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# Identification, Isolation and Analysis of Human Gut-Associated Lymphoid Tissues

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## Abstract

Gut-associated lymphoid tissues (GALT) are key intestinal immune inductive sites, including the Peyer's patches of the small intestine and different types of isolated lymphoid follicle (ILF) found along the length of the gut. Our understanding of human GALT is limited due to a lack of protocols for their isolation. Here we describe a technique which, uniquely amongst intestinal cell isolation protocols, allows identification and isolation of all human GALT, as well as GALT-free intestinal lamina propria (LP). The technique involves the mechanical separation of intestinal mucosa from the submucosa, allowing the identification and isolation of submucosal ILF (SM-ILF), LP-embedded mucosal ILF (M-ILF), and LP free of contaminating lymphoid tissue. Individual SM-ILF, M-ILF, and Peyer's patch follicles can be subsequently digested for downstream cellular and molecular characterization. The technique, which takes 4-10 hours, will be useful for researchers interested in intestinal immune development and function in health and disease.

## Introduction

The intestinal immune system can be divided into two major compartments: the gut-associated lymphoid tissues (GALT), which serve as the major sites of adaptive immune cell priming and differentiation; and the effector sites comprising the intestinal lamina propria (LP) and epithelium, in which primed adaptive immune cells localize and persist long-term to maintain intestinal homeostasis[1,2]. Human GALT includes the macroscopically visible Peyer's patches (PP) of the small intestine and tens of thousands of isolated lymphoid follicles (ILFs), which are distributed along the entire length of the gut[3]. Current techniques used to assess immune cell composition and function within the human intestine involve the digestion of tissue that contains both GALT, submucosa (SM) and LP, and thus do not allow accurate downstream analysis of these distinct immune compartments in isolation[4,5]. While interesting changes to the leukocyte composition of the intestinal mucosa have recently been described in inflammatory bowel disease (IBD)[6,7], it remains unclear whether such changes reflect alterations within the cellular composition of the intestinal LP, or are simply due to inflammation-associated expansion of lymphoid follicles. Here, we describe a protocol that overcomes these limitations by enabling the identification, isolation, and analysis of SM, GALT-free LP, and individual or pooled human GALT structures (Fig. 1).

### Development of the protocol

Using this protocol, we recently characterized ILF in the mucosa (M-ILF) and submucosa (SM-ILF), and identified their unique patterns of distribution along the length of the human intestine[8]. Comprehensive phenotypic and functional analysis highlighted major differences in adaptive immune subset distribution between GALT and GALT-free LP[8]. Finally, we identified specific roles for PP and ILF in the generation of regionalized adaptive immune responses in the small intestine and colon, respectively[8]. Collectively, these results highlight the importance of analyzing each of these compartments separately, not only to enhance our understanding of how intestinal immune

responses are initiated and regulated, but also to identify potential compartment-specific alterations that may contribute to intestinal diseases, such as IBD.

## Comparison with other methods

Historically, it has only been possible to analyze fixed intestinal tissues of human GALT using immunohistochemistry and electron microscopy[9–11], which has precluded high-dimensional phenotypic and functional analysis of GALT-derived immune cells. Although ILFs can be identified by counterstaining the intestinal mucosa with indigo carmine during endoscopy[12] or in biopsies using methylene blue (MB)[13], it is unclear whether such techniques are capable of identifying all SM-ILF and smaller ILF, or of distinguishing SM-ILF from colonic M-ILF and PP follicles from small intestinal M-ILF. Furthermore, the lymphoid compartment of the ILF obtained in this fashion appears similar to LP samples, suggesting contamination[13]. Finally, the numbers of ILF that can be sampled in this way are restricted by the ethical constraints of endoscopic procedures, limiting the breadth of analysis. As a different approach to identifying ILF in the human ileum, Meier et al removed the epithelium from surgical resections using EDTA, and quantified and isolated ILF under a microscope[14]. However, the possibility of LP contamination in these preparations was not addressed. Our experience suggests that, without separation of the mucosa from the SM, many M-ILF are not visible and the majority of SM-ILF cannot be identified.

The current protocol represents a marked improvement on previous approaches, as it allows for unambiguous identification, quantification and isolation of M-ILF and SM-ILF, as well as of PP and multi-follicular colonic GALT (Fig. 1, Extended Data Fig. 1A-C)[8]. This also extends to the isolation of GALT-free LP, which only occasionally accommodates small unorganized clusters of T cells (Extended Data Fig. 1D-F). Following enzymatic digestion, compartment-specific characteristics of GALT and GALT-free LP can be assessed in the absence of

contaminating tissue, which is not achievable using conventional isolation and digestion techniques. In the case of GALT, our protocol allows the isolation of single cells without the use of enzymes, where necessary. Importantly, this protocol enables the isolation of viable cells from individual ILF (Fig. 2A-C), allowing the analysis of local environmental effects on leukocyte compartments (Fig. 3A and B, Extended Data Fig. 2)[8]. Finally, removal of the SM from the LP also allows rapid and complete digestion of the LP. This is not possible using conventional techniques[15] without prolonged enzymatic digestion, which has a negative impact on both cell yield and cell viability[16].

## Applications

These methods permit the isolation of human GALT structures including PP follicles, M-ILF, and SM-ILF, as well as GALT-free LP. Our initial analysis of the adaptive[8] and innate immune cell composition of these sites (Fig. 3A and B, Extended Data Fig. 2) highlights their distinct features and the importance of studying them independently. In combination with state-of-the-art single-cell analysis tools, our isolation techniques can be used to characterize distinct human GALT structures, providing key insights into immune responses within these compartments and whether such responses differ between the different types of GALT or within different segments of the intestine. The ability to isolate cells from individual GALT, in combination with single cell analysis of TCR and BCR repertoires, should enable the tracking of adaptive immune cell clones as they undergo activation, expansion and differentiation in response to local environmental cues. Finally, our protocol can be applied to surgical tissue specimens removed from IBD patients with active disease (Fig. 4), allowing assessment of changes to GALT composition and function in disease. This is important because GALT have been implicated in the initiation and pathogenesis of IBD[17,18].

The protocol could also potentially be used to identify and isolate GALT in the intestinal compartments of livestock such as pigs, cattle, sheep and horses, all of which contain GALT-like structures[19]. Such studies should provide the scientific community with an increased understanding of intestinal immune responses with potential applications for the prevention and treatment of disease in animals and humans. Finally, we speculate that the technique may be applicable to other mucosal tissues that contain an underlying SM such as the oral cavity, the oropharynx, the gastric mucosa or the urogenital tract, allowing more comprehensive analysis of compartmentalized immunity in health and disease.

## Limitations

Given the paucity of ILF in randomly sampled biopsies[13,14], isolation of GALT using these methods is best suited to intestinal surgical material. However, our technique can be applied to remove contaminating SM and ILF from endoscopic biopsies (Extended Data Figure 3A-B), for the cases in which this occurs[13,14]. Furthermore, advanced endoscopic visualization methods can identify ILF[20], which could be assessed for composition and function using a modified version of this method. This would be useful for assessing other patient cohorts, such as IBD patients on distinct treatment regimens.

The sample processing time of this method is lengthy compared with standard intestinal cell isolation techniques because it involves separating the SM from the mucosa, staining of the SM for the identification of SM-ILF, and dissecting individual ILF from both SM and mucosa. Processing time depends greatly on the size of the tissue and the range of immune compartments to be analyzed. The impact of sample processing time on the phenotype and transcriptional profile of isolated cells remains to be addressed in depth, although cell viability of leukocytes ($CD45^+$) is high in all processed tissues (Fig. 2A).

The use of the MB counterstain to identify SM-ILF results in an increased fluorescent background of a fraction of SM-ILF-derived cells in flow cytometry, particularly in channels collecting light from fluorophores excited by red light (640 nm), consistent with the peak excitation wavelength of MB (668 nm)[21,22]. However, as most cells which stain with MB have disrupted surface membranes, these can be excluded from analysis using viability dyes. The issue can also be largely overcome by extensive washing of the SM prior to removal of SM-ILF (see Steps 9-11 and Table 1) (Extended Data Fig. 3C and D). Large SM-ILF can also be visualized and isolated without the use of MB (Step 8 and Extended Data Fig. 1A), allowing samples to be used for techniques such as immunohistochemistry (Extended Data Fig. 1B), for which background fluorescence signals can be problematic.

Determining accurate numbers of M-ILF may be problematic, as M-ILF smaller than 0.2 mm in diameter could be found using stereomicroscopy, but could not be confidently identified using the quantification method based on two-dimensional images used here (Extended Data Fig. 1C, and supplementary information). Similarly, for patients in which the background staining of vessels and other SM components is prominent, the frequency of smaller SM-ILF (>0.2 mm) might also be underestimated. However, other quantification techniques such as manual counting under the stereomicroscope may circumvent these issues. It is theoretically possible that “GALT-free” LP obtained using this protocol could occasionally contain tiny ILF or potential GALT precursor-structures undetectable by stereomicroscopy, although these would seem to contribute minimally to the overall immune-cell composition of the tissue[8]. Moreover, after peeling and removal of M-ILF where necessary, we were unable to find any highly organized lymphoid tissue remaining in the LP tissue by immunofluorescence staining, although occasionally small T cell-rich clusters of unknown identity remained (Extended Data Fig. 1D-F).

Finally, while the technique is also applicable to intestinal material from some patients with IBD, including patients with clear aphthoid ulcers (Fig. 4A-D), it is not always possible to separate the SM from the mucosa in highly inflamed and/or fibrotic areas of tissue.

## Overview of the protocol

Here we describe the implementation of the protocol when processing ileum and proximal colon tissues in parallel (Fig. 1). Initial processing of ileum and proximal colon tissue includes the removal of the serosa, fat and muscularis externa (Step 2), removal of mucus (Step 4) and trimming of the SM (Step 5). SM is then separated from the LP by peeling (Step 7), followed by identification and isolation of SM-ILF (Steps 8-12), M-ILF (Steps 14-16), GALT-free LP (Step 17) and, where required, PP follicles (Steps 18-23). Finally, the isolated intestinal compartments are digested into single-cell suspensions (Steps 24-48) for further analyses, such as flow cytometry. The techniques described in this protocol can be performed by anyone with a basic knowledge of gut anatomy. However, several parts of the protocol, in particular the peeling technique (Step 7), require extensive practice before tissue processing can be repeated consistently. For tissue preparation, even the most experienced researcher might need the assistance of another researcher if large quantities of tissue are to be processed.

## Experimental Design

### *Initial preparation of intestinal tissues*

Intestinal tissues are initially prepared in a similar manner to that used for conventional intestinal digestion protocols[15,23] (Fig. 1). The basal side of the tissue containing the muscularis externa, serosa, and fat is identified (Fig.1, Supplementary Video 1) and is removed by cutting beneath the muscularis externa layer with curved scissors and forceps (Step 2, Supplementary Video 2). Mucus is removed by washing the remaining tissue twice with medium containing the surfactant dithiothreitol (DTT); complete mucus removal from ileum tissues requires additional, gentle brushing with the rear surface of curved forceps/scissors between washes (Step 4). Treating the tissue with DTT also enhances the visibility of the SM and improves trimming of the tissue (Step 5). The next step involves trimming the basal flocculent SM to allow better penetration of MB and

isolation of SM-GALT at later stages. Tissue trimming is best initiated at the edge of the sample and continued in a contiguous wave across the tissue (Supplementary Video 3). In our hands, SM trimming did not impair SM-ILF integrity, as these structures are tightly associated with SM lying closest to the mucosa. Trimming of the SM is not necessary if the aim is only to isolate M-ILF and/or GALT-free LP, as it does not affect the ability to peel the SM from the mucosa (Step 7).

### *Separating mucosa from remaining submucosa*

Following the initial tissue processing described above, the mucosa is physically peeled away from the SM using forceps (Step 7). Starting from the tissue edge, the mucosa is held with forceps whilst pulling away at the SM with a second pair of forceps (Fig. 1, Supplementary Video 4-5)[8]. Visualizing the process under a dissecting stereomicroscope is required for most samples, especially for ileal tissue, which tends to be more fragile than colonic tissue. The grip on the mucosa should be changed frequently as peeling progresses, keeping it close to the peeling front to avoid tearing of the tissue, a particular issue with fragile samples. We recommend that new users test this step on their sample of choice, and note that this can be done on surplus tissue that has been stored overnight in R5 medium, which does not affect the peeling process.

### *Identification, isolation and analysis of individual immune niches*

The protocol now splits into three separate procedures, depending on the experimental design, which may be performed in parallel: (A) identification and isolation of SM-ILF, (B) identification and isolation of M-ILF and GALT-free LP, and (C) identification and isolation of PP and PP follicles.

(A) *Identification and isolation of SM-ILF (steps 9-12)*.

Peeled SM is counterstained with MB and washed first in PBS then in PBS containing EDTA. SM-ILF should become clearly visible as single blue follicles (Fig. 1), which can be excised with a scalpel or biopsy punch (Supplementary Video 6) for subsequent single-cell isolation with or without the use of enzymes[8] (Steps 24-31). Note, some colonic SM samples also contain double, irregular and multi-follicular GALT structures[8].

(B) *Identification and isolation of M-ILF and isolation of GALT-free LP (steps 14-17)*.

For identification of M-ILF, mucosal tissues are washed several times in HBSS containing EDTA to remove the overlying epithelium and improve transparency of the tissue. M-ILF are then removed either with a scalpel or with a biopsy punch. While some M-ILF are visible by eye after epithelial removal, a stereo microscope at 15-30x magnification must be used to identify all M-ILF. Colonic M-ILF are most easily identified from the basal side of the LP, whereas ileum M-ILF can be identified from both the basal and luminal sides (Fig. 1). For the ileum, we recommend gently brushing aside villi, as M-ILF may be concealed underneath. Having excised M-ILF from the mucosal tissue, M-ILF and the remaining GALT-free LP can now be digested (Steps 24-31 and Steps 40-48).

(C) *Identification and isolation of PP and PP follicles (Steps 18-23)*.

PP are defined in the literature as small intestinal GALT structures that contain more than 3-5 follicles[24–27], whereas M-ILF are always single follicles[8–10]. Human PP are however known to fracture and shrink with age[24,25], meaning that follicles on the edges of a fractured PP may be difficult to distinguish from M-ILF (Extended Data Fig. 4A). Yet, as PP are embedded both in the mucosa and SM (Extended Data Fig. 4B-C), they can be distinguished from mucosa-associated M-ILF, which peel away from the SM. Other distinguishing features can be found in Step 19 and in Extended Data Fig. 4D-F. PP follicles identified in this way can then be excised with a scalpel and single cells isolated with or without the use of enzymes (Steps 32-39) for analysis.

## Materials

### Biological materials

Human intestinal tissues

**CRITICAL** Sample processing should be initiated as soon as possible after isolation to ensure optimal tissue integrity and cell viability.

**! CAUTION** All experiments must be conducted with appropriate authorization from local ethics committees and data protection agencies. The experiments described here were approved by the Scientific Ethics Committee of the Copenhagen Capital Region (Approval # H-3-2013-118) and samples were acquired after informed consent according to the Declaration of Helsinki. Patients under 18 and over 85 were excluded from the study. Patients were enrolled prior to surgical resection for either colorectal cancer (n=74), volvulus (n=2), diverticulitis (n=2), stenosis (n=1) or Crohn's disease (CD; n=5) (Supplementary Table 1).

**! CAUTION** human tissue samples should always be handled wearing protective gloves and a lab coat, and the procedure should be carried out in a biosafety cabinet to avoid exposure to potentially infectious material.

### Reagents for sample preparation

DNAse I (Grade II from bovine pancreas; Sigma-Aldrich; cat. no. 000000010104159001).

DTT (1 M in water; Thermo Fisher; cat. no. 426380100).

EDTA buffer (0.5 M EDTA, pH 8.0; Hoefer; cat. no. GR123-100).

Fetal calf serum (FCS; Atlanta Biological; cat. no. S11150, heat inactivated at 65 $^{o}$C for 4 h before use).

10x HBSS (Thermo Fisher; cat. no. 14180046).

HEPES buffer (1 M; pH = 7.2-7.5; Thermo Fisher; cat. no. 10041703).

MB solution (1.5%; Sigma-Aldrich; cat. no. P6148).

PBS (no $Mg^{2+}$ and no $Ca^{2+}$; cat. no. 14190169).

Penicillin and streptomycin (P/S; 10,000 U/ml; cat. no. 15140122).

RPMI (HyClone; cat. no. SH30027.01).

TM-Liberase (Sigma-Aldrich; cat. no. 5401119001).

## Preparation of reagents

**DNAse I stock** - Reconstitute in sterile double-distilled water to a final concentration of 10 mg/ml and aliquot into 1 ml tubes. These aliquots can be stored for at least 6 months at -20ºC.

**DTT medium** - Preheat R5 medium to 37 ºC and add 4 µl DTT per ml R5. Prepare fresh.

**EDTA wash buffer** - Warm up HBSS medium to 37 ºC and add 10 µl EDTA buffer per ml HBSS medium. Prepare fresh.

**HBSS medium** - Add 100 ml of 10x HBSS, 8 ml of HEPES buffer and 10 ml of P/S to 882 ml of double distilled water. Can be stored for at least one month at 4ºC.

**0.1% MB in PBS** - Add 1 ml MB solution (1.5%) in 14 ml PBS. Prepare fresh.

**PBS with 5 mM EDTA** - Add 10 µl EDTA buffer per ml PBS. Prepare fresh.

**R5 medium** - Add 50 ml of FCS and 5 ml of P/S to 445 ml of RPMI. Can be stored for at least one month at 4ºC.

**TM-Liberase stock** - reconstitute powder with PBS to a concentration of 2.5 mg/ml and aliquot into 1 ml tubes. These aliquots can be stored for at least 6 months at -20ºC.

**TM-digest buffer** - Preheat R5 medium to 37 ºC and add DNAse I stock at a final dilution of 1:65 and TM-Liberase stock at a final dilution of 1:40. Prepare fresh.

## Equipment

Cell counter (Automated Hematology Analyzer, XP-300, Sysmex Corporation).

Centrifuge (Sorvall ST40A; Thermo Scientific; VWR).

Centrifuge (Scanspeed mini; Labogene; VWR).

15ml centrifuge tubes (15 ml, 120x17 mm, PP; Ref #62.554.502; Sarstedt).

50ml centrifuge tubes (50 ml, 114x28 mm, PP; #62.547.004; Sarstedt).

Eppendorf tubes (Micro tubes 1.5 ml; Ref #72.690.001; Sarstedt).

Curved forceps (Dumont 7 Forceps, curve tips, 0.03x0.07 mm, Biologie Tips, Inox 11.5 cm, Agnthos, Cat #11274-20).

Fume hood (Scanlaf Mars; Ninolab).

Heating block (HLC Ditabis; Ninolab).

Incubator (Forma Series II 3111; Thermo Scientific).

100 µm mesh filter (100 µm Nylon; Corning).

Microscope cabinet (Sample analysis safety enclosure, WAYSAFE SA640; Solotec Scientific).

Microscope camera (GXCAM, HiChrome-MET, 0.5X, 0.7-4.5, G T Vision Company).

Pasteur pipettes (Transfer pipette PE 1ml, Grad; Fisher Scientific).

Petri dish (Low Form; Polystyrene; sterile; 90 x 16.2mm; Fisher Scientific; Cat #12694785).

Curved scissors (Tip Shape: Sharp/Sharp; 11.5cm; Agnthos; Cat #03-029-115).

Stereo microscope (isiScope ZTL 350; VWR).

Scalpel blades (Carbon steel 22, sterile; Heinz Herenz Medizinalbedarf; Cat #1110922).

Scalpel handles (No. 04; Heinz Herenz Medizinalbedarf; Cat #1110705).

Water bath (12 L; VWR).

## Procedure

## Preparation of intestinal tissues ● TIMING 30-90 min

1) Store intestinal surgical tissue on ice in RPMI containing 5% FCS and 1% P/S (R5 medium) prior to processing.

   ▲ CRITICAL STEP As tissues will dry out quickly, they should be kept in appropriate buffered media at all times.

2) Place tissue with the mucosal side facing down (Fig. 1, Supplementary Video 1) in a Petri dish with ~5 ml ice cold R5 medium and trim off mesenteric fat, the serosal layer, and the muscularis externa, by gripping the muscularis externa with forceps and cutting just beneath it with inverted curved scissors (Fig. 1, Supplementary Video 2).

   ▲ CRITICAL STEP Avoid perforating the mucosa as this will slow down the later peeling step (Step 7) and leads to tissue loss.

3) Cut the remaining tissue(s) into 5-7 $cm^2$ pieces.

4) Remove remaining mucus and fecal material by incubating each tissue piece in 10 ml warm R5 medium with 4 mM DTT (DTT medium) at 37°C under constant agitation (350 rpm) on an orbital shaker, for 10 min. Then replace with fresh DTT medium, and repeat incubation step. For ileum tissue, carefully brush off mucus after the first wash with the rear surface of curved scissors or forceps without damaging the villi to achieve complete epithelial removal during the later stages of the protocol. DTT incubation also increases the visibility of the SM for trimming in step 5.

5) Place the tissue in the lid of a Petri dish with ~5 ml ice cold R5 medium and trim the SM under trans-illumination with scissors and forceps, cutting away as much of the SM as possible, which leaves a thin layer close to the LP (Fig. 1, Supplementary Video 3). Process one piece of tissue at a time, storing the remaining tissues in ice cold R5 medium. The SM should be removed stepwise from one edge of the tissue to the other by carefully

dragging the SM over inverted curved scissors whilst cutting, to avoid perforation of the tissue. The process is easily followed as the tissue becomes more transparent upon trimming. This trimming step is not required if the aim is only to analyze GALT-free LP, as the thickness of the SM does not affect the peeling of mucosa from SM.

6) (*Optional*) Detect and isolate visible GALT under a trans-illuminated stereo microscope. PP, irregular/multi-follicular structures of the colon, and larger SM-ILF, are occasionally visible under a stereomicroscope after trimming without methylene blue counter-staining (Extended data Fig. 4D-F and Extended Data Fig. 5); their visualization can be further improved by stretching the tissue with forceps. A groove in the luminal surface that is visible by stereomicroscopy is a useful guide to identify the presence of an underlying SM-ILF in colon[8,9] (Supplementary Video 7).

## Separation of mucosa and submucosa ● TIMING 10 min to 3 hours

The time required to separate mucosa and SM will vary based on tissue location, quality and quantity. In our experience, ileum samples in general take the longest time to peel, followed by the distal colon, and finally proximal colon. Inflamed and fibrotic tissues also take longer to process. This said, processing time can vary considerably even with tissues from the same site, patient group, and inflammatory/fibrotic state.

7) Place tissues (process one at a time) in the lid of a Petri dish with ~5 ml ice cold R5 medium, and use forceps to gently grip the edge of the mucosa and slowly pull away from the attached SM using a second set of forceps[8] (Fig. 1, Supplementary Videos 4 and 5). Some samples will contain areas where the SM and mucosa have separated after removal of mucus (Step 4), providing an ideal starting point for peeling. Histological analysis of these two layers demonstrates that this step is highly efficient at separating SM and mucosa (Extended Data Fig. 6A-F). While the majority of the muscularis mucosa (MM)

associates with the SM (Extended Data Fig. 6C-F), intact M-ILF remain associated with the mucosa (Extended Data Fig. 6G-H), and intact SM-ILF are exclusively found in the SM[8].

▲ CRITICAL STEP Removal of the SM as a single layer is important to ensure that the remaining LP is completely free of SM tissue.

▲ CRITICAL STEP Some colonic samples contain multi-follicular SM-GALT[8] that peel away with the SM fraction, but with substantial amounts of LP attached. These structures can be identified using MB counterstaining in the same way that SM-ILF are identified (see step 9-11). Some ileal samples contain PP and it is not possible to peel the SM from these areas. For identification and isolation of PP, see steps 18-21.

**? TROUBLESHOOTING**

## Identification and isolation of submucosal ILF ● TIMING 30-90 min

The identification and isolation of SM-ILF (Steps 9-11) may be performed in parallel with identification and isolation of M-ILF (Steps 14-16) and PP (Steps 18-21) to save time.

▲ CRITICAL The following steps describe the identification and isolation of SM-ILF using MB counterstaining. MB can affect later fluorescence analysis, as it has a pH-dependent absorption maximum of around 668 nm[22], with peak emissions around 688 nm[21,22]. As this may adversely affect immunofluorescence microscopy analysis, we advise that any structures needed for this technique should be isolated prior to counterstaining (Step 9). Although flow cytometric analysis can also be affected by MB, this can be mitigated or circumvented (see Step 9 and Table 1).

**? TROUBLESHOOTING**

8) (*Optional*) Identify any clearly visible follicles under a trans-illuminated stereo microscope (Fig. 1 and Extended Data Fig. 1A), and isolate using a tissue punch or scalpel, similar to isolation of MB counterstained SM-ILF (Step 12).

▲ CRITICAL STEP This will only provide larger SM-ILF that may not be easily distinguishable from irregular/multi-follicular GALT at this stage and is not applicable to all patient samples.

9) Stain the peeled SM with 0.1% MB in PBS (3-5 ml per tissue piece) for 2 min and immediately transfer to PBS. The staining solution can be reused for several tissues.

▲ CRITICAL STEP Although the phenotype, function[8] and viability (Fig. 2a) of $CD45^+$ cells from SM-ILF seem unaffected by MB, it has been shown that MB may cause DNA damage[28,29] so if this is to be avoided at all cost, visible SM-GALT should be isolated without MB staining (Step 8).

10) Wash the SM 2-3 times for 1 min each in horizontally-oriented 50 ml tubes with 25 ml PBS on an orbital shaker (350 rpm) until the buffer remains clear.

**? TROUBLESHOOTING**

11) Wash the SM 2-5 times for 5 min each in horizontally-oriented 50 ml tubes on an orbital shaker (350 rpm), with 10 ml PBS with 5 mM EDTA to visualize all follicles/GALT within the SM. After the last washing step, the washing buffer should be transparent and the blue or pale blue SM-ILF clearly visible (Fig. 1), allowing the identification of SM-ILF from the larger irregular/multi-follicular GALT[8]. Transfer washed tissues to R5 medium and store on ice. Photos can then be taken for later quantification and size estimation.

12) Identify SM-ILF under a microscope and isolate these using a scalpel or tissue punch[8] (Supplementary video 6).

▲ CRITICAL STEP Gentle stretching of putative follicles under the microscope is useful to distinguish between genuine follicles and staining artifacts, as follicles maintain their stain and shape when stretched.

▲ CRITICAL STEP Follicles can sometimes contain epithelial crypts visible under the microscope, which can be removed using a scalpel or peeled away using forceps as in Step 7.

▲ CRITICAL STEP Although it may save time, we advise to isolate SM-ILF away from SM for digestion even when performing bulk analysis of SM-ILF, as the highly vascular SM contains significant numbers of resident and blood-derived immune cells, which will contaminate SM-ILF preparations.

## Identification and isolation of mucosal ILF and GALT-free LP ● TIMING 60-90 min

This section describes the isolation of M-ILF and GALT-free LP after removal of the epithelium. The protocol can be adapted if epithelial cells and intraepithelial lymphocytes are to be isolated. Note that the use of MB does not significantly improve detection of M-ILF, as M-ILF and the surrounding LP take up similar amounts of the stain.

▲ CRITICAL We found that most ileum and distal colon samples contained M-ILF, but found their numbers in the proximal colon to be low[8].

13) (*Optional*) Complete removal of M-ILF from LP requires processing steps 14-15. However, some M-ILF may be identified and isolated without further processing, and isolation without processing should be performed if an intact follicle-associated epithelium is required. Transfer mucosa with the basal side facing upwards (colon) or downwards (ileum) to a Petri dish lid with ~5 ml ice cold R5 medium. Using a stereomicroscope, identify M-ILF and isolate them using a scalpel or a tissue punch (see Step 16).

▲ CRITICAL If the peeled mucosa still contains visible SM, ensure that M-ILF are distinguished from SM-ILF by only isolating follicles not surrounded or attached to SM.

**? TROUBLESHOOTING**

14) Transfer each piece of peeled mucosa to 50 ml Falcon tubes containing 10 ml 1x HBSS with 8 mM HEPES buffer and 1% P/S (HBSS medium) and shake it gently for 10-20 seconds to wash off previous media.

15) Remove HBSS medium and add 10 ml fresh warm HBSS medium with 5 mM EDTA (EDTA wash buffer) to each tube and incubate at 37ºC on an orbital shaker (350 rpm) for 10 min. Repeat 3-5 times, until the EDTA wash buffer remains clear.

**? TROUBLESHOOTING**

16) Transfer LP with the basal side facing upwards (colon) or downwards (ileum) to a Petri dish lid with ~5 ml ice cold R5 medium. Pictures may be taken for later quantification and size estimation of M-ILF at this stage. Identify M-ILF under a trans-illuminated microscope as round follicular structures obscuring the intestinal crypts[8] (Fig. 1) and excise them using a scalpel or tissue punch, making sure to remove as much associated LP as possible without disrupting the follicle (Supplementary video 8).

▲ CRITICAL STEP A stereo microscope with a magnification between 15-30x is preferable to identify all M-ILF accurately. In the distal colon, M-ILF should not be confused with “craters” left behind by detached SM-ILF (Extended Data Fig. 7). It may also be necessary to brush aside villi in the small intestine to identify and isolate otherwise hidden M-ILF or very small ILF. Follicles smaller than 0.2 mm can often be found using this strategy, but cannot be confidently identified and quantified based on a two-dimensional photo (Extended Data Fig. 1C). As previously reported,[27] we generally noted an absence of M-ILF in close proximity to PP.

**? TROUBLESHOOTING**

17) (*Optional*) If GALT and SM-free LP is required, make sure to remove any areas where SM remains attached to the LP using a scalpel.

## Identification and isolation of PP and PP follicles ● TIMING 50-160 min

▲ CRITICAL Not all ileum samples contain PP, although samples of terminal ileum from the region of the ileocecal junction often do. While PPs vary greatly in appearance and size between patients, some or all of the indicators outlined below (Step 19) should allow identification and isolation of PP and PP follicles in all samples where they are present. Apart from the last few centimeters of the terminal ileum, PP are located on the anti-mesenteric side of the small intestine[24–26].

18) Remove epithelia (as in steps 14-15) and identify clearly outlined PP (Extended Data Fig. 4D). Pictures of PP may be taken at this stage for later quantification and size estimation of the PP and its follicles.

19) If PP are not seen easily by the naked eye, look for several indicators that may suggest their presence, including: i) An area of tissue where peeling is not possible; ii) a distinct outlined area that is denser than the surrounding tissue, even after cutting away accessible SM; iii) an area of tissue containing fewer villi than surrounding tissue; iv) the presence of "black spots" in the SM and/or around follicular structures (Extended Data Fig. 4E-F).

20) (*Optional*) To improve visualization of PP follicles, counterstain with MB (steps 8-10) (Fig. 1).

    ▲ CRITICAL STEP Counterstaining of the tissues with MB inhibits epithelial detachment by EDTA and should always be performed after epithelial removal.

21) Cut away remaining LP surrounding the PP.

22) (*Optional*) Excise Individual follicles using a scalpel or a tissue punch. This may require the use of a stereomicroscope for samples where follicles are not easily discernible, and may not be possible for all follicles as some form fused or irregular follicular clusters.

Moreover, PP from some patients only contain indistinguishable deteriorated follicular tissue without clearly outlined follicles or subepithelial domes, making follicular isolation all but impossible.

23) (*Optional*) Cut out and isolate inter-follicular areas (interstitium) using a scalpel.

## Generation of single-cell suspensions ● TIMING 1-2h

In our hands, digestion of tissues with either Liberase TM (as demonstrated in this article) or Collagenase D (as demonstrated in our previous study)[8] provides large numbers of leukocytes for flow cytometry. Liberase TM is more efficient but it cleaves surface CD4, which Collagenase D does not[23]. As other enzymatic cocktails may be better suited for specific purposes, we recommend testing different protocols depending on the surface markers and cellular subsets of interest[30]. It is also possible to generate single-cell suspensions of GALT without the use of enzymes by simply mashing them through a 100 µm filter (Step 28/36). However, this approach is likely to provide fewer cells than enzymatic digestion, is not suited to isolate cells from GALT-free LP and may have differential effects on the yield of individual cell subsets.

**Digestion of SM-ILF, M-ILF, and PP follicles.**

▲ CRITICAL These steps may be performed on pooled or individual follicles.

24) Place follicle(s) in 200-500 µl R5 medium with 0.154 mg/ml DNAse I and 0.062 mg/ml Liberase™ (TM-digest buffer) in labeled 1.5 ml tube(s).

25) (*Optional*) To improve digestion and maximize cell numbers when only a few follicles can be obtained, transfer the medium and follicle(s) into the lid of a Petri dish and cut follicle(s) into two pieces. Pour medium and the halved follicle(s) back into the marked 1.5 ml tube(s).

26) Incubate at 37°C with constant agitation (350 rpm) on a heating block for 45 min.

27) Pour the resulting material through a 100 µm filter into a marked collecting tube.

▲ CRITICAL STEP When processing follicles individually, the digestion medium can alternatively be poured through a smaller 40 µm mesh filter and back into the 1.5 ml tube, followed by homogenization of remaining undigested tissue with a pipette tip and the pouring of 0.5-1 ml R5 medium through the filter (replacing Steps 27-28) to improve cell yield.

28) Homogenize the remaining undigested tissue through the filter using a syringe plunger or pipette tip and pour at least 5 ml of ice cold R5 medium through the filter and into the collecting tube, before placing the tube on ice.

29) (*Optional*) Transfer remaining undigested tissue (if present) from the filter into 200-500 µl TM-digest buffer and repeat steps 26-28.

30) Spin down cells at 400 *g* for 5-7 min at 4°C and resuspend pellet(s) in 1 ml R5 medium.

31) Count cells.

**Processing and digestion of PP.**

32) Place the PP in the lid of a Petri dish with 2-5 ml of TM-digest buffer.

33) Cut the PP into 1-2 $mm^2$ pieces.

34) Transfer tissue pieces along with 2 ml TM-digest buffer per 1 $cm^2$ of tissue into labeled 50 ml Falcon tube(s) and incubate at 37°C with constant agitation (350 rpm) on an orbital shaker for 45 min.

**? TROUBLESHOOTING**

35) Pour the resulting material through a 100 µm filter into a marked collecting tube.

36) Homogenize the remaining undigested tissue through the filter using a syringe plunger and pour at least 5 ml of ice cold R5 medium through the filter and into the collecting tube; place the tube on ice.

37) (*Optional*) If present, transfer any remaining undigested tissue from the filter into 3-5 ml of the TM-digest buffer and repeat steps 34-36.

38) Spin down cells at 400 *g* for 5-7 min at 4°C and resuspend pellet(s) in 1 ml of ice cold R5 medium.

39) Count cells.

**Processing and digestion of GALT-free LP and inter-follicular PP tissue.**

40) Place tissue(s) in the lid of a Petri dish with ~5 ml R5 medium.

41) Cut tissue(s) into 2-4 $mm^2$ pieces.

42) Transfer tissue pieces into labeled 50 ml tube(s) and remove excess R5 medium.

43) Add the appropriate digest buffer (0.5 ml digest buffer/$cm^2$).

44) Incubate at 37°C under constant agitation (350 rpm) on an orbital shaker for 45 min.

**? TROUBLESHOOTING**

45) Break up remaining undigested tissue pieces by repeated pipetting (~50 times) using a Pasteur pipette.

46) Pour/transfer at least 5 ml of ice cold R5 media through a 100 µm filter into a marked collecting tube and place the tube on ice.

47) Spin down cells at 400 *g* for 5-7 min at 4°C and resuspend pellet(s) in 1 ml of ice cold R5 medium.

48) Count cells

## Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

| **Step** | Problem | Possible reason | Solution |
|---|---|---|---|
| 7 | Difficulty initiating peel / fragile tissue(s) | Patient variation | Start at an edge, use only the tips of the forceps and/or use a stereomicroscope. Alternatively, grab more of the mucosa close to the edge of the tissue along with some SM, and "force" a peel by pulling hard at the SM with the other forceps. |
| 7 | Difficulty in peeling | Patient variation | Cut sample(s) into 0.5 mm wide and >2 cm long strips and peel from top to bottom. |
| 9 | High methylene blue background staining | Thick SM | Make sure to trim SM (Step 5). Alternatively, stretch the tissue with forceps to help identify SM-ILF. |
| 9 | Incomplete staining | Tissue may be folded | Unfold tissue and redo staining. |
| 10 | Entangled SM | Falcon tube placed upright | Place Falcon tube horizontally on the orbital shaker. |
| 13 and 16 | Unable to identify and isolate M-ILF | Small M-ILF / M-ILF covered by villi | Use a stereomicroscope (15-30x) to gently brush aside villi and/or stretch tissue, and look for small round and slightly denser areas devoid of crypts. For colonic samples, make sure to turn the tissue with the basal side facing up. Consider whether to leave a small amount of SM during Step 7 to enable correct orientation. |
| 13 and 16 | Distinguishing M-ILF from PP | Fractured or partial PP in the tissue sample | Make sure that no SM is attached to the follicle by looking at the follicle from the basal side. Be cautious of larger follicles at the edge of the tissue, as these could derive from a dissected PP cut during sample acquisition or an earlier processing step. |
| 15 | Incomplete epithelial removal | Patient variation / mucus incompletely removed | Make sure mucus was completely removed (Step 5), and that the tissue is physically moving around during the washing steps. Vortex samples for 1-3 seconds at the end of each wash and/or gently scrape off visible epithelium with the rear surface of curved scissors/forceps prior to the last wash. |
| 34 and 44 | Incomplete digestion | Tissues sedentary during digestion | Make sure tissue pieces are floating around during digestion, which is best achieved by using a 50 ml Falcon tube and adding no more than 15 ml per tube. The digestion step may also be repeated for remaining undigested tissue. |
| 34 and 44 | Incomplete digestion | Batch effect of enzymatic reagent(s) | Add more enzymes - titrate enzymatic solution if necessary. |
| 34 and 44 | Incomplete digestion | EDTA inhibition | Ensure EDTA buffer is washed off before digesting as EDTA may inhibit digest enzyme activity. |
| Flow cytometric analysis | MB background stain | Tissue stained with MB | Keep one of the channels excited by the red laser free (preferably the APC channel) and remove the cell population that stains double positive for this and one of the other channels excited by the red laser (e.g. Alexa 700 or APC-Cy7). Alternatively, use APC as a "dump channel", and remove all cells that stain positive in this channel. |

| | | | |
|---|---|---|---|
| Flow cytometric analysis | MB background stain | Tissue stained with MB | Even though MB spills over into all channels excited by the red laser, these can still be used provided each of the relevant fluorophores are conjugated to markers known to be mutually exclusive. E.g. APC on CD3 and APCCy7 on CD19. |

## Timing

Step 1, acquisition of sample: NA

Steps 2-3, removal of muscularis externa, serosa and fat: 2-10 min

Step 4, mucus removal: 20-25 min

Step 5, trimming submucosa: 5-25 min

Step 6, (*optional*) isolation of ILF (unpeeled): 5-30 min

Step 7, peeling: 10-180 min

Step 8, (*optional*) isolation of SM-ILF (unstained): 5-30 min

Steps 9-11, visualization of SM-ILF: 20-40 min

Step 12, isolation of SM-ILF: 10-50 min

Step 13, (*optional*) M-ILF isolation (with follicular epithelium intact): 5-30 min

Steps 14-15, epithelial removal: 45-60 min

Step 16, M-ILF isolation: 5-30 min

Step 17, LP preparation: 5-15 min

Steps 18-19, identification of PP and PP follicles: 2-10 min

Step 20 (*optional*) MB staining and washing of PP: 20-40 min

Step 21, removal of LP from PP: 5-10 min

Steps 22-23, (*optional*) isolation of PP follicles and interfollicular area: 10-120 min

Steps 24-48, digestion: 1-2 hours

### Anticipated results

We have routinely used these techniques to process ileum and colon resections from macroscopically normal intestinal areas of colorectal cancer, diverticulitis and volvulus patients[8]. Of these, more than 90% were processed successfully, with the remainder being either too fibrotic and/or too fragile to provide sufficient amounts of GALT and GALT-free LP for analysis within a

realistic timeframe. Our experience of processing surgical material from IBD patients is more limited, but we have been able to successfully separate GALT and GALT-free LP from four of five proximal colon resections from CD patients with active disease and presence of aphthoid ulcers (Fig. 4A-B). The overwhelming majority of tissues that we have analyzed come from elderly patients[8] (Supplementary Table 1) and it remains unclear whether the success rate for processing surgical tissues will differ in younger patients or in patients with other types of disease.

The concentration of M-ILF and SM-ILF varies considerably between patients and anatomical site[8–11]. In our experience, M-ILF are primarily present in the ileum and distal colon, whereas SM-ILF are found throughout the colon and rarely in the ileum[8]. For an approximate guide to the expected number of M-ILF and SM-ILF in different intestinal segments, please see our recent study[8]. ILF size is highly variable, even within the same patient, ranging from just under 0.1 mm in diameter to almost 1.3 mm (Extended Data Fig. 1C), sometimes distributed among irregular/double or 3+ follicle clusters[8]. It is not yet known if the distribution, number and size of ILF is similar in younger cohorts or cohorts with IBD. Based on the limited number of samples that we have analyzed (four patients), SM-ILF from CD patients appear consistently larger than those found in macroscopically normal colon from colorectal cancer patients, although their numbers appear similar (Fig. 4). Furthermore, three of the four (75%) successfully analyzed CD patients had several colonic GALT structures with three or more follicles, which is considerably higher than in the colorectal cancer patient controls[8]. Finally, tertiary lymphoid organs/tissues (TLO/TLT) have been associated with both CRC and IBD[31–34], but an agreement on how to distinguish TLO/TLT from GALT has yet to be reached. We anticipate that further studies on healthy, CRC and IBD patients may help elucidate this and potentially lead to novel insights into the distinct contributions of GALT and TLO/TLT to intestinal disease.

The number of live cell events that can be acquired on the flow cytometer from an individual ILF depends on ILF size, ranging from 100-20,000 cells for smaller ILF (<0.35 mm in

diameter) to 2,000-200,000 for larger ILF (>0.75 mm in diameter) (Fig. 2B and C). After Liberase TM digestion, we obtain 2 $x10^6$ - $5x10^6$ cells per $cm^2$ of tissue from GALT-free LP and 5 $x10^6$ - 20 $x10^6$ cells per $cm^2$ from PP. Most viable cells (~90-95%) in all GALT compartments are leukocytes, of which the vast majority are B and T cells, whereas GALT-free LP preparations contain more $CD3^-CD19^-$ leukocytes and $CD45^-$ cells[8]. Innate leukocyte subsets are also found within all tissues, although the proportions vary between niches (Fig. 3A-B).

**Data availability**

Source data tables are available for Figure 2, Figure 3, Figure 4, and Extended Data Figure 1. Replicates of the immunofluorescence images in Extended Data Figure 1, 4 and 5 are available on Figshare.com (10.6084/m9.figshare.13060592).

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## Author contributions statements

TF, PBJ, and WA designed our previously published study. TF and PBJ developed the protocol and performed experiments. UMM performed experiments and discussed data. HLJ, LBR and OHN provided patient samples and discussed data. PBJ, TF, UMM and WA wrote the manuscript.

## Competing interests

None.

## Additional information

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## Key reference using this protocol

Immune Profiling of Human Gut-Associated Lymphoid Tissue Identifies a Role for Isolated Lymphoid Follicles in Priming of Region-Specific Immunity, Fenton and Jorgensen et al., Immunity, 2020, DOI: 10.1016/j.immuni.2020.02.001.

## Figure Legends

**Figure 1 |** Experimental outline for preparing ileum and colonic tissues for isolation of GALT and GALT-free LP. Intestinal tissues are oriented with the basal side facing up (Supplementary Video 1) and then have their external layers cut away (Step 2, Supplementary Video 2) and mucus removed (Steps 3-4), before the remaining SM is trimmed using forceps and scissors (Step 5, Supplementary Video 3). Trimmed tissues are physically peeled apart using curved forceps (Step 7, Supplementary Video 4-5), leaving a layer containing the SM with associated SM-ILF, and a layer containing the mucosa with associated M-ILF. Intestinal samples are then split into A) SM for the isolation of SM-ILF using MB, B) mucosa for epithelial removal and M-ILF and LP isolation, and C) PP for epithelial removal and follicle isolation. For A), SM is first stained with MB followed by washing in PBS and PBS containing EDTA to reveal SM-ILFs, which may then be isolated. For B), the epithelium is removed using a wash buffer containing EDTA revealing M-ILF, which can then be isolated. For C), epithelium is first removed with a wash buffer containing EDTA, which may be followed by MB staining to clearly outline PP follicles and associated interfollicular area (interstitium) for isolation. Single cells may then be isolated from the tissues (Steps 24-48). Scale bar, 5 mm. Follicles indicated with ►. GALT, gut-associated lymphoid tissue. LP, lamina propria. M-ILF, mucosal isolated lymphoid follicle. ME, muscularis externa. MM, muscularis mucosa. PP, Peyer's patch. SM, submucosa. SM-ILF, submucosal isolated lymphoid follicle.

**Figure 2 |** Expected viability and cell numbers for flow cytometric analysis. (**a**) Percentage of viable $CD45^+$ cells in indicated GALT and GALT-free LP preparations as assessed by flow cytometry. (**b** and **c**) Number of live $CD45^+$ cells in (**b**) large (>0.75 mm), intermediate (0.75-0.35 mm) and small (<0.35 mm) colon SM-ILF, and (**c**) ileum and sigmoid colon M-ILF. Results are from (**b**) 9 pooled patients (4 proximal colon and 5 distal colon samples) and (**c**) 7 pooled patients (3 ileum and 4 sigmoid colon samples). Each symbol represents a single follicle. Bars represent

the mean and SD. See supplementary information and source data table for experimental details. LP, lamina propria. M-ILF, mucosal isolated lymphoid follicle. PP, Peyer's patch. SM-ILF, submucosal isolated lymphoid follicle. Prox, proximal. Dist, distal.

**Figure 3 |** Expected proportions of selected innate immune cell subsets in ileal and colonic GALT and GALT-free LP preparations. (**a**) Percentage of innate immune subsets among total $CD45^+$ cells in ileum and proximal colon GALT and GALT-free LP (n = 6; paired samples). (**b**) Percentage of innate immune subsets among total $CD45^+$ cells in distal colon GALT and GALT-free LP (n = 6 for mast cells, granulocytes and ILC3, and n = 3 for DCs and Mo/Mac; paired samples). Bars represent the mean and SD. p-values were calculated using 2-way ANOVA with Tukey's multiple comparison test. * $p<0.05$; *** $p<0.001$. See supplementary information and source data table for experimental details. DCs, dendritic cells. ILC3, innate lymphoid cells type 3. LP, lamina propria. M-ILF, mucosal isolated lymphoid follicle. Mo/Mac, monocytes and macrophages. PP, Peyer's patch. SM-ILF, submucosal isolated lymphoid follicle.

**Figure 4 |** Applying the protocol to intestinal tissues from Crohn's disease (CD) patients. (**a**) Pictures of the luminal surface of the mucosa (at Step 5) from a lightly inflamed area of the proximal colon from a CD patient with clearly visible aphthoid ulcers (indicated with ►), magnified examples of which are shown in inserts (red and yellow boxes). (**b**) Representative picture of a MB counterstained proximal colon SM from a CD patient. (**c**) Average size and (**d**) number of SM-ILF per $cm^2$ of proximal colon SM. Each symbol represents an individual patient sample. (●), cecum; (▲), ascending colon tissue. †, Non-diseased is pooled tissues from 30 colorectal cancer samples taken >10 cm from the tumor site and are taken from data generated in our previous study[8]. See source data table for details. Bars represent the mean and SD. The p-value was

calculated using Mann-Whitney test. *** p>0.0001. Scale bar = 5 mm and 1 mm for inserts. SM-ILF, submucosal isolated lymphoid follicle.