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Supplementary information

Identification, isolation and analysis of human gut-associated lymphoid tissues

In the format provided by the authors and unedited

Supplementary Table

Supplementary Table 1 | Patients included in this study.

	Non-diseased†	Crohn's Disease
Number	79	5
Age	70.2 ± 9.3	48.2 ± 19.6
Sex		
Female	44	4
Male	35	1
Site		
Terminal ileum	56*	-
Recto-sigmoidal resection	23	-
Right-sided hemicolectomy	56*	-
Prox. Site:		
Cecum	14**	4
Ascending	21**	1
Transverse	25**	-
Diagnosis		
Volvulus	2	-
Diverticulitis	2	-
Stenosis	1	-
Adenocarcinoma	74	-
UICC score:		
0	13	-
1	16	-
2	22	-
3	18	-
4	5	-

† = Macroscopically normal intestinal tissue >10 cm from tumor (if present).
* = Paired samples.
** = Multiple sites sampled from some patients.

Supplementary Video Legends

Supplementary Video 1 | Turning over full-thickness colon and ileum wall at Step 1, showing the basal and luminal side of each tissue.

Supplementary Video 2 | Cutting off fat, serosa and muscularis externa (Step 2) of colon.

Supplementary Video 3 | Trimming submucosa (Step 5) of colon.

Supplementary Video 4 | Initiation and peeling (Step 7) of colon.

Supplementary Video 5 | Initiation and peeling (Step 7) of ileum.

Supplementary Video 6 | Excision of methylene blue stained submucosal isolated lymphoid follicles from the peeled submucosa using a tissue punch (Step 12).

Supplementary Video 7 | Identification of a submucosal isolated lymphoid follicle from the basal side, and its association with a mucosal invagination on the luminal side, before the initiation of peeling (Step 6).

Supplementary Video 8 | Excision of mucosal isolated lymphoid follicles from peeled sigmoid colon lamina propria using a scalpel (Step 12).

Supplementary Methods

Quantification of GALT numbers and size

GALT size and number for **Fig. 4** and **Extended Data Fig. 1C-D** were analyzed as previously described[8]. SM-ILF and M-ILF size and number were assessed from images of MB counterstained SM or (unstained) peeled LP, respectively, using ImageJ (v 1.51k) software, assessing at least 2.5 cm^2 SM/LP per patient. Follicle area was obtained using the “circle” and

"ROI Manager" tools, providing a pixel count, followed by conversion of this pixel count into an area (mm^2) based on a size bar, and finally using this to calculate the diameter. Only SM-ILF that had a circular shape with no irregularities were categorized as a SM-ILF. Other structures were categorized as either double/irregular or 3+ GALT based on their morphology and number of visible follicles[8]. The area of the double/irregular or 3+ GALT were calculated similar to SM-ILF, but with the "polygon" tool replacing the "circle" tool.

Flow cytometry

Flow cytometry was used in this study as an auxiliary method to determine leukocyte viability (**Fig. 2A**), cell numbers for individual isolated lymphoid follicles (**Fig. 2B-C**), the percentage of total leukocytes made up by various innate lymphoid cell subsets (**Fig. 3A-B** and **Extended Data Fig. 2**), and the effect of MB (**Extended Data Fig. 3**).

Digested cells were spun down (400 *g* for 5 min) and blocked in Brilliant stain buffer containing 3% mouse serum for 10 min, before the antibody mixture (prepared in brilliant stain buffer) was added for another 30 min. Samples were washed twice in PBS containing 2% FCS (FACS buffer), before being spun down (400 *g* for 5 min) and resuspended in FACS buffer (300 µl), and filtered through 40 µm mesh filters into FACS tubes. For intracellular RORγt staining, the Foxp3 Transcription Factor Staining Buffer Set wash used. First, digested cells were spun down (400 *g* for 5 min) and the pellets resuspended in Fixation buffer (100 µl) for 30 min. Samples where then washed in Perm buffer before being spun down (500 *g* for 5 min) and resuspended in Perm buffer (50 µl) containing 10% mouse serum for 15 min. Perm buffer (50 µl) containing anti-human/mouse RORγt PE antibody, at a final concentration of 1:20, was added and left to stain for 30 min. The samples were then washed twice in Perm Buffer and resuspended in FACS buffer (300 µl) and filtered through 40 µm mesh filters into FACS tubes. The samples where acquired using a custom Fortessa Flow cytometer and FACS Diva software, and data analyzed using FlowJo software. Compensation was performed using OneComp and UltraComp eBeads.

Flow cytometry reagents

Brilliant stain buffer (BD; Cat. No. 566349).

Foxp3 / Transcription Factor Staining Buffer Set (Invitrogen, Thermo Fisher; cat. no. 00-5523-00).

Mouse serum (Sigma-Aldrich; cat. no. M5905-10ML).

Primary antibodies: CD45 BUV393 (BD; clone: HI30; cat. no. 563792; RRID:AB_2744400), CD3 BV510 (BD; clone: UCHT1, cat. no. 563109; RRID:AB_2732053), CD19 APC (BD; clone: HIB19, cat. no. 561742; RRID:AB_10894000), CD19 BV786 (BD; clone: SJ25C1; cat. no. 563325; RRID:AB_2744314), cKit PE-CF594 (BD; clone: YB5.B8; cat. no. 562407; RRID:AB_11154595), cKit PE-Cy5 (BD; clone: YB5.B8; cat. no. 559879; RRID:AB_397354), CD127 APC-R700 (BD; clone: HIL-7R-M21; cat. no. 565185; RRID:AB_2739099), HLA-DR FITC (BD; clone: G46-6; cat. no. 560944; RRID:AB_10562569), CD14 PE (Invitrogen; clone: 61D3, cat. no. 12-0149-42; RRID:AB_10598367), CD64 BV421 (BD; clone: 10.1; cat. no. 562872; RRID:AB_2737856), RORγt PE (eBioscience; clone: AFKJS-9; cat. no. 12-6988-82; RRID:AB_1834470), rat IgG2a PE Isotype Control (eBioscience; clone: eBR2a; cat. no. 12-4321-80; RRID:AB_1834380).

Viability dyes: 7-AAD (Invitrogen; cat. no. 00-6993-50), SYTOX™ Green (Thermo Fisher Scientific; cat. no. R37168), Fixable viability stain 440UV (BD; cat. no. 566332), Fixable Viability Dye eFluor™ 780 (eBioscience; cat. no. 65-0865-14). **! CAUTION** 7-AAD, CyTOX, Fixable viability stain 440UV and Fixable Viability Dye eFluor™ 780 are hazardous reagents and should be handled using protective gloves, eye protection and clothing.

Brilliant stain buffer containing 3% mouse serum - 3 µl mouse serum per 97 µl brilliant stain buffer. Prepare fresh.

FACS buffer - 2 µl mouse serum per 98 µl PBS. Can be stored for at least one week at 4°C.

Fixation buffer – add 1-part Fixation Permeabilization Concentrate and 3 parts Fixation/Perm Diluent. Prepare Fresh.

Perm buffer – Add 1-part Permeabilization Buffer 10x and 9 parts double distilled water. Prepare fresh.

Flow cytometry equipment

FACS tubes (5 ml Polystyrene Round-Bottom Tube, 12x75 mm style; Fisher Scientific, Sweden)

FACS tubes with 40 µm mesh filter (5 ml Polystyrene Round-Bottom Tube with Cell-Strainer Cap, 12x75 mm style; Corning; Mexico).

Flow cytometer (Fortessa; BD).

OneComp eBeads™ (Invitrogen; cat. no. 01-1111-42).

UltraComp eBeads™ (Invitrogen; cat. no. 01-2222-42).

Software

FACS Diva Software (v8.0.2).

FlowJo (v10.2).

Immunohistochemistry

Confocal laser microscopy was used in this study as an auxiliary method to identify SM-ILF morphology in peeled SM (**Extended Data Fig. 1B**), to discern why PP could not be separated from the SM (**Extended Data Fig. 4B-C**), and to investigate the efficiency of separating LP and SM (**Extended Data Fig. 6**).

To prepare tissues for microscopy, intestinal samples including unseparated LP with SM, PP, as well as GALT-free LP and SM or separated tissue layers were fixed for 2-6h with 4% PFA in PBS at 4°C and 120 rpm. Fixed tissues were washed once and stored in histology wash/stain buffer. For imaging, washed tissues were either embedded in 4% low melting agarose in PBS and cut into 100 µm sections with a swinging blade microtome, or stained as whole tissues. For staining, sections and whole tissues were incubated overnight in 500 µl wash/stain buffer containing fluorescently labelled antibodies against epithelial cell adhesion molecule (EpCAM), alpha smooth muscle actin (αSMA), CD3, CD19 and CD45 in the indicated combinations, together with 4,6-diamidino-2-phenylindole (DAPI) to highlight cell nuclei. After additional washing in 5 ml histology wash/stain buffer, tissues were mounted on glass slides using ProLong Gold. Whole tissues were cleared overnight and subsequently mounted in Omnipaque® (iohexol) on glass slides. Images were acquired using a LSM700 or LSM710 confocal laser microscope and processed using the Zen v2.3 or Imaris v8 software.

Histology reagents

4,6-diamidino-2-phenylindole (DAPI; Thermo Fisher; cat. no. D1306; RRID: AB_2629482).

Histology wash/stain buffer - 474ml PBS + 25ml FCS (5%) + 1ml Triton X-100 (0.2%). Can be stored for at least one week at 4ºC.

Low-melting point agarose (Invitrogen, cat. no. 16520-100).

Paraformaldehyde (PFA, Sigma-Aldrich, cat. no. P6148). **! CAUTION** PFA is a hazardous reagent and should be handled in a chemical fume hood using protective gloves, clothes and mask.

Primary antibodies: Anti alpha smooth muscle actin Alexa Fluor® 488 (Abcam; clone 1A4; cat no. 184675; RRID: AB_2832195), anti EpCAM – Alexa Fluor® 594 (Biolegend; clone 9C4; cat no. 324228; RRID: AB_2563209), and anti CD45 – Alexa Fluor® 647 (Biolegend; clone HI30; cat no. 304018; RRID: AB_389336), CD45 - Alexa Fluor® 488 (Biolegend; clone HI30; cat no.

304017; RRID: AB_389314), anti CD3 - Alexa Fluor® 647 (BD Biosciences, clone UCHT1; cat no. 557706; RRID: AB_ 396815), anti CD19 – PE-CF594 (BD Biosciences, clone HIB19; cat no. 562321; RRID: AB_11153848).

ProLong Gold (Thermo Fisher, cat. no. P36930).

Triton-X100 (Sigma-Aldrich, cat. no. T8787).

Omnipaque (iohexol; GE Healthcare, cat. no. Y530).

Histology equipment

Falcon® 24 well clear flat bottom plates (Corning, cat. no. 353047).

Swinging blade microtome (Vibratome VT1200S; Leica).

Confocal laser microscope (LSM700 and LSM710; Zeiss).

Polysine Adhesion Slides (Thermo Fisher; cat. no. 10219280).

Glass cover slips (Hounisen; cat. no. 0422.2432).

Software

Imaris (version 8).

Zeiss Zen (v2.3).