were transferred to a UV transilluminator chamber (365 nm, high setting) for 30 minutes. Excess bleaching buffer was removed, and slides were washed twice with PBS (5 minutes each).

Slides were washed twice with PBS-T (25% Triton) and twice with PBS (5 minutes each). In a humidity chamber, slides were incubated with Image-iT FX Signal Enhancer (Invitrogen, I36933) for 15 minutes,

followed by two washes with PBS-T (25% Triton) and one wash with PBS (5 minutes each). A cocktail solution of all conjugated antibodies was prepared in PBS Antibody Stabilizer (Candor Bioscience, 131125) with 10% rabbit serum. Slides were incubated with the antibody cocktail in a humidity chamber for 1 hour and 30 minutes in the dark at room temperature. Slides were then washed twice with PBS-T (25% Triton) and once with PBS (5 minutes each). A secondary staining solution was prepared with PBS Antibody Stabilizer (Candor Bioscience, 131125), 10% goat serum, 0.1% Hoechst 33342 (Thermo Fisher Scientific, H3570), and 1% Streptavidin ArgoFluor 890 (Rarecyte, 52-1026-802). Slides were incubated with the secondary solution in a humidity chamber for 30 minutes in the dark at room temperature. Slides were then washed once with PBS-T (25% Triton) and once with PBS (5 minutes each). Slides were coverslipped with ArgoFluor Mounting Medium (Rarecyte, 42-1214-000) and left to dry overnight.

Image acquisition

Images were acquired at 20× magnification using the Orion imaging system (Rarecyte) equipped with a Nikon CFI Plan Apo 20x Lambda D Objective (NA 0.80), a solid-state laser with 402, 455, 488, 527, 553, 639, 690, 750, 808 laser lines and a Teledyne Prime BSI Express Camera. The list of filters is provided in [Supplementary table 1](#). Images were processed with Artemis software (version 4.1.0.28).

Legendplex

14 d.p.i half tumors were collected from macrophage-depleted mice (see above) were incubated overnight at 37°C in MC38 culture media (see above). Culture supernatants were collected and subjected to LEGENDplex™ Mouse/Rat Free Active/Total TGF-β1 assay (BioLegend, 740489) and LEGENDplex™ MU Proinflamm. Chemokine Panel 1 assay (BioLegend, 741295) following manufacturer's instructions. Fluorescence intensities were acquired using Cytotflex analyzer.

Histology

Once the samples have been placed in cassettes, they are dehydrated for 4 hours at 45°C in alcohol baths, then clarified in xylene for 4 hours before being transferred to paraffin at 60°C. All these steps are carried out on the HistoCore Leica automated system.

Formalin-fixed paraffin-embedded tissues are placed on GordiamKey cold plate and sectioned at 3 μm using a Microm Microtech HM340E microtome, then transferred to a VWR W20 histology water bath before drying. Hematoxylin-Eosin-Safran stainings on lung, liver and tumor draining lymph nodes were performed using the automated stainers Leica histoCore SPECTRA ST. The slides are dewaxed with xylene and alcohol then, rehydrated with distilled water. After that, the coloration is made with the application of hematoxylin (Dako, CS70030-2) for 4 minutes, eosin (CELLAVISION, ZR312740-1000) for 6 min and Safran (CELLAVISION, ZR369200-1000) for 2 minutes. The slides are passing into alcohol and xylene before mounting with the mounting medium HistoCore SPECTRA CV X1 (Leica 3801733). For MC38 tumors, 5-μm cryosections were collected and stained in PicroSirius Red solution for 60 minutes. For B16F10 tumors, Picrosirius Red (Clinisciences, SRS250) was applied according to manufacturer's instructions on formalin-fixed paraffin-embedded tissue sections of 3 μm. Immunohistochemistry was carried out on Permaslides Plus HistoCore slides (Leica, 3800455CL) using the Leica Bond-Max automated stainer. CD3 labeling (Dako RTU IR503) was achieved following epitope retrieval at pH 9 for 20 minutes, with the Bond Polymer Refine Red Detection (Leica, DS9390) development of mixed red for 60 minutes, and application of Protein Block (Agilent, X090930-2). F4/80 staining (Cell Signaling Technology, 70076, 1:400) was performed using the Polymer Refine Detection (Leica DS9800) with identical epitope retrieval, protein block and DAB development conditions.

Slides were digitized using the NanoZoomer S360 (Hamamatsu) slide scanner, with acquisition at 20× (0.46 μm/pixel) or 40× (0.23 μm/pixel) resolution NA: (0.75). Image acquisition was performed using NZAcquire and visualization/analysis with NDPview2.

3D reconstruction of tumors by Kratoscope autofluorescence imaging

Tumors were harvested, fixed in 4% paraformaldehyde overnight at 4°C and subsequently dehydrated in successive increasing concentration of ethanol baths. Samples were then embedded in 5% Oil Red-O-stained paraffin (Diapath, C0512) as described in Mallocci *et al*⁸³. Serial block face imaging of the samples was performed using the Kratoscope system (Kaer Labs, France) to detect native tissue autofluorescence. Briefly, the tumors were mounted on an automated microtome and sectioned into 10

µm thick sections. The tissue block was imaged using a 405nm excitation LED. The successive imaging-sectioning cycles generated a stack of images reflecting the autofluorescence throughout the whole sample and was used to perform 3D reconstruction of the total tumor volume. Individual 2D sections were collected during the process for downstream histological staining.

Image analysis

Cell counting was performed using QuPath cell detection tool. Regional analyses were performed by dividing whole tumor images into 100 µm x 100 µm tiles (for a total of 1867864 ROIs covering 14 CD64^{WT} and 19 CD64^{DTR} tumors) and extracting cell number or staining densities or intensities using QuPath software (v0.4.4). Subsequent analysis was performed using R software (v4.3.1). For each tile, cell or staining enrichment was determined as the cell or staining density (threshold determined using QuPath) was superior to the mean cell or staining density of positive regions (cell number or staining density > 0). Percentages of positivity were quantified using QuPath software.

Solidity measurements were performed using Fiji software. Briefly, tumor masks were drawn by hand (delineating the whole tissue and removing the skin), and the shape descriptor plug-in was applied.

GLCM Analysis

The gray level co-occurrence matrix⁸⁴(GLCM) algorithm was applied to characterize the texture of collagen. For each sample, a minimum of 5 regions (318 µm × 318 µm) from the core and border were manually selected. For each region, the GLCM was computed, and textural homogeneity was extracted. The gray level co-occurrence matrices and homogeneity properties were calculated using functions from the Scikit-Image Python library⁸⁵: `skimage.feature.graycomatrix` and `skimage.feature.graycoprops`, respectively. The GLCM, with 256 intensity levels, was computed with the following parameters: distances from 1 to 100 pixels with a step of 1 pixel (1 pixel = 0.45µm), and angles of 0°, 45°, 90°, and 135°. The textural homogeneity was then calculated as the mean of the homogeneity values along all the angles and all the distances of the GLCM.

Topographical feature extraction from SHG images

SHG images were analyzed using the OrientationJ plug-in (developed by EPFL, Switzerland) within the ImageJ software. This function computes the structure tensor for every Gaussian-shaped window (with a size of 20-60-100-140 pixels) by determining the continuous spatial derivatives along the x and y axes through Gaussian interpolation. The structure tensor is a symmetric matrix computed in regions spaced 40 pixels one from the other. The tensor can be characterized by three quantities (derived from the two eigenvectors and eigenvalues): the energy (trace of the tensor, related to the size of the ellipsis associated to the tensor), the coherency (difference of the eigenvalues normalized by the trace, related to the aspect ratio of the ellipsis) and the orientation (angle formed by the major axis of the ellipsis with the x-axis of the image). Intensity mean and variance, density and density variance, and distance of the region from the border were extracted using ImageJ software. The number of cells (either Ly6G⁺ or CD3ε⁺) in 100 µm x 100 µm windows were extracted from Qupath software⁸⁶.

Principal Component Analysis was performed using *prcomp* function in R (v4.3.1) software.

Random-forest classification

Random Forest predictions were performed using a custom-made Python code. 70% of the initial data was used for training the model, and the remaining 30% for the test set. In addition, for the prediction of T cell rich regions in CD64^{WT} and CD64^{DTR} tumors at day 14 A stratified 10-fold cross-validation was performed on the training set, during which a RandomSearch (N=50) was conducted to find the best combination of parameters based on the performance obtained on the validation set.

For evaluating random forest performances, we use various metrics based on the true positives (TP), true negatives (TN), false positive (FP) and false negative (FN) which are derived from the predicted versus observed target class.

Accuracy is the proportion of correctly classified instances out of the total instances, formulated as: $\text{Accuracy} = (\text{TP} + \text{TN}) / (\text{TP} + \text{TN} + \text{FP} + \text{FN})$.

Balanced accuracy corresponds to the macro-average of the recall score per class, here it is formulated as: $\text{Balanced Accuracy} = (1/2) \times (\text{TP} / (\text{TP} + \text{FN}) + \text{TN} / (\text{TN} + \text{FP}))$,

The Precision corresponds to the proportion of true positive out of all positive predicted instances denoted by: $\text{Precision} = \text{TP} / (\text{TP} + \text{FP})$,

The Recall measures the proportion of true positive out of all observed positive instances where: $\text{Recall} = \text{TP} / (\text{TP} + \text{FN})$,

The Area Under the Receiver Operating Characteristic Curve score (ROC AUC) denoted as AUC is a measure of the model's ability to discriminate between positive and negative classes across different probability thresholds.

The Matthews correlation coefficient (MCC) takes into account the TP, TN, FP and FN rate and is especially useful for imbalanced datasets. It is formulated as:

$$\text{MCC} = (\text{TN} \times \text{TP} - \text{FN} \times \text{FP}) / \text{square-root} ((\text{TP} + \text{FP}) \times (\text{TP} + \text{FN}) \times (\text{TN} + \text{FP}) \times (\text{TN} + \text{FN})).$$

The F1 score corresponds to the harmonic mean of precision and recall, providing a balance between the two metrics, with: $\text{F1} = (2 \times \text{Precision} \times \text{Recall}) / (\text{Precision} + \text{Recall})$.

FACS analysis of the blood

80 μL of blood was collected submandibularly in 10 μL of 100mM EDTA (ThermoFisher Scientific, 15575020) diluted in PBS and centrifuged 10 min at 2000xg at 4°C. The pellet was resuspended in 7 mL of RBC lysis buffer 1X diluted in water and incubated on ice for 5 min. After adding at least 7 mL of FACS buffer, centrifugation 7 min at 1500rpm, cells were resuspended in FACS buffer and plated in a 96 well plate. After centrifugation (5min, 1500rpm), cells were incubated 10 min with Fcblock solution diluted in FACS buffer (BD, 553142, 1:100), washed with FACS buffer and incubated with following antibodies : anti-SiglecF PE/Dazzle 594 (Biolegend, 155530,1:400), anti-CD115 APC-Cy7 (Biolegend, 135531, 1:200), anti-GR1 BV510 (BD, 563040, 1:200), anti-B220 PE (BD, 553090, 1:200), anti-CD4 FITC (BD, 553047, 1:300), anti-CD8 APC (BD, 553035, 1:300), anti-NK1.1 PerCP-Cy 5.5 (BD, 551114 , 1:200). After washing with FACS buffer, 10 μL of counting beads (Invitrogen, 01-1111-42) were added. Flow cytometry was performed on an Aurora Analyzer (Cytex) and analyzed with FlowJo software (version 10.8.1).

Atomic force microscopy

30 μm thick cryosections were prepared at -20°C from tumors embedded in OCT compound and immediately transferred to a poly-L-lysine coated glass-bottom dish. PBS containing Complete (TM) EDTA-free Protease Inhibitor (Sigma) and 10% horse serum was added on top of the sections as soon as they melted. CD3e staining was then performed in PBS with protease inhibitors, 10% horse serum and 0.2% triton X-100 for one hour at room temperature. The atomic force microscopy measurements were done on the same day.

Atomic force microscopy analysis was performed on A JPK NanoWizard II system (Bruker Nano GmbH) with a CellHesion module mounted on a Zeiss LSM510 confocal microscope (Carl Zeiss NTS Ltd.). We used silicon nitride cantilevers (spring constant: 0.06 N/m, spherical 4.5 μm diameter tip; Novascan Technologies). The cantilever spring constant and deflection sensitivity were calibrated in fluid via the thermal noise method (Hutter and Bechhoefer, 1993). We used a 10x objective connected to a webcam attached to the microscope to visualize the samples and to determine the regions of interest. In each location, indentations distributed in a 5×5 points grid (25 μm ×25 μm) were performed. The elastic modulus for each force curve was calculated using JPK software (JPK DP version 4.2) assuming a Hertz model of impact.

Binding motifs enrichment analysis

The 5000 bp upstream of the differentially expressed genes ($\text{Log}_2(\text{FC}) > 0.25$, adjusted p-value < 0,05) between CD64^{DTR} and CD64^{WT} tumors in fibroblasts and total cancer cells (C1 + C2) or between C2 and C1 independently from macrophage depletion were extracted using the Ensemble browser. Binding motif enrichment analysis was performed on extracted promoter regions using the AME tool from the MEME suite.

Supplementary figures

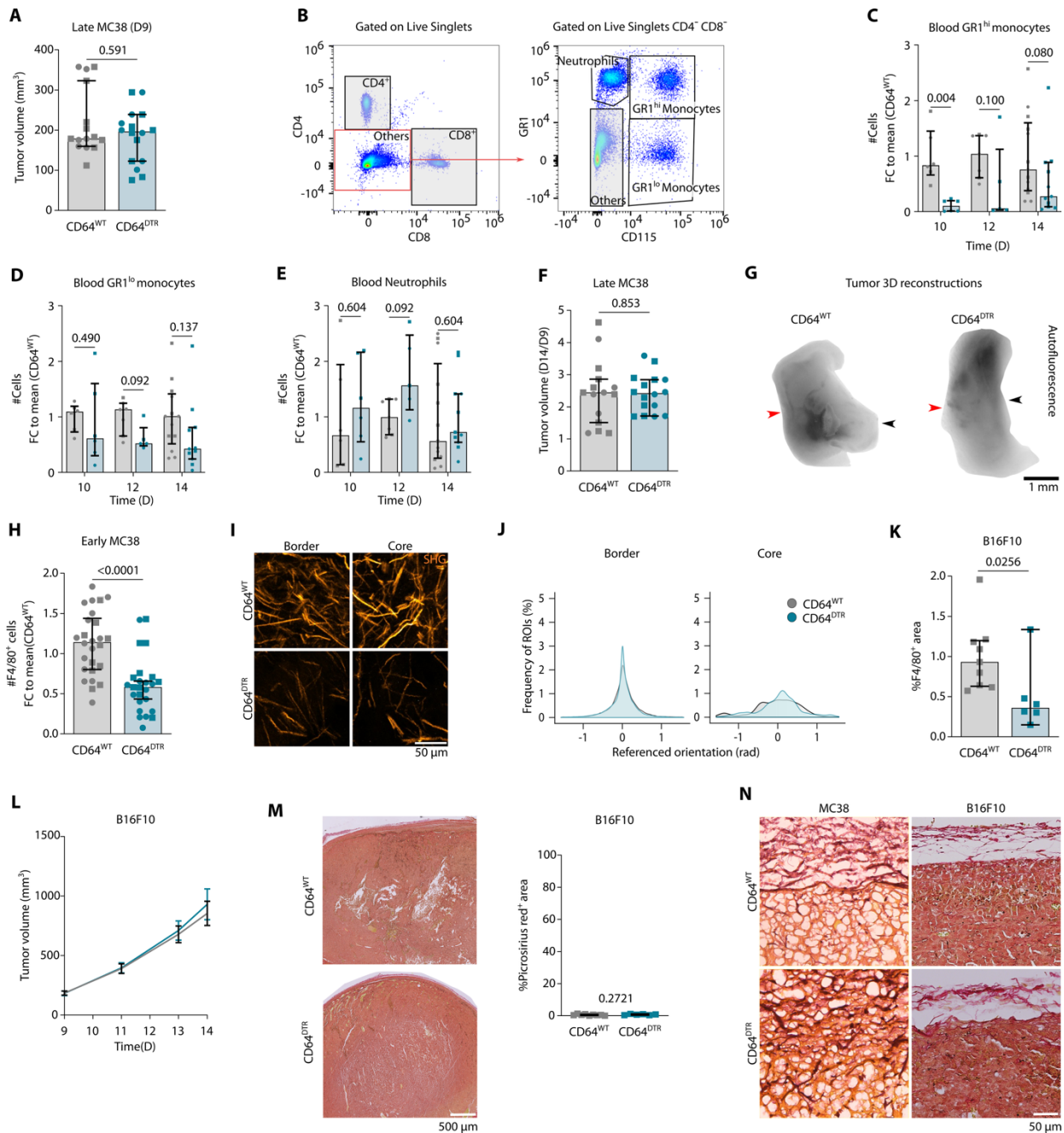


Figure S1: Macrophage depletion disrupts fibrillar collagen topography in tumors.

(A) Tumor volume of CD64^{WT} and CD64^{DTR} tumors at D9. (B) Gating strategy of flow cytometry analysis of the blood. (C) Number of CD115^{high}GR1^{high} monocytes, (D) CD115^{high}GR1^{low} monocytes, (E) and CD115-GR1^{high} neutrophils in the blood of CD64^{DTR} and CD64^{WT} mice at D10, 12 and 14. (F) Volume of CD64^{WT} and CD64^{DTR} tumors at D14 normalized to their volume at D9. (G) Autofluorescence images of 3D reconstructions of CD64^{WT} and CD64^{DTR} tumors at D14 (black arrow = tumor border; red arrow = skin). (H) Fold change of the number of F4/80⁺ cells per mm² in CD64^{DTR} over CD64^{WT} tumors at D8 (Early depletion). (I) SHG signal of CD64^{WT} and CD64^{DTR} tumors depleted from D3 to D7 observed at D8 (Early depletion). (J) Distribution of 79.5 μ m x 79.5 μ m ROIs referenced orientation at the border (0-1000 μ m depth) and in the core (>1000 μ m depth) of the tumor (n = 13 CD64^{WT}, 16 CD64^{DTR}; 66092 and 107635 ROIS for CD64^{WT} and CD64^{DTR} tumors, respectively). (K) Fold change of % F4/80⁺ area in CD64^{DTR} over CD64^{WT} B16F10 tumors at D14 (Depletion D9-13). (L) Tumor growth curves from D9 to D14 of CD64^{WT} and CD64^{DTR} B16F10 tumors (n=18 CD64^{WT}; n=15 CD64^{DTR}). (M) Picrosirius red (PR) staining and %PR⁺ (fibrillar collagen) area in CD64^{WT} and CD64^{DTR} B16F10 tumors at D14. (N) PR staining at the edge of CD64^{WT} and CD64^{DTR} MC38 and B16F10 tumors at D14 (Late depletion, D9-D13). In all graphs, each dot represents one tumor. Squares indicate males; circles indicate females. Statistics: (A, F, H, K, M) median $\pm$ IQR, Mann-Whitney test. (C-E) median $\pm$ IQR, two-way ANOVA.

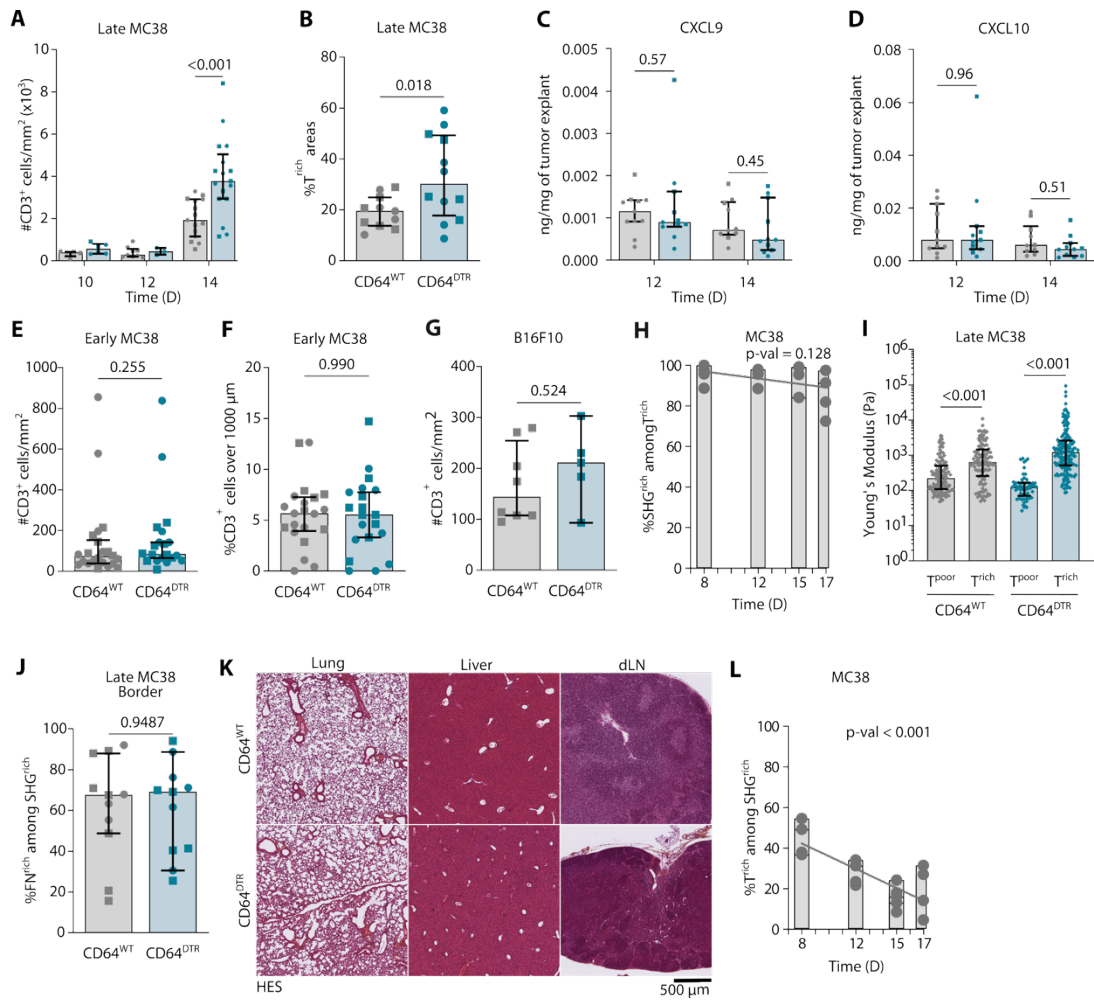


Figure S2: Collagen remodeling is associated with increased T cell infiltration in specific network topographies (Part 1).

(A) Number of CD3⁺ cells/mm² in CD64^{WT} and CD64^{DTR} tumors at D10,12,14. (B) %T^{rich} areas in CD64^{WT} and CD64^{DTR} tumors at D14. (C) ELISA quantification of CXCL9 and (D) CXCL10 in tumors supernatants from in CD64^{WT} and CD64^{DTR} tumors at D12 and D14. (E) Number of CD3⁺ cells/mm² and (F) %T cells over 1000 μ m from tumor border in CD64^{WT} and CD64^{DTR} tumors at D8 after early depletion. (G) Number of CD3⁺ cells/mm² in B16F10 CD64^{WT} and CD64^{DTR} tumors at D14. (H) Linear regression between %T^{rich} regions showing enrichment in SHG signal and tumor resection day in MC38 tumors. (I) Young's Modulus of T^{rich} regions and T^{poor} regions in CD64^{WT} and CD64^{DTR} tumors at D14 measured by atomic force microscopy (one dot per indentation, n= 5 CD64^{WT}, n= 4 CD64^{DTR} tumors). (J) %SHG⁺ areas positive for fibronectin (FN) at the edge of CD64^{WT} and CD64^{DTR} tumors at D14. (K) Representative images of Hematoxylin-Eosin-Safran staining of lungs, liver, and draining inguinal lymph node of CD64^{WT} and CD64^{DTR} mice bearing MC38 tumors at D22 (n=8 CD64^{DTR}, n=6 CD64^{WT}). (L) Linear regression between %SHG^{rich} regions showing enrichment in T cells and tumor resection day in MC38 tumors. In all graphs, one dot per tumor unless otherwise indicated. Squares indicate males; circles indicate females. Statistics: median $\pm$ IQR, Mann-Whitney test for all graph unless otherwise indicated.

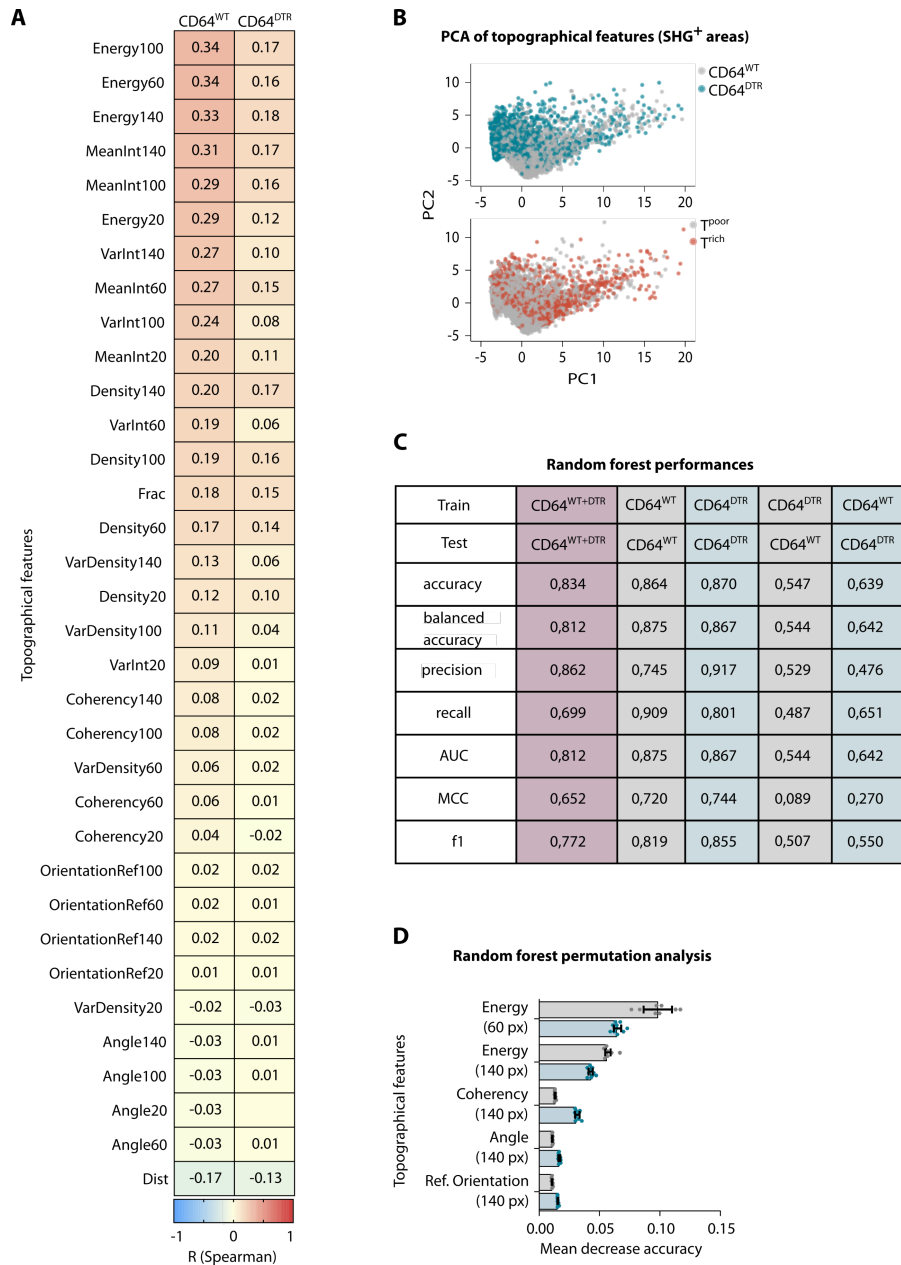


Figure S3: Collagen remodeling is associated with increased T cell infiltration in specific network topographies (Part 2).

(A) Spearman correlation coefficients calculated between fibrillar collagen topography features and the mean number of T cells in 100 μm x 100 μm tiles in CD64^{WT} and CD64^{DTR} tumors at D14. **(B)** Principal Component Analysis of local fibrillar collagen topography features in CD64^{WT} and CD64^{DTR} tumors at D14 highlighting: mice genotypes (left) and T^{rich} or T^{poor} classifications (right) (only 0.1% of ROIs are represented here). **(C)** Tables of random forest model performances. **(D)** Permutation analysis showing the importance of energy (60 px and 140 px), coherency (140 px), angle over tumor edge (140 px), and referenced orientation to a 140 px ROI in predicting T cell enrichment in CD64^{WT} and CD64^{DTR} datasets (one dot represent one model; median $\pm$ IQR, Mann–Whitney test). For all graphs: n = 14 CD64^{WT}, 18 CD64^{DTR}; 770766 and 1097098 ROIS for CD64^{WT} and CD64^{DTR} tumors respectively.

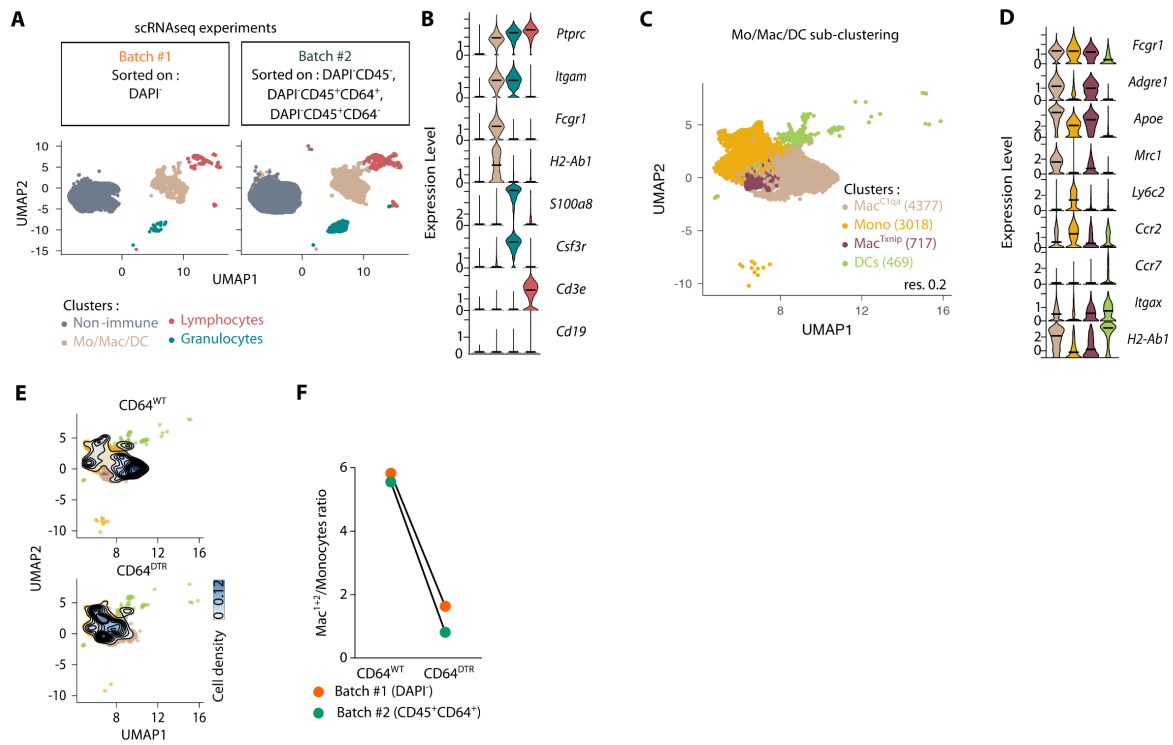


Figure S4: Macrophage depletion induces a pro-fibrotic program in cancer and stromal cells (Part 1).

(A) UMAP of merged scRNAseq data from CD64^{WT} and CD64^{DTR} tumors from two independent experiments. **(B)** Expression levels of classical markers of non-immune cells, monocytes/macrophages/DCs, lymphocytes and granulocytes (line at median). **(C)** UMAP showing sub-clustering of Mo/Mac/DC cluster from CD64^{WT} and CD64^{DTR} tumors. **(D)** Expression levels of classical markers of monocytes/macrophages/DCs (line at median). **(E)** UMAP of Mo/Mac/DC clusters showing cell densities in CD64^{WT} and CD64^{DTR} tumor datasets. **(F)** Ratio between number of macrophages (Mac(*C1qa*) + Mac(*Txnip*)) and monocytes in batch #1 and batch #2 DAPI⁻CD45⁺CD64⁻ samples from CD64^{WT} and CD64^{DTR} tumors (one dot represents one tumor).

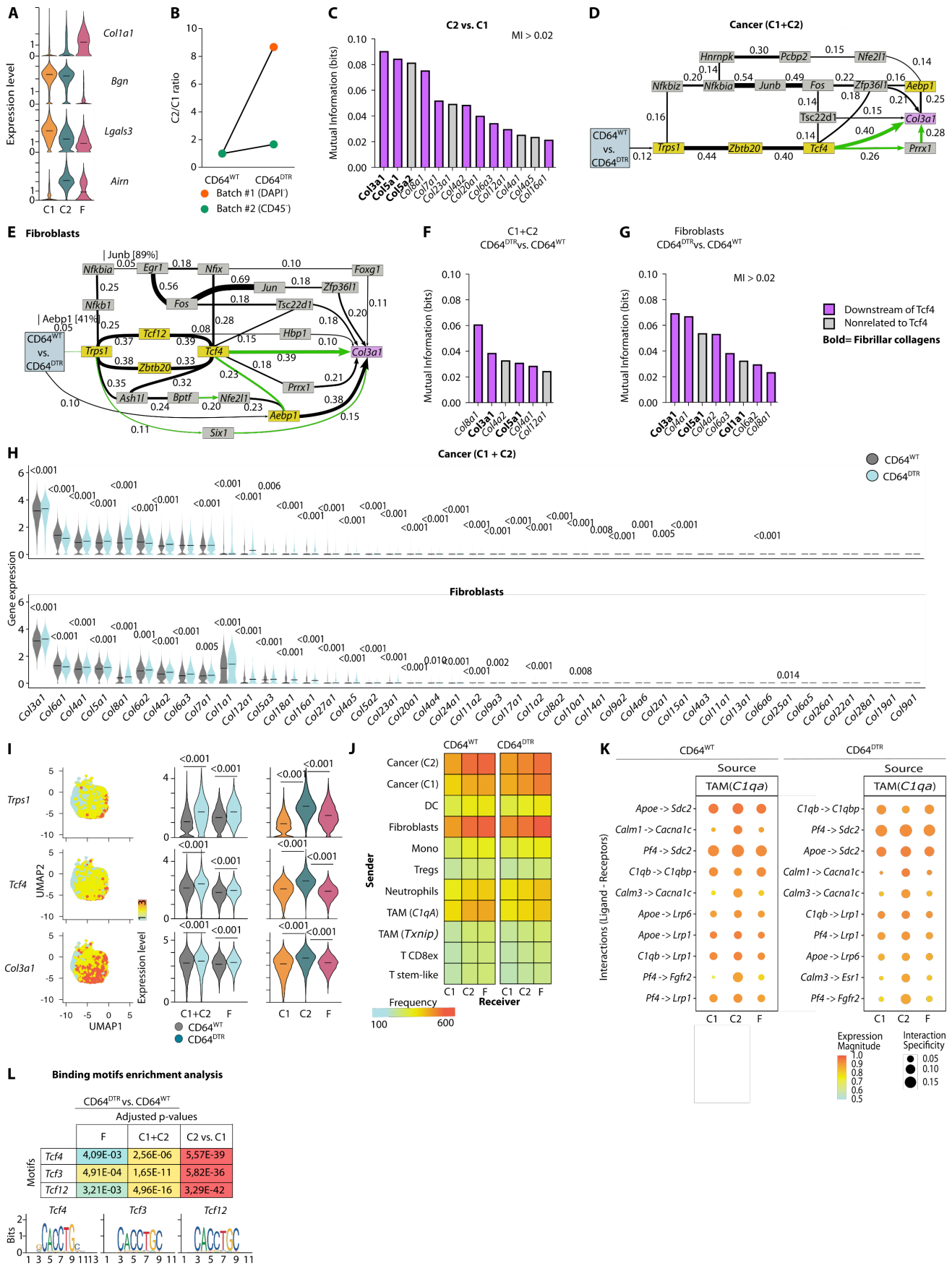


Figure S5: Macrophage depletion induces a pro-fibrotic program in cancer and stromal cells (Part 2).

(A) Expression levels of classical markers used to identify cancer (*Bgn*, *Lgals3*, *Airn*) and fibroblast (*Col1a1*) clusters. **(B)** Ratio of C2 over C1 in batch #1 and batch #2 DAPI⁺CD45⁻ samples from CD64^{WT} and CD64^{DTR} tumors. **(C)** Mutual information of collagens expression with the C2 cancer cluster signature (Violet: found downstream of *Tcf4* by MIIC analysis, bold: fibrillar collagens). **(D)** Transcriptional network induced by macrophage depletion in cancer cells (C1+C2) and **(E)** in fibroblasts and identified by MIIC analysis (Top 250 TFs + 44 collagen genes). **(F)** Mutual information of collagens expression with macrophage depletion in cancer cells (C1+C2) and in **(G)** Fibroblasts. **(H)** Collagen gene expression in cancer cells (C1+C2) and fibroblasts (Wilcoxon test). **(I)** Expression levels of *Trps1*, *Tcf4* and *Col3a1* in non-immune clusters in CD64^{WT} and CD64^{DTR} tumors (Line at median, Wilcoxon test). **(J)** LIANA analysis showing frequency of ligand-receptor interactions received by cancer cells (C1, C2) and fibroblasts (F) and **(K)** Ligand-receptor pairs between TAM (*C1qa*) and non-immune clusters (the color indicates the expression magnitude and size the interaction specificity) assessed by scRNA-seq. **(L)** *Tcf4*, *Tcf3* and *Tcf12* adjusted p-values from binding motif enrichment analysis of differentially expressed genes between CD64^{WT} and CD64^{DTR} tumors in fibroblasts (F) and cancer cells (C1+C2), and between C2 and C2; and Logo of *Tcf4*, *Tcf3* and *Tcf12* binding motifs from JASPAR database.

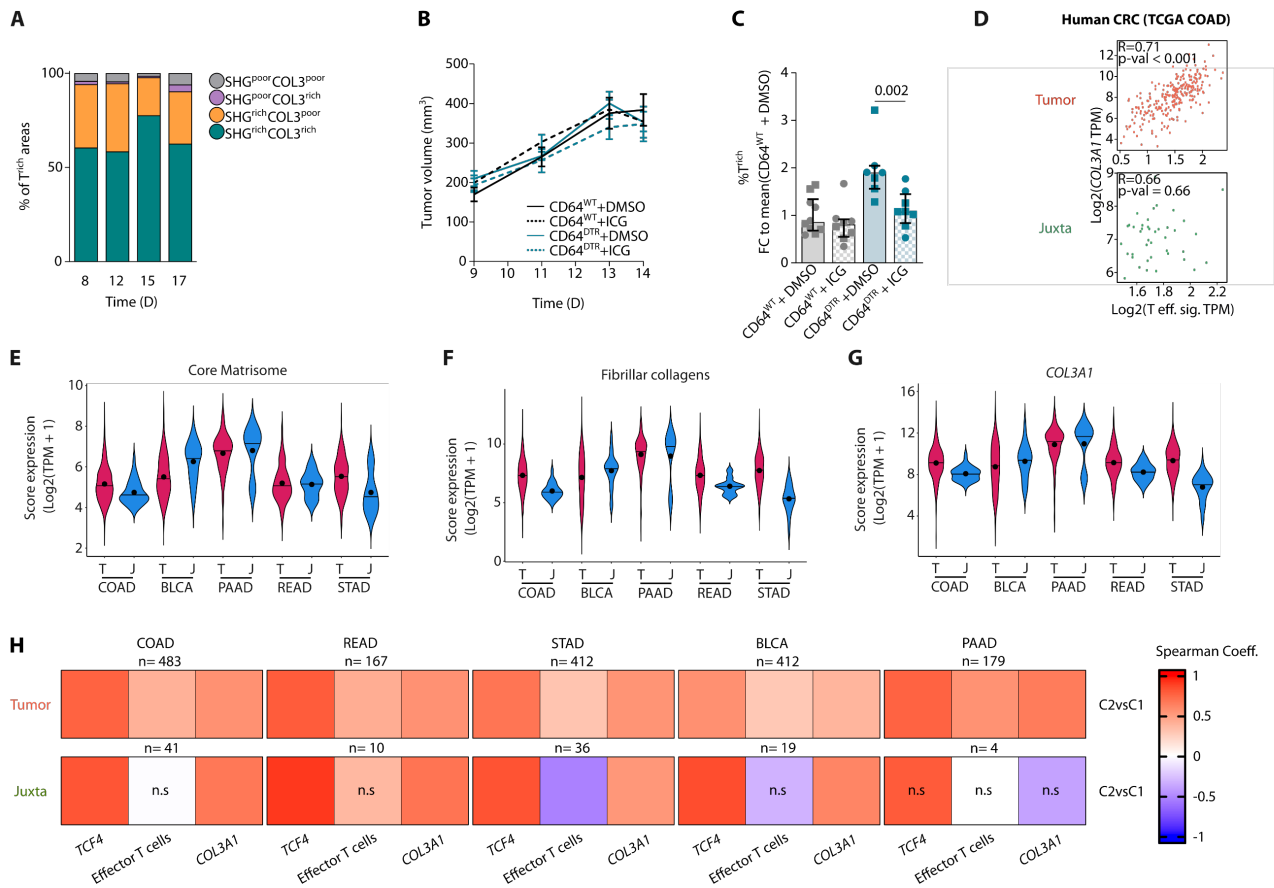


Figure S6: Collagen 3-rich networks driven by Tcf4 support tumor local T cell infiltration in tumors.

(A) Distribution of SHG^{rich} COL3^{rich}, SHG^{rich} COL3^{poor}, SHG^{poor} COL3^{rich}, SHG^{poor} COL3^{poor} among T^{rich} regions (100 μ m x 100 μ m tiles) in WT tumors at D8, 12, 15, 17 (n=14, median). (B) Tumor growth curves from D9 to D14 of CD64^{WT} and CD64^{DTR} tumors treated with TCF4 inhibitor ICG-001 or DMSO (n=14 CD64^{WT} + DMSO, n=14 CD64^{WT} + ICG-001, n=13 CD64^{DTR} + DMSO, n=12 CD64^{DTR} + ICG-001, mean $\pm$ SEM). (C) %T^{rich} areas in CD64^{WT} and CD64^{DTR} tumors at D14 treated with TCF4 inhibitor ICG-001 or DMSO (each dot represents one tumor; Squares indicate males; circles indicate females, median $\pm$ IQR, Mann-Whitney test). (D) Spearman correlation of COL3A1 expression and effector T cell signatures in TCGA COAD tumors and juxta-tumoral tissues bulk RNA sequencing dataset. (E) Expression levels of Core matrisome, (F) fibrillar collagen signatures, and (G) COL3A1 in TCGA COAD, READ, STAD, BLCA and PAAD tumors and juxta-tumor tissues bulk RNA sequencing dataset (n=, line at median; dots at mean). (H) Spearman correlation between expression of TCF4, COL3A1 and effector T cell signature and between C2 signature (C2 vs. C1) in TCGA COAD, READ, STAD, BLCA and PAAD tumors and juxta-tumor tissues bulk RNA sequencing dataset.

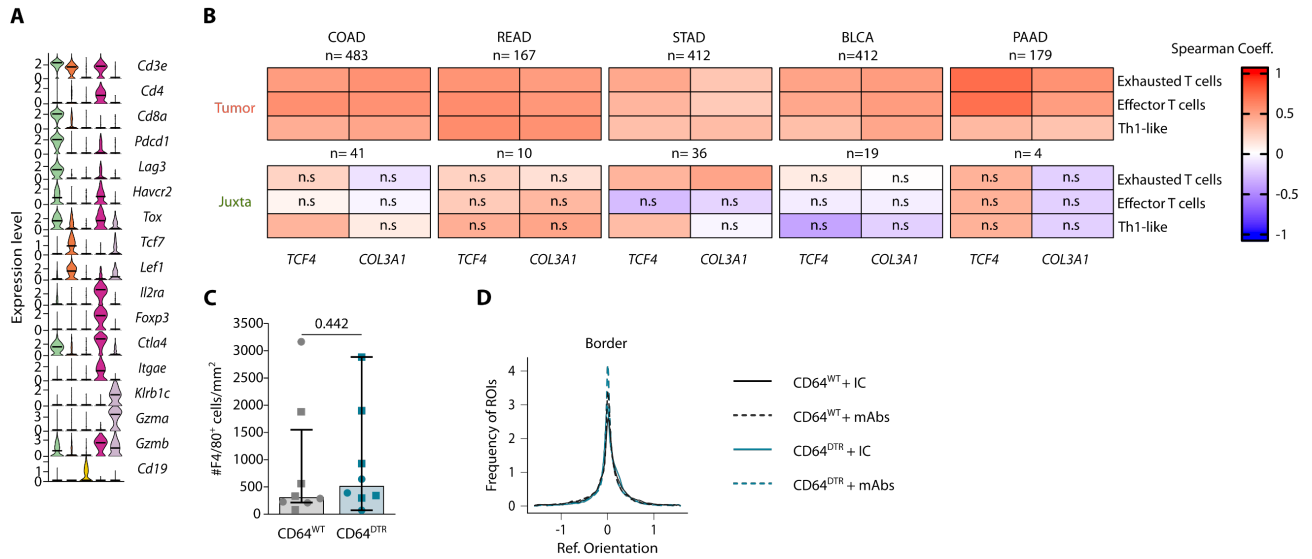


Figure S7: Fibrillar collagen topography directs T cell infiltration without affecting their transcriptional state.

(A) Expression levels of classical lymphocyte markers used to identify clusters. **(B)** Spearman correlation between *TCF4*, *COL3A1* expression and exhausted, effector and Th1-like T cell signatures in TCGA COAD, READ, STAD, BLCA and PAAD tumors and juxta-tumor tissues bulk RNA sequencing dataset. **(C)** #F4/80⁺ cells/mm² in CD64^{WT} and CD64^{DTR} tumors at D22 (Squares indicate males; circles indicate females; median ± IQR, Mann–Whitney test). **(D)** Distribution of 79,5 μm x 79,5 μm ROIs referenced orientation at the border of CD64^{WT} and CD64^{DTR} tumors at D22 treated with anti-PD-1 + anti-CTLA-4 antibodies or ICs (n=8 CD64^{WT} (120383 ROIs), 9 CD64^{DTR} (189500 ROIs)).

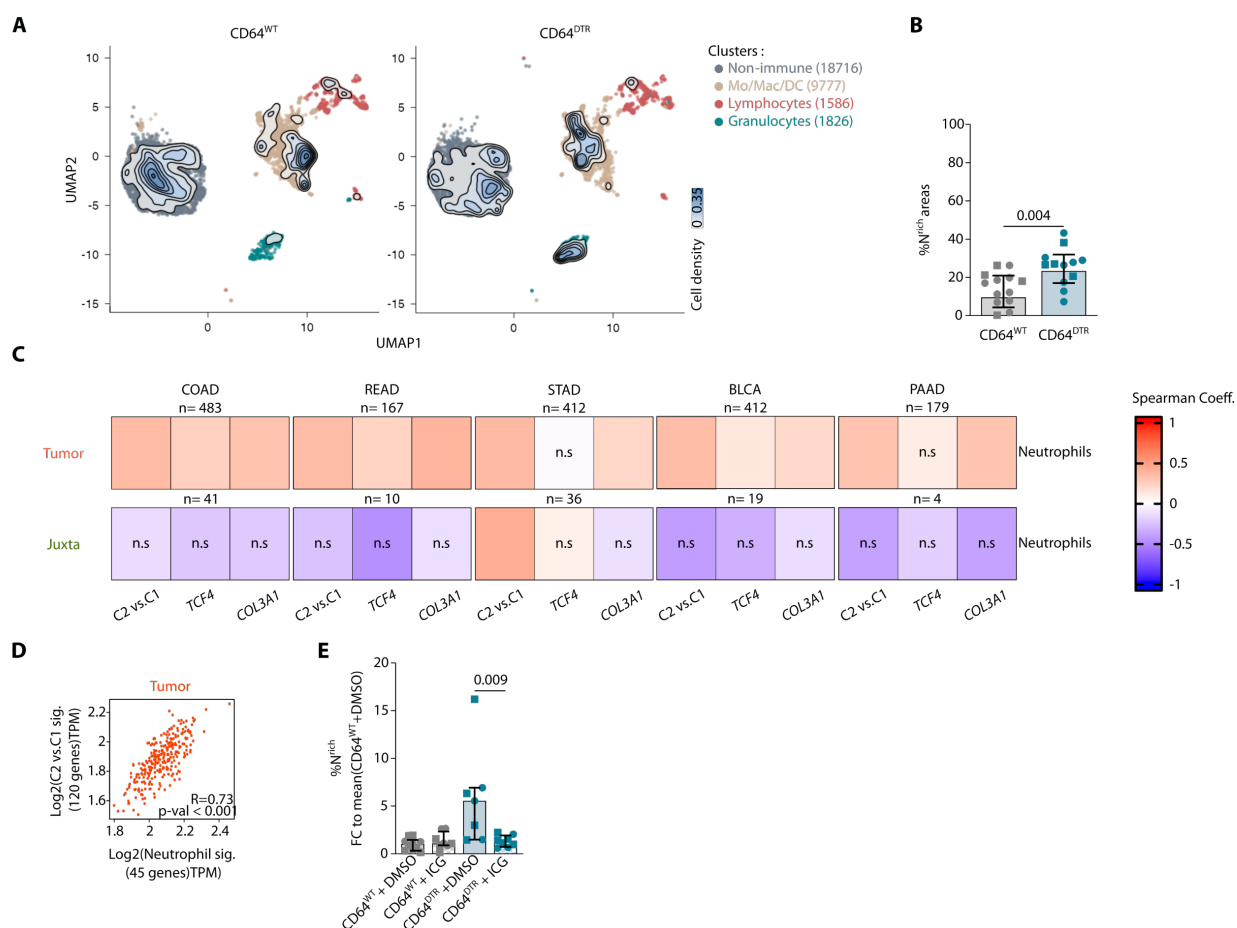


Figure S8: Fibrillar collagen topography dictates tumor permissiveness to immune infiltration.

(A) UMAP of the complete scRNAseq dataset showing cell densities in CD64^{WT} and CD64^{DTR} tumors datasets. (B) %Neutrophil^{rich} areas (100 μ m x 100 μ m tiles) in CD64^{WT} and CD64^{DTR} tumors at D14. (C) Spearman correlation between expression of C2 vs. C1 signature, *TCF4*, *COL3A1* and neutrophil signature in TCGA COAD, READ, STAD, BLCA and PAAD tumors and juxta-tumor tissues bulk RNA sequencing dataset. (D) Spearman correlation between expression of neutrophil signature and C2 signature (C2 vs. C1) expression in TCGA COAD tumors bulk RNA sequencing dataset. (E) %Neutrophil^{rich} areas (100 μ m x 100 μ m tiles) in CD64^{WT} and CD64^{DTR} tumors at D14 treated with TCF4 inhibitor ICG-001 or DMSO normalized to the mean of CD64^{WT} + DMSO condition. (B, E): each dot represents one tumor. Squares indicate males; circles indicate females. Statistics: median $\pm$ IQR, Mann-Whitney test.

Supplementary tables

Channel	Excitation (nm)	Fluor	Transmission band (nm)	Center wavelength (nm)	Wavelength bandwidth (nm)
1	402	–	425 – 522	452	10
2	445	–	465 – 605	498	10
3	488	ArgoFluor 520	510 – 655	520	10
4	488	ArgoFluor 555L	510 – 665	555	10
5	527	ArgoFluor 548	550 – 710	548	10
6	527	ArgoFluor 580L	550 – 710	580	10
7	553	ArgoFluor 660L	572 – 710	672	10
8	553	ArgoFluor 572	572 – 768	572	10
9	553	ArgoFluor 602	572 – 768	602	10
10	553	ArgoFluor 624	572 – 768	624	10
11	639	ArgoFluor 658	660 – 894	658	10
12	639	ArgoFluor 676	660 – 894	676	10
13	639	ArgoFluor 698	660 – 894	698	10
14	690	ArgoFluor 706	718 – 894	714	10
15	690	ArgoFluor 724	718 – 894	724	10
16	690	ArgoFluor 760	718 – 894	758	10
17	750	ArgoFluor 782	718 – 894	782	10
18	750	ArgoFluor 812	718 – 894	812	10
19	808	ArgoFluor 845	840 – 894	848	10
20	808	ArgoFluor 874	840 – 894	874	10

Table S1: Configuration of the Orion lasers and filters.

Supplementary data files

Data file S1 - Tabulated data

Data file S2 - SHG topography raw data - MC38 CD64DTR late depletion

Data file S3 - SHG topography raw data - MC38 CD64DTR early depletion

Data file S4 - Differential analysis family top10

Data file S5 - Differential analysis myeloid cluster top10

Data file S6 - Core Matrisome and fibrillar collagen genesets

Data file S7 - Differential analysis non-immune clusters top10

Data file S8 - Differential analysis C2 vs. C1 LOG2FC 1.

Data file S9 - Motifs enrichment analysis.xlsx

Data file S10 - Signatures for human COAD analysis.

Data file S11 - Differential analysis lymphocyte cluster top10

Data file S12 - DEG CD3 clusters.

Data file S13 - SHG topography raw data - MC38 CD64DTR late depletion + Immunotherapy