Dissecting immune and stromal niches along the length of the human intestine

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Preface

This thesis has been submitted to the department of Health Technology at the Technical University of Denmark in order to obtain a PhD degree. The presented research was conducted from June 2015 to May 2019 under the Supervision of Professor William Winston Agace (DTU) and Ole Haagen Nielsen (Herlev Hospital); first at the Veterinary Institute, then the Department of Nanotechnology and finally at the Department of Health Technology.

The Thesis consists of a two-part introduction covering 1) the structure, morphology and cellular content of the immune niches found in the human intestine, and 2) a general overview of the stromal cell compartment in the human intestinal lamina propria, followed by a discussion of the three included manuscripts. Lastly, the three manuscripts are included, of which Manuscript I was submitted (in an updated format) in July, and is now undergoing revisions.

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Popular science summary

The intestinal mucosa contains the most diverse and largest number of immune cells in the body. Immune cells in the intestine are localized within two main compartments. The first comprises the specialized lymphoid organs collectively known as gut-associated lymphoid tissues (GALT) which are the sites where adaptive immune responses are initiated, and the second is the connective tissue of the gut wall (the "lamina propria") and epithelium where adaptive immune cells localize and carry out many of their functions. These two compartments contain different immune cells and carry out distinct functions, highlighting the importance of studying these structures independently. Most of the studies assessing the function of GALT have been performed in animal models and our understanding of the structure, immune composition and function of human GALT remains limited. In the current thesis we developed a novel technique to identify and isolate human GALT from surrounding intestinal tissue. We subsequently used these methods to assess the immune cell compartment of GALT and lamina propria free of GALT. Our results suggest a role for small GALT structures termed isolated lymphoid follicles in contributing to regional immune responses within the intestine.

Effective immune responses require many different cell types to act in concert. Amongst these are the stromal cells (SCs) that are best known for providing the scaffolding that holds tissues together, but more recently implicated in the regulation of immune responses. In the second part of the project, we set out to better understand SCs in the intestine, using recently developed methods that allow genes to be analyzed in individual cells. In this way, we identified several distinct populations of SCs in the human small and large intestine and used the information to identify where they reside in the gut wall, as well as predict their possible functions. These populations included the SCs known as myofibroblasts that have been associated with inflammatory reactions and we found these to be more prevalent during intestinal inflammation.

Together our results pave the way for more detailed analysis on the function of GALT and SC subsets in intestinal homeostasis and disease, potentially enabling the development of new treatments for inflammatory bowel disease such as Crohn's disease and ulcerative colitis.

Populærvidenskabeligt resumé

Tarmens slimhinde indeholder det mest forskelligartede immunsystem og det største antal af immunceller i kroppen. Tarmens immunceller er primært lokaliseret til to typer væv i slimhinden. Det første udgør specialiserede lymfoide organer kaldet ”gut-associated lymphoid tissues” (GALT) (tarm-associerede lymfoide væv) hvor adaptive immunresponser initieres, hvorimod det andet udgøres af et bindevævslag kaldet ”lamina propria” og epitelvævet, hvor adaptive immunceller udfører mange af deres funktioner. Disse to typer væv indeholder forskellige immunceller og udfører specifikke funktioner, hvilket understreger vigtigheden af at studere disse to vævstyper hver for sig. De fleste studier der har studeret funktionerne af GALT er blevet udført i dyremodeller, og vores forståelse af strukturen, immuncellesammensætningen og funktionen af human GALT er derfor begrænset. I denne afhandling udviklede vi en ny teknik til at identificere og isolere human GALT fra omkringliggende væv. Herefter brugte vi denne metode til at undersøge immuncellesammensætningen af GALT og lamina propria separat fra hinanden. Vores resultater antyder en rolle for små GALT strukturer kaldet isolerede lymfoide follikler i regionale immunresponser i tarmen.

Effektive immunresponser kræver at mange forskellige typer celler interagerer sammen. Blandt disse celler findes stromale celler (SC), der bedst kendes for at give ophav til bindevævet, der holder vævet sammen, men har fornyligt også vist sig at være involveret i reguleringen af immunresponser. I den anden del af projektet forsøgte vi at forstå SC i tarmen ved at gøre brug af en nyligt udviklet metode, der gør det muligt at se på genudtrykket for individuelle celler. På denne måde identificerede vi flere specifikke populationer af SC i tynd- og tyktarm, og brugte denne information til at identificere hvor i tarmvæggen de opholder sig, samt forudsige deres mulige funktioner. Disse populationer inkluderer de SC, der er kendt under navnet ”myofibroblaster” og er associeret med inflammationsreaktioner, og som vi fandt var mere udbredt i den betændte tarmvæg.

Tilsammen muliggør disse resultater mere detaljerede analyser af GALT og SC funktioner under normale forhold og i tarmsygdomme, hvilket potentielt vil gøre det muligt at udvikle nye behandlingsmetoder imod inflammatoriske tarmsygdomme så som Crohn’s sygdom og colitis ulcerosa.

Manuscripts included in the thesis

Manuscript I:

Immune profiling of human gut associated lymphoid tissue identifies a role for isolated lymphoid follicles in regionalized adaptive immune responses

Thomas M Fenton*, **Peter B Jørgensen***, Kristoffer Niss, Yoni S Ruben, Urs M Mörbe, Lene B Riis, Clement da Silva, Julien Vandamme, Henrik L Jakobsen, Søren Brunak, Aida Habtezion, Ole H Nielsen, Bengt Johansson-Lindbom, William W Agace

* Joint first author

Manuscript II:

Identification, isolation and flow based immune profiling of human isolated lymphoid follicles

Peter B Jørgensen*, Thomas M Fenton*, Lene B Riis, Urs M Mörbe, Henrik L Jakobsen, Ole H Nielsen, William W Agace

* Joint first author

Manuscript III:

Generation of a stromal cell atlas of the human intestinal lamina propria

Peter B Jørgensen*, Kristoffer Niss*, Urs M Mörbe, Ann-Kristine Kamps, Thomas M Fenton, Lene B Riis, Grigory Nos, Henrik L Jakobsen, Søren Brunak, Ole H Nielsen, William W Agace

* Joint first author

Review not included in the thesis

Intestinal barrier integrity and inflammatory bowel disease: Stem cell-based approaches to regenerate the barrier.

Holmberg FEO, Pedersen J, **Jørgensen P**, Soendergaard C, Jensen KB, Nielsen OH.

Journal of Tissue Engineering and Regenerative Medicin.

April 2018;12(4):923-935.

Abbreviations

APRIL	A proliferation-inducing ligand
αSMA	α smooth muscle actin
BECs	Blood vessel endothelial cells
BMP	Bone-morphogenic proteins
cDC	Classical dendritic cell
CCL	C-C motif ligand
CCR	C-C motif receptor
CD	Crohn's disease
CXCL	Chemokine C-X-C motif ligand
CXCR	CXC receptor
CP	Cryptopatch
DC	Dendritic cell
DSS	Dextran sodium sulfate
ECM	Extracellular matrix
FAE	Follicle-associated epithelium
FBs	Fibroblasts
FDCs	Follicular dendritic cells
FRCs	Fibroblastic reticular cells
GALT	Gut-associated lymphoid tissue
GC	Germinal center
GPR	G-protein coupled receptor
Gremlin	GREM
HEV	High endothelial venule
Hh	Hedgehog
IBD	Inflammatory bowel diseases
ICAM	intercellular adhesion molecule
IEL	Intraepithelial lymphocyte
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
ILF	Isolated lymphoid follicle
Ihh	Indian hedgehog
iMCs	Intestinal mesenchymal cells
iSC	Intestinal stromal cell
LECs	Lymphatic endothelial cells
LGC	Lymphoglandular complex
LI	Large intestine
LN	Lymph node
LP	Lamina propria
LT	Lymphotoxin
LTi	Lymphoid tissue inducer
LTo	Lymphoid tissue organiser
LTβR	lymphotoxin β receptor
LYVE-1	lymphatic vessel endothelial hyaluronan receptor 1
M	Microfold
MAdCAM-1	Mucosal addressin cell adhesion molecule 1
MFBs	Myofibroblasts
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MM	*Muscularis mucosa*
MMPs	Matrix metalloproteinases

NOD	Nucleotide-binding oligomerization domain-containing protein
OSMR	Oncostatin M receptor
PC	Plasma cell
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDPN	Podoplanin
PP	Peyer's patches
PRR	Pattern recognition receptor
RA	Retinoic acid
SCs	Stromal cells
SED	Subepithelial dome
SI	Small intestine
SILT	Solitary isolated lymphoid tissue
SLO	Secondary lymphoid organ
SM	Submucosa
SMCs	Smooth muscle cells
TCZ	T cell zone
T_{CM}	T central memory
T_{EM}	T effector memory
T_{fh}	T follicular helper
TGFβ	Transforming growth factor β
Th	T helper
TLO	Tertiary lymphoid organ
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T_{reg}	Regulatory T
UC	Ulcerative colitis
VCAM	Vascular cell adhesion molecule
vWF	von Willebrand factor
WNT	Wingless-related integration site

1. Chapter 1: The human intestinal immune system

1.1 Intestinal architecture – regional specialization

The small intestine (SI) and the large intestine (LI) make up the gastrointestinal tract and is a continuation from the pylorus (stomach) (**Figure 1**, bottom), specializing in the digestion and absorption of food and fluids. The length and thickness of the intestinal tract is significantly different between the SI and LI, with the former in humans being on average 460 cm long and 2-4 cm in diameter, and the latter being 150 cm long and 4-5 cm in diameter[1]. The SI and LI are covered by a single layer of columnar epithelial cells that provide a physical and innate immune barrier between the host and external environment. The SI can be subdivided into three segments; the duodenum which is directly attached to the stomach; a long middle segment termed the jejunum; and the most distal segment connecting the SI with the LI, termed the ileum. After initial digestion and mechanical processing in the mouth and stomach, food constituents pass to the duodenum where further enzymatic digestion helps in the breakdown for later uptake by absorptive epithelial cells in the duodenum, jejunum and ileum. Anatomically the SI contains macroscopically visible mucosal folds and long, finger-like projections called villi that extend from the crypts of Lieberkühn (**Figure 1**, bottom). Both SI and LI contain intestinal crypts, but only the SI contains villi, which decrease in height from the jejunum to the ileum. The absorptive epithelial cells of the SI villi are covered by tiny membrane projections called microvilli that constitute the brush-border and serve to increase the surface area for optimal food digestion and absorption. Indeed, collectively the gut mucosa has an estimated surface area of approximately 32 square meters[1–3] and thus represents by far the largest body surface that is exposed to the outside environment. At the distal part of the ileum, intestinal content passes through a sphincter called the ileocecal valve into the cecum, the first part of the LI, to which the appendix is attached (**Figure 1**, bottom). The content then passes along the length of the LI, moving from the cecum/ascending colon into the transverse colon, down the descending colon, into the S-shaped sigmoid colon and finally into the rectum where it is later expelled from the body (**Figure 1**, bottom).

The gut wall is protected by mucus, which consists of a single layer in the SI and a double layer in the LI. The mucosa is the tissue immediately below the lumen, consisting of the epithelium, a layer of connective tissue termed the lamina propria (LP) and a thin layer of smooth muscle cells called the *muscularis mucosa* (MM). The epithelium contains several cell types including absorptive enterocytes and goblet cells responsible for mucus production. The lamina propria is made up of connective tissue and in the SI contains large amounts of vasculature and a central lymphatic vessel called the lacteal, whereas the LP of the LI is less dense and has very few lymphatic vessels under homeostatic conditions[4,5] (**Figure 1**, top). Below the MM sits the highly vascular submucosa (SM),

bordered by two layers of muscle termed the *muscularis externa* or *muscularis propria*, and attached to this we find a connective tissue layer termed the serosa[3,6].

The content of the intestinal lumen changes along the length of the intestine as nutrients are digested and extracted in the SI, with primarily water and undigested carbohydrates entering the LI. As a result, there are significant differences in microbial diversity and number between the SI and LI, both being highest in the LI[2]. In parallel, there is considerable variation in the levels of biologically active microbial and food metabolites, including short chain fatty acids, vitamin A and aryl hydrocarbon receptor ligands. These influence several aspects of local tissue homeostasis including immune cell composition and function[2,7].

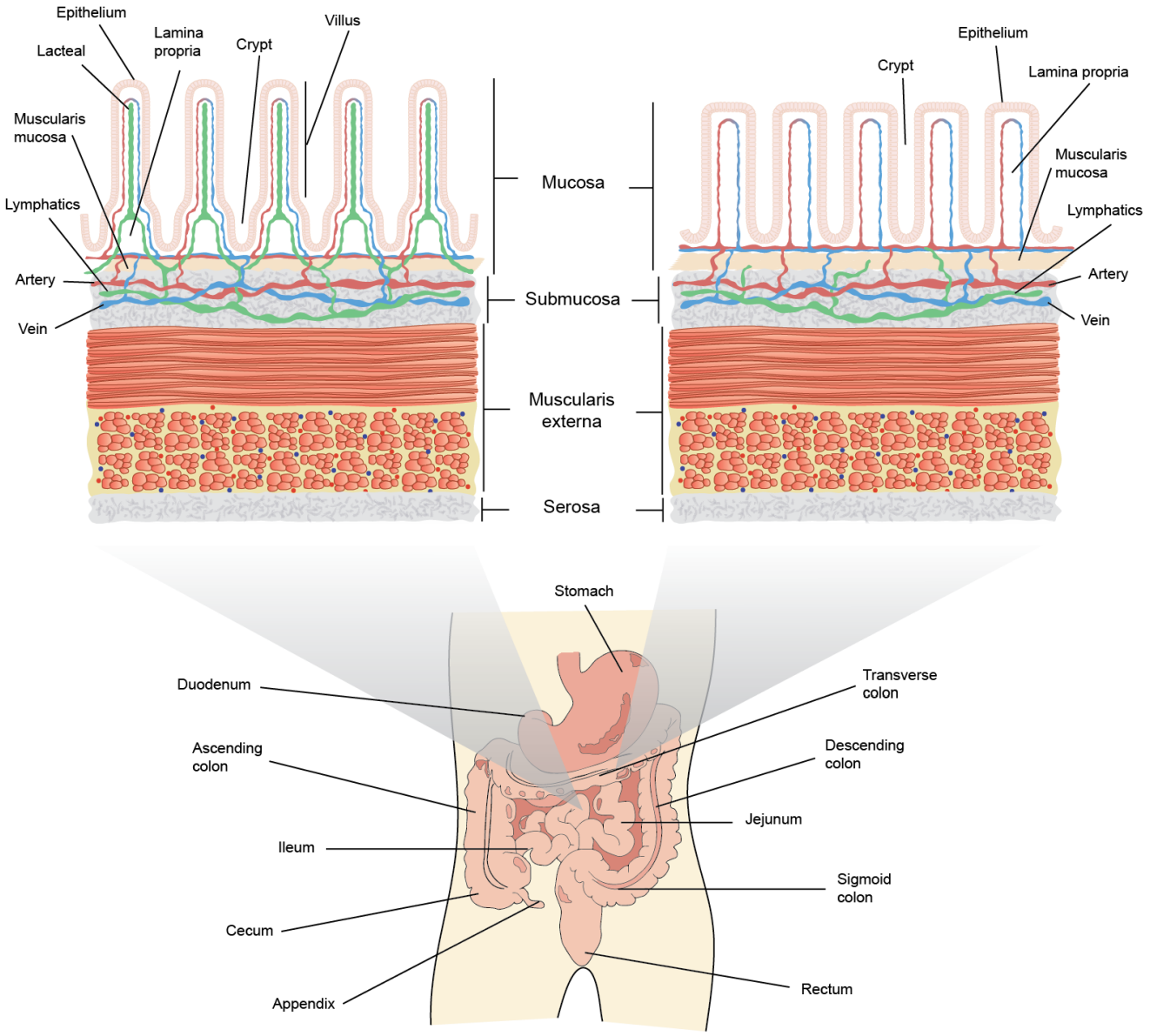

Figure 1 Human intestinal anatomy.

The human intestine (bottom) starts at the stomach and ends at the rectum, and is made up of several distinct segments. The start of the small intestine attached to the stomach is the duodenum, followed by the jejunum and later the ileum. The ileum then merges with the first part of the large intestine, the cecum, at the ileo-cecal valve. The appendix is a secondary lymphoid tissue at the blind end of the cecum. From the cecum, the large intestine continues as the ascending colon, followed by the transverse, descending, and sigmoid colon, and finally the rectum. A closer look at the small intestinal wall (top left) and colonic wall (top right) reveals that these are divided into distinct layers. From top to bottom they are made up of the epithelium, lamina propria and *muscularis mucosa* that together make up the mucosa, the vascular connective tissue of the submucosa, two layers of muscle (*muscularis externa*), and finally the connective tissue of the serosa. Whereas the small intestine (top left) has villi and a central lymphatic vessel in the lamina propria called the lacteal, the colon (top right) only has crypts and very few lymphatic vessels in the lamina propria.

1.2 The intestinal immune system

The intestinal immune system is constantly exposed to foreign material in the form of food or microbial derived products generated by the trillions of beneficial microbes that inhabit the intestinal lumen. It must respond appropriately to these challenges in order to maintain tissue homeostasis while at the same time generate protective immune responses to pathogens that utilize the intestine as a port of entry. As a result it is perhaps unsurprising that the intestine contains the largest number and diversity of immune cells in the body[2,8]. The various immune cell subsets can be found in either the organized gut-associated lymphoid tissues (GALT), comprising the Peyer's patch (PP), and solitary isolated lymphoid tissues (SILT), or in the mucosa itself[2]. The GALT and gut-draining mesenteric lymph nodes (MLN) act as the primary inductive sites for intestinal immune responses, from where primed lymphocytes migrate back to the intestinal LP or epithelium and act as the effector cells that control the microbiome, mediate tolerance against harmless antigens and defend against pathogens where appropriate[9]. It is paramount that these immune responses are controlled as they may otherwise lead to inappropriate responses to commensals and food antigens, leading to pathological conditions such as food allergies and inflammatory bowel diseases (IBD) (**Box 1**). However, the exact cellular interactions and pathways leading to such conditions remain elusive.

Box 1: Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) describe a series of chronic incurable intestinal diseases, the two most prevalent forms being ulcerative colitis (UC) and Crohn's disease (CD), which usually begin in young adults. At present they affect approximately 0.5% of Western populations, but their incidence is on the rise globally, especially in developing countries, providing a substantial economic burden to society[225]. IBD are thought to stem from a combination of an imbalance in the interactions between microbiota and the immune system, environmental factors, and genetic predisposition[226]. Whereas UC only affects the colon, progressing continuously upwards from the rectum and is confined to the mucosa[227], CD can be found throughout the gastro-intestinal tract, can affect several areas, leaving others unaffected (skip lesions), and can affect all parts of the gut wall[228]. Both diseases are thought to be initiated after a breach of the epithelial barrier, leading to excessive and sustained cytokine production, together with immune cell recruitment and activation[227,228].

1.3 Intestinal lymphoid structures

GALT is found all along the length of the gut and comprises large aggregates such as the PPs of the SI or appendix of the LI, or as smaller structures collectively termed SILT scattered all along the intestine[2]. The distribution of GALT varies greatly down the length of the healthy intestine, generally becoming more prevalent towards the distal parts of both the SI and LI[2,10]. In mice, SILT are found as a spectrum from immature structures called cryptopatches (CPs), to larger and more mature structures called isolated lymphoid follicles (ILFs)[11,12]. Similar SILTs are present in the human gut and have classically been termed lymphoglandular complexes or lymphoid aggregations[10,13], which have been likened to PPs and multi-follicular patch-like structures in mice LI[14], although this notion has not caught on in the literature. Nevertheless, I will for the sake of simplicity here refer to these as either SILT or ILFs; no matter if human and mouse SILT are homologous structures. It should also be noted that the following descriptions of human GALT will be based on the present literature, and so will not include new insights on structure, composition and function that we provide in **Manuscript I and II**.

The tissues of the GALT are classical secondary lymphoid organs (SLO), concentrating different immune cell subsets within distinct anatomical compartments, thus providing a microenvironment suited for antigen uptake and presentation, with subsequent immune cell interactions[15]. Each organ comprises follicles containing B cells and germinal centers (GCs) surrounded by areas containing T cells and the specialized blood vessels called high endothelial venules (HEVs) that are involved in the recruitment of lymphocytes into the tissue. Like other lymph nodes (LNs), MLN possess afferent lymphatics that allow entry of leukocytes and lymph which arrive in the subcapsular sinus. This then connects to a system of conduits formed by fibroblastic reticular cells (FRCs) that allow lymph borne cells and small molecules to drain to the antigen presenting cells and HEV present in the T cell zone (TCZ). Lymph from the subcapsular sinus and conduits then drains via the medullary sinus where antigen can be sampled by specialized antigen presenting cells, before exiting via efferent lymphatics into the thoracic duct and then the bloodstream via the subclavian vein[16]. The GALT differ from MLN in several respects, including their mucosal localization, specialized mechanisms of antigen acquisition, and the absence of a capsule or afferent lymphatics[2,17].

Whereas PPs and the similar larger structures in the LI, often referred to as colonic patches, appear around day E12.5-15[18–21], SILTs do not develop until after birth[14,20]. As is the case for LN development, interleukin (IL)-7 expressing lymphoid tissue inducer (LTi) cells, which are a type of innate lymphoid cell (ILC), are required for PP development. Initially, LTi are recruited by the chemokine CXCL13 expressed by the stromal cells known as lymphoid tissue organizer (LTo) cells, whereupon lymphotoxin (LT) $\alpha 1\beta 2$ on LTi cells interacts with LTβ receptor (LTβR) on LTo cells, inducing further expression of these proteins. As a result, a positive feedback loop is created,

recruiting more LTi cells to the developing PP. At day E17.5, T and B cells begin to enter the PP, completing the development of the PP[20,22]. The development of colonic patches follows a similar path, although LTα1β2-LTβR interactions are only required for the full development of these structures and not for the initial LTi-clustering[20]. SILT development also requires LTi-clustering and LTα1β2-LTβR interactions, leading to the formation CPs that consist of LTi, LTo, dendritic cells (DCs), and rare B and T cells. Importantly, SILT development is not pre-programed and presumably requires signals from the diet. CPs can then mature into B cell rich ILFs, a process that requires CXCL13 in the SI, but not in the LI[20]. Moreover, whereas SI ILF development in mice is promoted by microbiota, this seemingly inhibits LI ILF development[19,20].

Human GALT development is much less studied, although it appears that all the structures develop before birth, with PPs developing between 14 and 19 weeks after conception[22] and SI and LI SILT being visible as early as 22 weeks post-conception[10,23]. Aggregates of LTi-like cell have been identified as early as 11 weeks after conception, but whether these are true LTi cells remains unresolved[22].

In the following paragraphs the primary focus will be on human GALT. However, most of our knowledge on their structure and function derives from studies in mice, but only few studies have tried to translate these observations to humans. Furthermore, another issue hampering progress in GALT research has been the inability to separate GALT from the surrounding LP, which is a vastly different immune environment[23–25]. Hence the field is in need of techniques for GALT isolation as well as translational studies, and these are some of the main issues resolved in **Manuscript I and II**.

1.3.1 Types, location, and number of gut-associated lymphoid tissues

PPs are found only in the SI, where they appear as large multi-follicular structures situated on the anti-mesenteric side and stretching from the SM to the mucosa. PPs increase in size and number from the duodenum to the distal ileum, where they form a lymphoid ring[2,26]. The number of PPs in mice varies from strain to strain[14], but is usually around 6-12[27], whereas humans may have more than 300, with numbers increasing even after birth unlike in mice[28]. Whereas mouse PPs contain 6-12 follicles[14], human PPs can vary greatly in size from small ones of around 5 follicles to large ones consisting of as many as 1000 follicles[28]. Both the number and size of PPs peaks around early adolescence and then decreases with age[29].

The mouse LI also contains multi-follicular structures located in the SM, termed the cecal patch, colonic patches and the rectal patch[14,21]. Multi-follicular structures of this kind are rare in healthy human LI, apart from the appendix, although some have been described in the rectum[10], suggesting the presence of human rectal patches

or disease-associated "rectal tonsils"[30–34]. Similar multi-follicular structures have also been observed occasionally in the distal colon[31], suggesting that structures similar to mouse colonic patches may exist in humans. A recent comprehensive study of human GALT suggested that such colonic structures were exclusive to young children[35].

The mouse gut may contain more than 1000 SILTs scattered throughout the SI and LI, most of which are found as CPs in young mice, with the proportion of mature ILFs increasing with age[14,36]. The human gut has been estimated to harbor as many as 30,000 SILTs[37,38]. However, the exact number may be very dependent on the health status of the individual, as they are much more numerous in patients with cancer, IBD, or other diseases, especially close to a tumor or an ulcer[10,13,39,40]. Although differing greatly in numbers between individuals, the number of LI SILT in humans was reported to never exceed 0.32 per square millimeter of submucosa, no matter the health status[10]. The presence of CPs in the human intestine is still controversial[23], although a single study did identify small lymphoid structures highly enriched for the $cKit^{+}CCR6^{+}$ LTi-like cells that characterize CPs in mice[41].

Mouse SILTs are found primarily as single follicles and are located solely in the mucosa in contrast to the SI/LI patches[14,21]. Although most SILT in human SI and a few in rectum are also limited to the mucosa, those in human LI may penetrate through the MM into the SM and indeed the largest part of most LI SILT lies in the SM[10,13].

1.3.2 Structure and composition of gut-associated lymphoid tissues

PPs are the most well described GALT structures. They consist of four main compartments: the dominating follicular area containing mostly B cells; interspersed T cell regions; a subepithelial dome (SED) region that lies between the follicles and the specialized follicle-associated epithelium (FAE) facing the lumen. Unlike LNs, PPs do not have afferent lymphatics, but have HEVs and efferent lymphatic vessels allowing the entry and egress of lymphocytes. Particulate luminal matter including bacteria is taken up into PPs via specialized epithelial cells in the FAE called microfold (M) cells that have short microvilli and which are more numerous in mice than human PP[42] (**Figure 2**, bottom). Together with a lower mucus-expression in the FAE this allows for efficient antigen sampling by M cells which then transfer the material to neighboring leukocytes present within or immediately underneath the FAE[26]. In particular the SED is rich in dendritic cells that can interact with and prime antigen-specific T cells, which may then migrate towards the follicular area and initiate a GC reaction (**Box 2**), where antigen-specific B cells are activated, proliferate, and undergo somatic hypermutation to make high affinity antibodies[36]. Mouse LI patches are similar in structure to PPs, albeit smaller in size with fewer follicles and smaller GCs[21]. The poorly described multi-follicular structures of the human rectum also appear to have an analogous structure [30–34], whereas the rare colonic patches are yet to be properly described. R-similar with the lymphatic story

CPs do not show this defined compartmentalization of structure, consisting mainly of loosely arranged LTi cells, activated stromal cells and dendritic cells[20,43]. However, when mouse CPs mature into ILFs, lymphocytes are recruited and they acquire an SED, a B cell follicle with a GC and a FAE with M cells. Mouse ILFs do not appear to contain HEVs or efferent lymphatics, and have only few scattered T cells[14,20,43]. In contrast, SILT in the human SI contain T cell zones and HEV-like vessels (**Figure 2**, top), and some may penetrate the MM and connect to underlying lymphatics[13]. The majority of submucosal SILT in human LI also has both T cell zones and HEV-like vessels, are connected to the lymphatics, do not contain a SED, and have a less clear FAE with fewer M cells[10,39,44] (**Figure 3**, bottom). The mucosal-restricted SILT of the human rectum are similar to mouse ILFs in having a clear FAE with numerous M cells and no apparent connection to the lymphatics, but differ by having T cell zones and potentially contain HEVs[10] (**Figure 3**, top). Whereas virtually all follicles in PPs contain GCs[27], this is only the case for a small proportion of SILT under homeostatic conditions[10,13,14].

Mouse ILF (~0.15 mm in diameter) are generally smaller than human SILT, while CPs are even smaller at around 0.08 mm in diameter[14]. Human LI SILT are generally in the range of between 0.1 and 1 mm in diameter, with those in healthy SI being estimated at around 0.25 mm on average[10,13]. Along with the predominantly submucosal localization, presence of draining lymphatics and HEVs, not seen in mice SILT, are why some have likened human SILT to mouse PPs and LI patches[14,45].

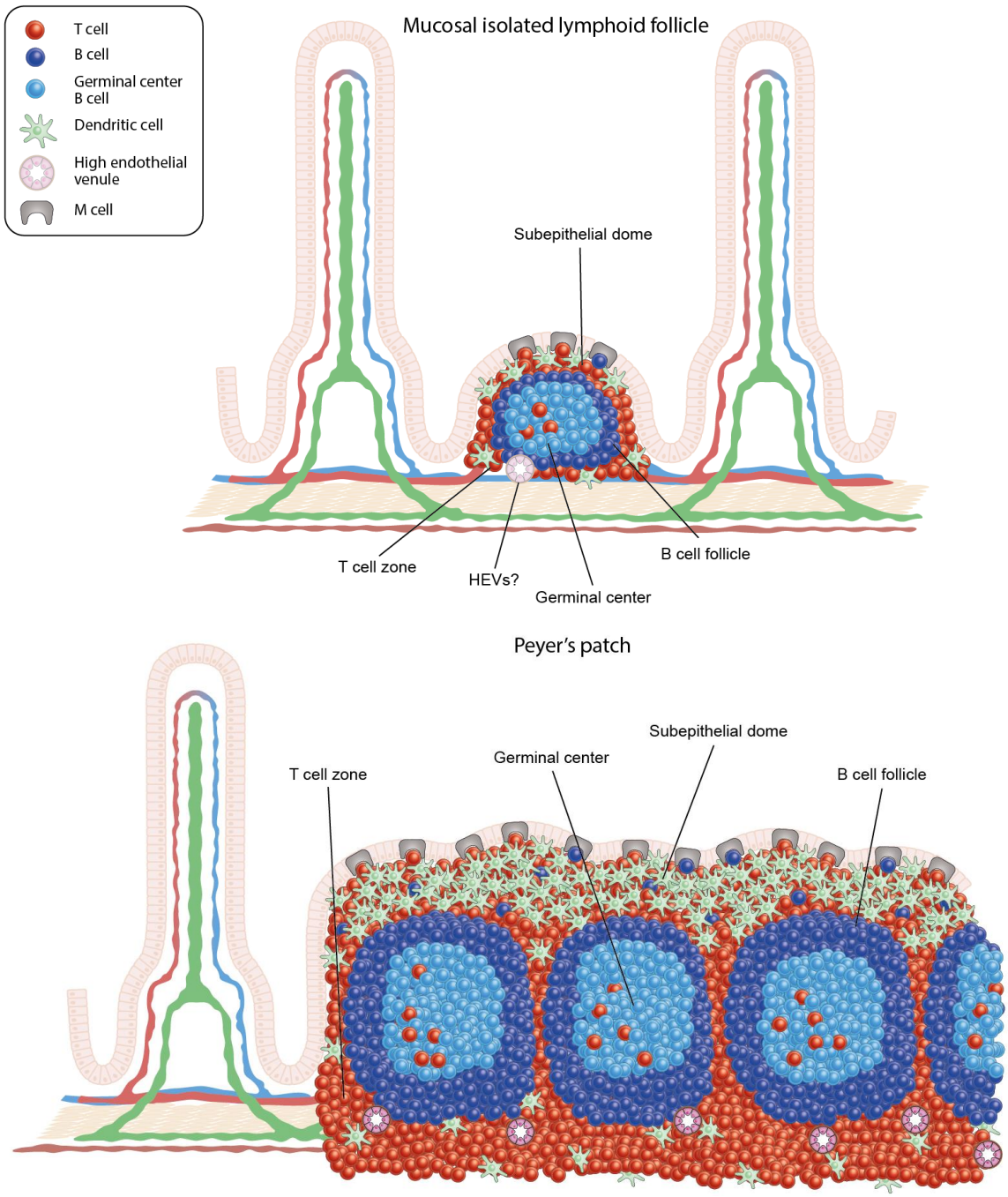

Figure 2 Gut associated lymphoid tissues associated with the human small intestine.

The human small intestine (SI) contains both small isolated lymphoid follicles (ILFs) (top) and larger multifollicular structures called Peyer's patches (PPs) (bottom). PPs stretch across the full depth of the intestinal wall from submucosa to epithelium, while most ILFs in the SI are confined to the mucosa, with only a few, larger ILFs penetrating the *muscularis mucosa*. Both structures contain large numbers of T and B cells, have a subepithelial dome with a high concentration of dendritic cells, and a follicular associated epithelium containing M cells. The T cells in PPs are organized in a defined T cell dependent area surrounding the B cell follicle(s), while they are scattered around the B cell follicle in ILFs. B cell follicles often contain a germinal center with associated T follicular helper cells (red cells inside germinal centers). Whereas PPs have high endothelial venules (HEVs) and draining lymphatics, HEVs have yet to be shown in ILFs and draining lymphatics are only seen in larger ILFs that penetrate the *muscularis mucosa* (not shown here).

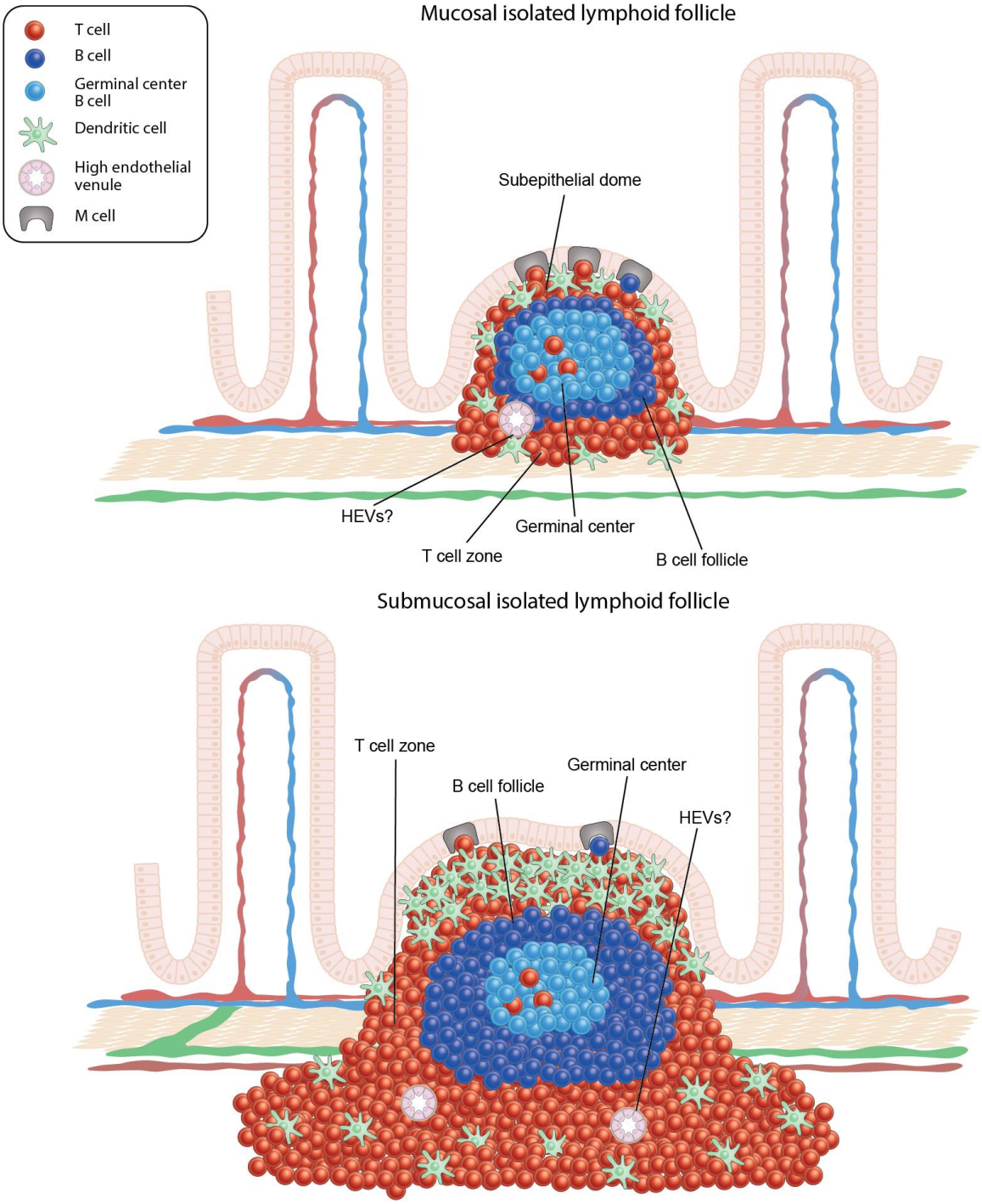

Figure 3 Gut associated lymphoid tissues associated with the human large intestine.

The human large intestine contains mucosal-restricted isolated lymphoid follicles (M-ILFs) (top) and submucosal-associated isolated lymphoid follicles (SM-ILFs) (bottom). They both have large numbers of T and B cells, the former found mainly in the T cell zone, and the latter found within the B cell follicle, which may contain a germinal center. M-ILFs have a subepithelial dome, but do not have draining lymphatics, whereas SM-ILFs are connected to the lymphatics and only penetrate into the lamina propria in small and less defined areas. It is still to be determined whether any of these structures have high endothelial venules (HEVs).

1.3.3 Cellular composition of gut-associated lymphoid tissues

The cellular composition of the different compartments of the GALT has been described most in PP. Large numbers of lymphocytes are found in the FAE[46] and in humans, these are present prior to birth[42]. They consist of roughly equal numbers of T and B cells[46–48], with $CD8^+$ T cells being found in slightly higher numbers than $CD4^+$ T cells, although not to the same extent that they dominate the epithelial compartment of conventional villus mucosa[46]. The $CD4^+$ T cells in FAE are primarily of a memory phenotype expressing CD45RO and they associate closely with M cells in the so-called "pocket" formed by the basolateral membrane of the M cell; in contrast, the $CD8^+$ T cells present are situated outside the M cell pocket and intermingled more generally between epithelial cells[46,48]. In humans, M cell-associated B cells are also primarily of a memory phenotype, expressing IgM but not IgD[48], whereas B cells in mouse FAE are dominated by IgD-expressing cells[42].

The SED of PP contains a large and heterogeneous population of leukocytes, with many $CD4^+$ T cells, dendritic cells (DCs), a small number of macrophages and B cells expressing many isotypes (IgM, IgG, and IgA) except IgD[49,50]. $CD4^+$ T cells dominate the T cell zones surrounding the follicles , where there are also scattered DCs[46,49]. More recent studies have shown that the T cell population of human PPs contain more naïve, central memory (T_{cm}) and regulatory T cells (T_{reg}) and less effector memory T cells (T_{em}) than the surrounding LP[26,35].

The B cell areas in human PPs comprise three distinct areas, a thin outer marginal zone dominated by IgD^- memory B cells, which is not found in mice PPs, a thin mantle zone dominated by IgD^+ naïve B cells, situated below the marginal zone, and the innermost GC[50–52]. The GC can further be divided into a dark zone dominated by proliferating B cells and a light zone that contains non-dividing B cells, follicular dendritic cells (FDCs) and $CD4^+$ T follicular helper (T_{fh}) cells[27,50] (**Box 2**). The B cell follicles of PPs differ from those of non-intestinal SLOs by having large numbers of B cells expressing IgA in addition to those expressing IgD and IgM[50,51]. The few studies of mouse LI patches suggest that these are very similar to PPs, with a TDZ containing $CD4^+$ T cells and some DCs in the T cell zone, surrounding B cell follicles containing a FDC network[14,21]. The cellular composition of human LI patches is unknown.

The cellular composition of SILT is also poorly described. Flow cytometry and immunohistochemistry have estimated that human ileal SILT[25,40] contain a B:T cell ratio of 1:1[25] or 1:1.8[40], respectively, with the proportions of $CD4^+$ and $CD8^+$ T cells being 60% and 13 %[25] or 80% and 20 %[40] of the T cell compartment, respectively. Parallel flow cytometric studies of human LI SILT suggest a B:T cell ratio of 0.6-0.8, with T cells mainly being $CD4^+$ (69%) and only small numbers (13%) of $CD8^+$ T cells[25]. One of the studies further showed that T_{fh} or T_{fh}-like cells could be found in ileal SILT, located primarily in the light zone of GC[40]. However, all these numbers were very variable, both within and between the studies. Furthermore, the only study which

compared the cellular compartment of SILT and LP surprisingly observed only minor differences between these tissues[40], suggesting imprecise identification and purification of SILT.

In summary, our knowledge on human GALT structure and cellular composition is very limited, relying in many cases on just a few studies, with especially SILT being poorly described. However, they do conform to the general architecture of SLO and to a large extend resemble similar and more well-described GALT in mice, although important differences are seen. **Table 1** provides an overview and comparison of mouse and human GALT. An in depth description of human GALT is the main focus of **Manuscript I**, and is also touched upon in **Manuscript II**, and so will be tackled further here.

Box 2: The germinal center reaction

When B cells encounter their antigen in gut-associated lymphoid tissues (GALT) they move to a region between the B cell follicle and T cell zone termed the interfollicular region were they encounter activated $CD4^+$ T cells specific to the same antigen. This interaction can give rise to early response antibody-producing plasmablasts, before the B cells and associated T cells move to the center of the B cell follicle. Here the B cells start expressing increased levels of CD10, CD20 and CD38, continue proliferating, and form a mature germinal center with a light zone and a dark zone[17,203,229]*. In the dark zone, the B cells proliferate and undergo somatic hypermutation before migrating into the light zone, where they interact with T_{fh} cells and antigen bearing follicular dendritic cells, resulting in the selection of the B cell clones with highest affinity for the antigen; a process that can be repeated several times. Isotype switching from IgM to IgG or IgA subclasses occurs and the selected B cells can differentiate into either memory B cells or plasmablasts, which may then leave the lymphoid tissue and migrate back to the gut*[52,203]*.*

Table 1. Gut-associated lymphoid tissues in mice and humans; based on present literature.

CP = cryptopatch; HEV = high endothelial venule; ILF = isolated lymphoid follicle; LI = large intestine; PP = Peyer's patch; SED = subepithelial dome; SI = small intestine; SILT = solitary isolated lymphoid tissue SM = submucosa; TDZ = T cell dependent area.

	Feature	Mouse	Human
SI GALT	**PP**	6-12 follicles, most concentrated in jejunum	5-200 follicles, most concentrated in terminal ileum
	CP	More than ILF	No
	ILF	Location: Mucosa Diameter: ~150 μm High B:T ratio SED: Yes TDZ: No HEV: No Efferent lymphatics: No	Location: Both exclusively mucosal ILF and ILF penetrating into SM Diameter: ~250 μm Low B:T ratio (1-1.8) SED: Yes TDZ: Small HEV: (Yes?) Efferent lymphatics: Only in SM-associated ILF
LI GALT	**Appendix**	No	Yes
	LI patches	One in cecum, several in colon, and one in rectum	Few in distal LI
	CP	Yes	No
	ILF	Location: Mucosa Diameter: ~150 μm High B:T ratio SED: Yes TDZ: No HEV: No Efferent lymphatics: No	Location: Primarily SM penetrating into LP; some exclusive to mucosa in rectum Diameter: 100-1000 μm Low B:T ratio 0.6-0.8) SED: Mucosa-restricted SILT TDZ: Yes HEV: (Yes?) Efferent lymphatics: Yes

1.3.4 Lymphocyte entry and gut homing

Lymphocyte entry, positioning within GALT, and homing to the mucosa is of utmost importance for a proper intestinal immune response. For lymphocytes this is a twostep process, where they are activated in GALT or MLN, and later recirculate back to the mucosa to affect their functions. There is a strong correlation between localization and activation state of lymphocytes, with naïve lymphocytes being restricted to secondary lymphoid organs such as the GALT and activated lymphocytes localizing mainly in the LP and epithelium[27,53]. Migration of lymphocytes into tissues is a multistep process where lymphocytes interact with endothelial cells, eventually allowing lymphocytes to enter the tissue. Generally, engagement of lymphocyte adhesion molecules with vascular ligands makes the lymphocytes tether and roll along the surface of the endothelia. Here chemokines mediate cellular changes inducing firm integrin-mediated arrest, and later allow lymphocytes to migrate through the vascular wall and into the tissues (diapedesis)[54]. In GALT, Lymphocytes enter through HEVs by expressing L-selectin and the mucosal vascular addressin cell adhesion molecule 1 (MAdCAM1)-receptor integrin α4β7, with the latter being especially highly expressed by naïve B cells, explaining the prevalence of these cells in PPs[27,54]. Chemokines are also essential for entry of lymphocytes into GALT. C-C motif ligand (CCL) 19 and CCL21 produced by GALT stromal cells attract C-C chemokine receptor (CCR) 7-expressing naïve T cells[55,56], whereas chemokine C-X-C motif ligand (CXCL)13 produced by FDCs attracts CXC receptor (CXCR)5-expressing B cells[27]. Once the lymphocytes have entered the GALT, they will then move to the T cell zones, controlled by CCL19/21 gradients, or the B cell follicle controlled by CXCL13[27].

Upon activation by gut-derived DCs in the GALT or MLN, T cells can then either become T_{fh} cells, upregulating CXCR5 and migrating to the B cell follicle, or they leave the GALT/MLN as effector cells[27,53,57]. In the latter case, the activated T cells acquire expression of the CCR9 chemokine receptor and α4β7 integrin under control of retinoic acid produced by gut derived DC. After leaving the GALT/MLN via efferent lymphatics and thoracic duct, the T cells enter the bloodstream at the subclavian vein and after arriving in the intestine, they are stimulated to exit into the LP via recognition of MAdCAM-1 on mucosal blood vessels by α4β7 and by ligation of CCR9 by epithelial derived CCL25[53,54]. Similar mechanisms govern the migration of B cells from the GALT into the mucosa, but it should be noted that CCR9 is only involved in preferential homing of lymphocytes activated in the PP or small intestine draining segment of MLN to the small intestinal mucosa and more to the epithelium than the LP. The mechanisms responsible for the equivalent processes in the LI remain to be established fully, although recognition of CCL28 by CCR10 has been implicated in migration of plasmablasts to the colon and G-protein coupled receptor (GPR) 15 appears to control recirculation of some activated T cells to the LI; the ligand for GPR15 remains unknown[55].

Interestingly, B cells activated in a single mouse PP may also be able to recirculate into the GC of other PPs, potentially creating a synchronized immunological response along the length of the intesine[58]. This may also be the case in humans, where memory B cells of similar clonal origin have recently been identified in both SI and LI GALT of the same patient[59].

1.3.5 The intestinal IgA response

The dominant Ig isotype in the intestine is IgA, which is expressed by 79% of plasma cells (PCs) in the LP of the SI and by 90% in the LI. Unlike that found in serum, IgA is secreted into the intestine as a dimer, where it acts as a non-inflammatory Ig. Two IgA isotypes are found in humans, with IgA1 dominating in the jejunum, whereas IgA2 dominates in the colon[60]. This difference may be down to IgA2 being more resilient to bacterial proteases making it better suited for the bacterial-rich environment of the LI, or because IgA1 and IgA2 PC may express different homing receptors. In contrast to other, non-intestinal tissues, IgG producing PCs are rare in the healthy intestine (~5% total), but the production of IgG is significantly increased during infection or IBD[2,51,60,61].

Several factors have been implicated in the preferential IgA class switching that occurs in the GALT. The main factor is transforming growth factor-β (TGFβ), but other factors such as IL-10, IL-4, retinoic acid (RA), and epithelial-expressed "a proliferation-inducing ligand" (APRIL) also plays a role. In humans, IgA-switching in GALT is dependent on classical T cell help and the IgA is of high affinity and hypermutated, being derived from conventional B2 B cells. However a substantial proportion of IgA in mice is of low affinity and is produced from innate B1 B cells via T cell independent switch mechanisms[27,51,62]. It has been suggested that murine SILT may induce T cell independent B cell responses due to their low number of T cells, whereas the larger lymphoid patches in both SI and LI patches may induce T-cell dependent B cell responses[63]. There has been controversy about whether IgA-switching can occur in the intestinal LP itself[27,42,51,62], as mice lacking GALT still contain IgA^+ PCs in their intestinal LP[27] and some workers have reported AID expression in LP from mice[64] and human colon[65]. However others have shown that this is exclusive to SI GALT in mice[66], and to SI and LI GALT in humans[62], although in the latter study it was unclear which type(s) of GALT and which part(s) of the LP were analyzed. The factors controlling preferential switching to either IgA1 or IgA2 remain largely unknown[51,62], although a combination of IL-10 and APRIL can induce IgA2-switching by human B cells *in vitro*[65].

PPs are the main site for the generation of IgA^+ B cells that home to the SI[27,51], while the cecal patch in mice may induce both LI and SI-homing in IgA^+ B cells[67]. Removal of the cecal patch leads to fewer IgA^+ B cells in the LI, while DCs from cecal patches but not PPs are also capable of driving CCR10 expression by B cells, underlining that specific SI or LI homing is determined by the local environment within individual compartments

of the GALT[67]. Interestingly, the PC defects produced by deletion of PPs or cecal patches lead to compensatory expansion of the SILT in SI or LI respectively, indicating that SILT may also contribute to the production of IgA^+ B cells in mice[11,67]. Whether human SILT plays a similar role in the generation of IgA^+ B cells remains unexplored, but we address this subject in **Manuscript I**.

1.4 Intestinal lamina propria and epithelium – effector sites of the gut

The intestinal LP and epithelium are the main battlegrounds of the intestine where large numbers of innate and adaptive immune cells maintain tissue homeostasis in the face of opportunistic pathogens and other insults. The intestinal epithelial layer contains large numbers of lymphocytes termed intraepithelial lymphocytes (IELs)[68]. While the overwhelming majority of IELs in both mice and humans are $CD8^+$ T cells, many of which are unique subsets, IEL composition varies greatly between species and location along the intestine[42,68,69]. In both mice and humans, the IEL to intestinal epithelial cell ratio is around 1:5-1:10 in the SI and approximately 1:40 in the LI[42]. Human IELs are mostly conventional $CD8\alpha\alpha^+TCR\alpha\beta^+$ T cells that are thought to have undergone initial activation in lymphoid tissues and subsequently migrated to the epithelium, where they can survive long-term. In contrast, the majority of IELs in mice are unconventional $CD8^+$ T cells that express the αα homodimeric form of CD8 and many express $TCR\gamma\delta$[42,69]. While IELs have been implicated in protection against microbial invasion, epithelial surveillance, and potentially IBD, the role of individual subsets remains largely unclear[42,68,69].

The LP is also dominated by T cells with an effector/memory phenotype, but whereas the IEL compartment mainly contains $CD8^+$ T cells, the intestinal LP has approximately twice as many $CD4^+$ T cells as $CD8^+$ T cells. Although all $CD4^+$ T cell subsets can be found in the gut, the two main subsets are IL-10 producing T_{reg} and IL-17 producing T_h17 cells[2,53]. Interestingly, the proportions of these cells in intestinal sections seems to be inversely related, with T_h17 being more prevalent in the SI and T_{reg} numbers increasing going down towards the colon. On the other hand, both Interferon (IFN)γ producing T_h1 and the rare IL-4, IL-5, IL9 and IL-13 producing T_h2 cells are distributed equally through the SI and LI[2].

The LP also contains large numbers of $CD38^{hi}$ effector B cells in the form of plasmablasts or the more mature PCs, together with a small number of scattered $CD20^+CD27^+MHCII^+$ memory B cells[51]. PCs are found throughout the intestinal LP, but are more frequent in the region around the crypt base and their numbers increase towards the distal end of the gut[2,51].

Mononuclear phagocytes, in the form of the monocyte-derived macrophages and conventional DCs (cDCs), are found throughout the LP, where they are important sentinels of the intestinal barrier. Together they

survey the environment for microbes and other threats, with cDCs having the ability to process and present resulting antigens to T cells. The acquisition of antigen prompts cDCs to leave the tissue and migrate to MLNs, where they can present their antigen and induce gut homing in antigen-specific T cells. As such these cells play an essential role in the activation and differentiation of intestinal adaptive immune responses[70–73]. Several subsets of intestinal cDC have been described that appear to play distinct roles in controlling different types of adaptive immune responses in the intestine[74]. Several Macrophage subsets are also recognized, but in contrast to cDC these are non-migratory cells. The main function of these cells is phagocytosis of microbiota and dying cells without initiating an overt inflammatory response, but they may also play indirect roles in tolerance-induction and help promote or maintain further differentiation of antigen-specific T cells by presenting antigens. The monocyte/macrophage compartment alters significantly in favor of pro-inflammatory subsets during IBD, suggesting a role for these cells in disease pathology[75].

Granulocytes can also be found in the healthy intestine, although neutrophils are rare under these circumstances and the majority consists of eosinophils and some mast cells. These cells may help protect against pathogens, but can also influence local immune homeostasis, with for instance eosinophils playing a role in the steady state production of IgA[76–79]. The intestinal LP also harbors all major ILC subsets, although the exact function and impact of these ILCs remains largely unknown[2,80–82].

1.5 Summary and outlook – Chapter 1

The human intestine is uniquely responsible for allowing uptake of nutrients and fluids, tolerating food and commensal antigens, while simultaneously keeping opportunistic pathogens at bay. This is handled by a huge and varied community of immune cells, separated neatly into inductive compartments in the form of patches and SILT that contain naïve lymphocytes, and the effector compartments of the LP and epithelia that containing diverse populations of leukocytes. The different compartments of the GALT remain poorly understood, in part due to the difficulty of obtaining such structures for analysis. Thus the development of tools/techniques for their isolation is paramount for accelerating progress in this field. In mice SILTs have been shown to play a role in generating IgA^+ B cells, but this has yet to be shown for human SILT, even though SILTs are the predominant form of GALT in the human colon. As IBD involve an inappropriate inflammatory response, potentially initiated in GALT, the study of these structures under such conditions may help in the treatment of these diseases. Finally, the field of intestinal mucosal immunology is dominated by research in mice whose intestine differs anatomically, functionally and immunologically from that in humans, making it important to recapitulate the findings in the human setting. **Manuscript I and II** performs such translational studies, with the focus on GALT and their general immune cell composition, structure and role in IgA generation.

2. Chapter 2: Intestinal stromal cells

2.1 Stromal cells

The term "stroma" or "stromal cells" (SCs) in tissues refers to cells that support the functional parts of the organ, but do not themselves play a direct role in these functions[83]. In the literature, the term intestinal SCs (iSCs) has been used to refer to mesenchymal derived cells such as fibroblasts (FBs), smooth muscle cells (SMCs) and pericytes[83–85], but has also included blood vessel and lymphatic endothelial cells, and even glia and neurons[86,87]. Unless otherwise stated, here I will refer to iSCs as all $CD45^-$ (non-hematopoietic) $EpCAM^-$ (non-epithelial) cells of unknown/unspecified/mixed origin in the intestine and will mostly discuss those in the LP. Although I will cover all known SCs of the intestinal LP, the main focus will be on intestinal mesenchymal cells (iMCs), defined as $CD45^-EpCAM^-$ non-endothelial ($CD31^-$), non-glial, and non-neuronal cells and comprising FBs, myofibroblasts (MFBs), pericytes, SMCs, and mesenchymal stem cells.

Historically SCs have been thought of as passive bystanders in immune responses, simply providing the framework for professional immune cells to operate. However, this concept is changing and SCs are now known to play direct roles both in inflammation and in immune homeostasis in organs such as the intestine. Such discoveries have prompted a general increase in SC research, with substantial interest particularly focusing on the iSCs of the LP. As well as providing structural support, these cells deliver signals for epithelial proliferation and differentiation, and play crucial roles in tissue repair and wound healing[88,89]. They are also important in the pathology of inflammation and colorectal cancer where they can both support and inhibit tumor-growth, induce angiogenesis, and may even control metastasis[90–92]. iSCs also play a role in the induction of fibrosis by secretion of extra cellular matrix (ECM) proteins[93–95]. In addition, recent research is beginning to reveal several immunological functions of iSCs under steady state conditions, including microbial sensing, leukocyte recruitment and antigen presentation[83,96]. However, due to the lack of good markers to distinguish and locate distinct iSC subsets, specific functions can often not be assigned to any particular subset, limiting our understanding of the immunobiology of these cells[83,96]. Therefore an important aspect of my project was to identify, assign protein markers, and locate individual iSC subsets, which is the main subject of **Manuscript III**.

2.2 Heterogeneity of intestinal lamina propria stromal cells

Recent publications have started to describe extensive heterogeneity within the iSC compartment of the intestinal LP, although the functions of iSC subgroups remain largely unknown[83,84]. Most previous work has relied on electron microscopic, histological, and immunohistochemical approaches[84], and a lack of good iMC-specific transgenic mouse models has also hampered further progress in the field[83,84]. It is also important to realize that large parts of human iSC research have relied on *in vitro* culture systems that are known to affect SC function and phenotype[97,98]. Furthermore, the biopsy material that has formed the basis of most previous research was likely to contain iSCs from the SM and GALT as well as LP, resulting in analysis of heterogeneous pools of iSCs of unknown anatomical origin. Importantly, the intricately associated SCs of GALT consist of several subsets each with important immunological roles (**Box 3**), making it of utmost importance to distinguish between these and true LP resident SCs. Although many of these subsets can be distinguished from other iSCs by their expression of CD157 in mice[99–101], no complementary surface marker yet exists for human GALT/LN stroma.

In the following paragraphs I will provide a background to the various iSC subsets known to be present within the intestinal LP at the start of my project, as well as their suggested location(s) and phenotype (**Figure 4 and 5**). Of note, *Kinchen et al.*[102], similar to our approach in **Manuscript III**, recently assessed iSC heterogeneity using single cell sequencing. These findings will not be discussed here but in **Manuscript III** in the context of our findings.

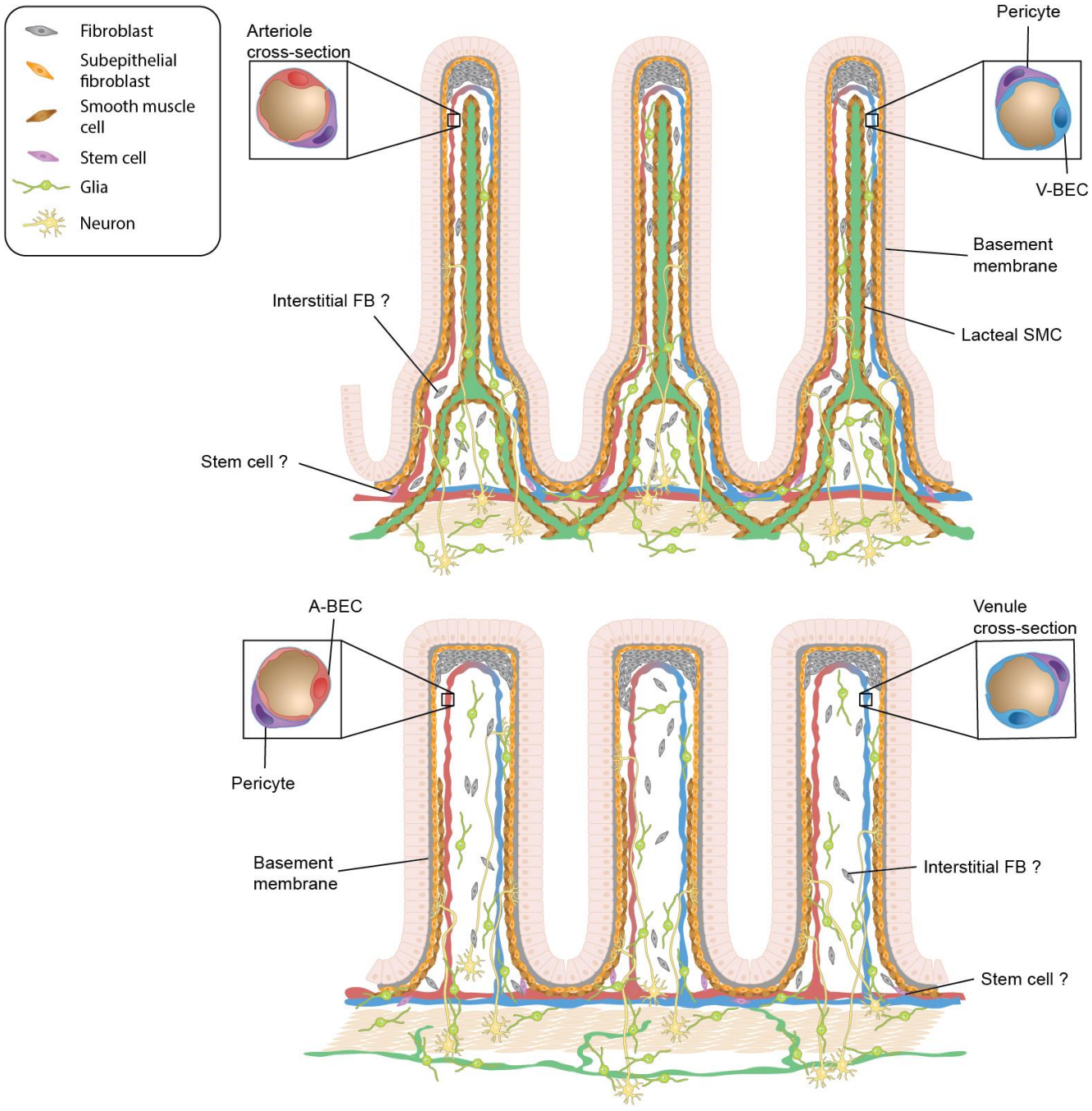

Figure 4 The human intestinal stromal cell compartment.

The stromal cells of the lamina propria (LP) are found in several distinct niches. Subepithelial fibroblasts (FBs) make up a single layer of cells situated in close association with the basement membrane (grey) underlying the epithelium. A single layer of smooth muscle cells (SMCs) is found as a "second layer" beneath the subepithelial FBs that does not quite reach the top of the colonic crypts or small intestinal villi. Instead the top of colonic crypts and small intestinal villi are populated by a dense cluster of FBs. The central area of the LP is populated by glial cells and presumed interstitial FBs, the former supporting neurons situated in the crypt, in the *muscularis mucosa*, or deeper tissues. Mesenchymal precursors may be present in the lower crypt area. The vascular niche (enlarged inserts) contains both arteriolar and venular blood vessel endothelial cells (A-BECs and V-BECs, respectively) surrounded by pericytes, whereas the lymphatic niche contains lymphatic endothelial cells (not shown) and lymphatic-associated SMCs ("lacteal SMCs") in the small intestine.

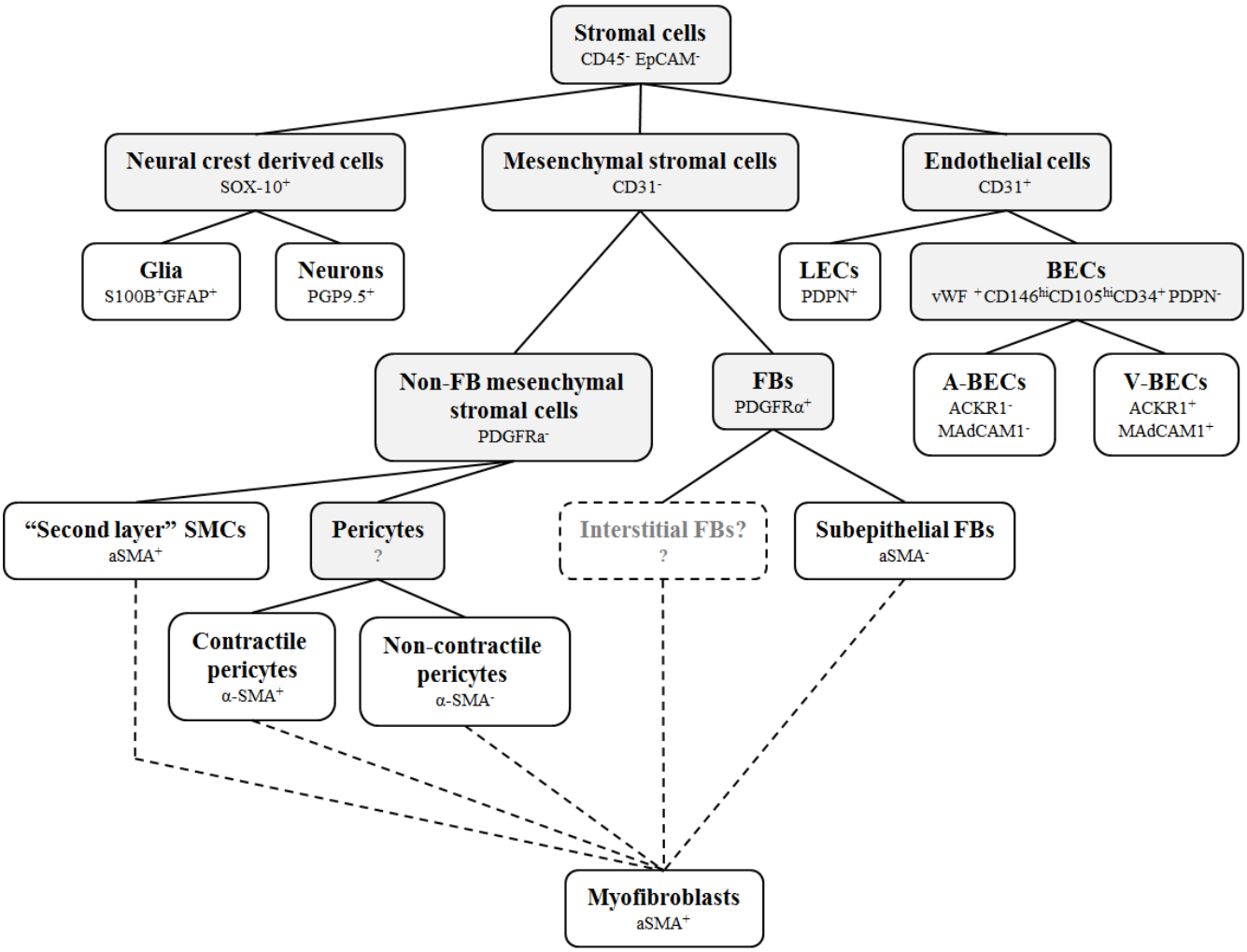

Figure 5. The human intestinal stromal cells and their marker expression.

Overview of the different populations of CD45-EpCAM- stromal cells (SCs) found in human intestine, showing independent lineages (grey boxes) and their subsets (white boxes), together with their expression of specific markers. Intestinal lamina propria (LP) SCs are found in three main linages, the neural crest-derived linage, the mesenchymal stromal cell linage, and the endothelial cell linage. These branch off into further subsets as shown in the figure. Interstitial cells outlined with a dotted line have yet to be described in human LP, whereas myofibroblasts are thought to derive from one of the other mesenchymal SC populations, although the exact origin remains to be determined; abbreviations: FB = fibroblast, BECs = blood vessel endothelial cells, A-BECs = arteriolar BECs, V-BECs = venular BECs, LECs = lymphatic endothelial cells, SMCs = smooth muscle cells.

2.2.1 Subepithelial fibroblasts, myofibroblasts and smooth muscle cells

The main iSC population of the intestinal LP consists of the spindle-shaped FBs found in all connective tissues. In the intestine, FBs are found as either subepithelial FBs, contractile subepithelial MFBs that express α smooth muscle actin (αSMA) or as isolated FBs within the central part of the connective tissue (interstitial area) of the LP[84,103,104]. The subepithelial FBs/MFBs form a continuous layer and change shape from a flattened phenotype in the crypts to a more stellate shape towards the tip of the villus. FB/MFB numbers are higher in the crypts and decrease towards the tip of crypts/villi, leaving gaps that allow leukocytes to probe into the epithelial layer[103]. The FBs/MFBs have been ascribed many roles, including production of the basement membrane that provides epithelial support, wound healing, cancer support and in regulating immune responses[84]. Upon activation by a mechanical insult or through stimulation with e.g. chemokines and cytokines, resting fibroblasts secrete large amounts of ECM proteins, ECM-remodeling proteins, cytokines, chemokines, and growth factors[93]. During resolution of the damage, activated FBs de-differentiate to the quiescent state or undergo apoptosis, whereas a failure of resolution may lead to chronic activation of the FBs eventually resulting in fibrosis[92,94].

The relationship between subepithelial FBs and MFBs is controversial, with MFBs often simply being classified by the ubiquitously expressed marker αSMA[93,96]. A stricter definition of MFBs using ultrastructural features put forward by *Eyden et al.* questioned the presence of true MFBs in the LP under homeostatic

Box 3: Stromal cells of secondary lymphoid organs

Several subsets of SCs are recognized in secondary lymphoid organs such as the GALT and LNs. These include several types of endothelia, FRCs of the T cell zone, FDC associated with GC B cells, and marginal zone reticular cells (MRCs) of the periphery, as well as less known subsets of blood-vessel associated pericytes and adventitial cells[99,100]. Several unique functions have been associated with each subset. FRCs are known to form the conduit system, provide survival factors such as IL-7, and position T cells within the T cell areas by their expression of CCL19/21[27,100]. They also take part in peripheral tolerance, either through expression of self-antigens or suppressive factors[230] and can play an active role in activating or restraining responses to infections[231,232]. FDCs are invaluable for the humoral response in that they provide CXCL13 to position B cells and T_{fh} cells within the GC, provide B cell survival factors such as IL-6 and BAFF, and continuously present antigen bound to their complement receptors[233]. MRCs are poorly described, although they provide structural support and take part in conduit formation, and have been suggested to play roles in T cell antigen capture and barrier defense[234,235]. Blood vessel and lymphatic vessel endothelial cells control lymphocyte and fluid-borne antigen influx[235]. CD34+ blood vessel adventitial cells have been shown to contain SC precursors[100], but whether they or some of the other minor populations have more direct immunological roles remains to be determined.

conditions[104,105]. They found that the subepithelial iSC compartment contained FBs and SMCs but not MFBs[104]. This is consistent with the later finding that αSMA$^-$ subepithelial FBs staining for platelet-derived growth factor receptor (PDGFR)α were the sole constituents of the subepithelial niche, surrounded by αSMA$^+$ cells in the lower two-thirds of colonic crypts[106]. Moreover, electron microscopic studies have confirmed the presence of more than one cell layer in crypts and lower part of villi[103,104,107]. Thus, it has been suggested that the subepithelial niche from the mid villus upwards consists of a single layer of PDGFRα$^+$αSMA$^-$ subepithelial FBs, but in the crypt and lower region of the villi this is surrounded by a second layer of PDGFRα$^-$αSMA$^+$ SMCs. However this idea remains controversial[103–106] and assigning specific functions to the two putative layers is difficult.

Subepithelial FBs may be even more heterogeneous, as a population of podoplanin$^+$ (PDPN - gp38)$^+$ CD34$^+$αSMA$^-$ subepithelial FBs is present in the lower LP around the colonic and small intestinal crypts in mice, whereas subepithelial FBs further up are PDPN$^+$CD34$^-$αSMA$^{+/-}$ [108]. As their position suggests, the PDPN$^+$CD34$^+$ cells have been shown to express factors that support the epithelial stem cell niche, a property not shared by their PDPN$^+$CD34$^-$ counterparts[108]. A three-dimensional electron microscopic study of rat SI has also revealed the presence of four types of subepithelial iMCs based on their ultrastructural appearance; two of these are found mainly in the area around the crypts, one in both the crypts and villus, and one solely in the villus[107]. As human subepithelial iMCs express epithelial-supportive genes dependent on their position in the colonic crypt[109], it seems likely that there may be analogous subsets of human subepithelial iMCs. However, as human LP subepithelial iMCs rarely express CD34[110], other markers are needed if such subsets are to be identified accurately in man.

2.2.2 Interstitial fibroblasts

Presumed interstitial FBs were shown to exist in the intestinal LP by early electron microscopic studies. They were described as stellate-shaped cells in the central part of the LP and were often connected to either vascular cells, axons or subepithelial FBs through finger-like projections[103,111]. However, these putative FBs may at least in part have been confused with glial cells, which are also stellate shaped, connect with similar cells, and occupy the same niche[112]. Moreover, as no consensus has been reached on how to properly define an interstitial FB in the intestinal LP[104], their presence, functions and marker expression remains unknown. Therefor identifying markers allowing for their identification among the other SCs is needed to further our understanding of these cells.

2.2.3 Blood vessel endothelial cells

Another important SC niche is the vasculature, which in the LP comprises microvessels (arterioles, capillaries, and venules), which line the subepithelial compartment, and contains blood vessel endothelial cells (BECs) and closely associated pericytes[110]. BECs make up the wall of blood vessels (endothelium), allowing transfer of nutrients and oxygen into tissues from the bloodstream, with metabolites and waste passing in the opposite direction. The endothelium also interacts with immune cells and plays an active role in their recruitment via the expression of chemokines and adhesion molecules enabling the extravasation of leukocytes through adhesion, rolling, and diapedesis[113,114]. BECs are also thought to play other roles in immunity, as they are able to sense microbiota through TLRs and may act as antigen presenting cells through expression of MHC molecules[113–115].

BECs are generally characterized as $CD31^{+}PDPN^{-}$ [98,113,114,116], and in the human LP are von Willebrand factor $(vWF)^{hi}CD31^{hi}$ $CD146^{hi}CD34^{hi}CD105^{hi}$, with vWF being specific for BECs[117]. This study also noted endothelial heterogeneity, as venule BECs specifically expressed CD234 (also known as atypical chemokine receptor 1 - ACKR1/DARC) and a subset present in areas rich in infiltrating leukocytes, expressed MECA-79, the PNAd ligand for CD62L[117]. Venule BECs of the intestinal LP are also known to express mucosal addressin cell adhesion molecule 1 (MAdCAM-1), important for gut specific recruitment of lymphocytes via its binding to ITGα4β7[118].

2.2.4 Pericytes

Pericytes are supportive cells surrounding and associating closely with the endothelia. They play important roles in angiogenesis and vascular homeostasis, and their loss/reduction during disease may lead to increased vascular leakage and exacerbation of pathology[110,119–121]. Pericytes may also sense microbiota through TLRs[119], and in the LP are thought to act as a tissue stem cell for iMCs[110,119]. Pericytes are associated with all blood vessels of the intestinal LP, but have yet to be characterized phenotypically. No specific marker exists for pericytes, although they can usually be distinguished from other SCs by their expression of at least two of PDGFRβ, NG2, CD13, desmin or αSMA, together with an endothelial marker such as CD31[122]. Other markers that may be present on pericytes include RGS5, CD105 and CD146, although capillary pericytes may lack αSMA[119,122].

2.2.5 Adventitial cells

Adventitial cells comprise a third layer of cells situated around the pericyte/vascular SMC layer of larger arteries and veins, where they are thought to contain tissue-specific mesenchymal stem cells[110,123,124], potentially capable

of reconstituting the SC compartment of an entire tissue as has been shown for mouse LNs[100]. Adventitial cells are $CD34^{+}CD146^{-}$ and constitute the main iSC population in the human intestinal submucosa[110], but as larger vessels are absent from the intestinal LP[110], these cells will not be considered further here.

2.2.6 Lymphatic stromal cells

Lymphatics are blind-ended vessels that play important roles in the intestine, draining the tissue of absorbed nutrients and water, as well as activated lymphocytes and the dendritic cells needed for inducing adaptive immune responses. Lymphatic endothelial cells (LECs) line lymphatic vessels and can be identified by their expression of CD31, PDPN, and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), the latter two distinguishing them from BECs[125,126]. LECs produce chemokines (e.g. CCL21) involved in recruiting lymphocytes and DCs into the vessels[127,128]. They may also sense pathogens through the expression of TLR4[115] and can scavenge inflammatory chemokines via expression of the decoy chemokine receptor ACKR2, reported to be involved in resolution of inflammation[127,128].

Although lymphatic vessels are found in both SI and LI, the lymphatic network within the SI LP is extensive, while the colonic LP is essentially devoid of lymphatic vessels in steady state[4,5,84]. However, new vessels may form during inflammation or neoplasia[84,129], with lymphangiogenesis being a feature of both ulcerative colitis (UC) and Crohn's disease (CD)[130]. The SI LP also contains special lymphatic vessels termed lacteals that extend centrally through the villous core and are key to the systemic distribution of absorbed nutrients[131]. The lacteals are unusual when compared with lymphatic capillaries in other tissues, as they are surrounded by a layer of αSMA^{+} cells that may be a unique population of SMC[131]. Interestingly, iSCs associated with the lacteal may be the only iSCs in the SI to undergo detectable proliferation under steady state conditions[132,133].

2.2.7 Neural crest derived cells

The enteric nervous system is second only to the brain in terms of size, and is capable of influencing everything from gut motility to blood flow, and even immune and inflammatory processes. It is organized into nerve plexuses containing both neurons and supportive glial cells, found in all layers of the gut wall, including the mucosa[86]. Enteric glial cells (EGCs) are the main supportive cells for enteric neurons, but may also have direct effects on gastrointestinal motility and epithelial barrier integrity[134], potentially playing a protective role in CD[86,134,135]. EGCs can sense pathogens via TLR2[136] and TLR4[137], produce and respond to cytokines, and may act as antigen presenting cells by expressing MHC molecules[134,135]. Enteric neurons are known to express TLRs (3, 4, and 7) in

both mice and humans[138], may be regulated by microbial metabolites and toxins, and may react to various factors secreted by other cells of the gut wall, directly or through their axons[139].

EGCs are located mainly in the interstitial areas around crypts, but can also be found closely associated with the epithelium or in contact with vessels and other LP cells[112]. Although these locational differences might suggest heterogeneity in the glial population of the mucosa, a morphological study concluded that all mucosal EGCs in mice were of the same type, whereas those in the other parts of the gut wall were more heterogeneous[140]. EGCs in the mucosa have a stellate morphology, with approximately four long protrusions, and their principal phenotypic markers are GFAP and s100β, both of which are considered to be EGC-specific[86,134]. EGCs and neurons are also the only cells in the intestine to express the transcription factor SOX10[140].

Enteric neurons are seen in large clusters in the plexuses of the SM and in the *muscularis externa* but are rarely observed in the LP. When present, they are generally found individually or as small groups of large round cells situated close to or within the MM, with long axons and specifically expressing PGP9.5[86,106,141,142].

2.3 Intestinal mesenchymal cell precursors

While multiple iMC subsets are found throughout the intestinal LP, their turnover rate and whether their maintenance requires input from a common multipotent precursor or by subset specific precursors in the steady state remains unclear. This is in part due to the lack of specific markers needed for carrying out lineage tracing, as well as their low turnover rate[83]. It has been suggested that pericytes may be a source of tissue-specific multipotent mesenchymal SC precursors. However, the evidence for this is still indirect, relying mostly on *in vitro* studies or non-specific Cre-recombinase driven models, risking contamination with other iMC-subsets and making it hard to draw firm conclusions[122]. More firm evidence has been provided for the vascular associated $CD34^+$ adventitial cells[110,123,124], but unlike in mice such cells are usually not detected in histological sections of intestinal LP in humans[110]. Recently a tissue-specific gremlin (GREM) 1 expressing precursor of subepithelial iMCs was identified in adult mice. This study used *Grem1*-$CreER^T$ mice to perform lineage tracing studies, where fluorescent labeling was turned on by an inducible Cre in *Grem1* expressing cells, enabling these cells to be tracked over time. After inducing expression during early adulthood, *Grem1*-expressing cells were found initially in the region of the intestinal LP between the crypt and the villus known as the isthmus; these then gave rise to the entire subepithelial iMC niche within a year[143]. Interestingly, in humans the only known *GREM1/2* expressing cells are αSMA^+ MFB/SMCs of the lower crypts and MM of the colon[109], suggesting that an equivalent tissue stem cell might be present in these locations in man. It has also been suggested that circulating bone marrow mesenchymal stem cells may act as precursors for iMCs[83,84], but whether this occurs during homeostasis is still to be shown.

Although the precursors of steady state iMCs remain largely unknown, several candidates have been proposed to replenish iMCs under stress conditions such as wound healing and inflammation. These include: 1) resident FBs, 2) resident SMCs, 3) bone marrow-derived mesenchymal stem cells, 4) $CD45^+$ circulating precursor cells known as "fibrocytes" or 5) epithelial cells undergoing damage-induced epithelial-mesenchymal transition[144–146]. A role for bone marrow-derived precursors in both mice and humans has been demonstrated by the presence of Y-chromosome expressing iSCs in sections of the mucosa from irradiated female recipients of male bone marrow[147,148]. However, direct evidence is yet to be provided for any of the other candidates[85,144–146].

2.4 Mesenchymal stromal cells in the support of epithelial cells

The epithelium of the SI and LI consists of a single layer of epithelial cells moving continuously from the base of the crypt to the villus/crypt top, from where they are shed. This process takes 2-5 days in humans and is dependent on constant replacement by self-renewing stem cells found near the bottom of the crypt. As a result, epithelial cells at different stages of differentiation are found in distinct anatomical regions along the crypt-villus axis. Epithelial cells formed in the stem cell zone continue to divide as they move up through the transit amplifying zone in the mid-crypt, before differentiating progressively as they move on to the top of the villus/crypt. As well as conventional absorptive epithelial cells (enterocytes), the stem cells give rise to Paneth cells, goblet cells and neuroendocrine cells[149–151] (**Figure 6**).

iSCs are found underlying the epithelium in each region of the crypt-villus unit, where in concert with epithelial cells, they form the basement membrane between the epithelium and LP. Current evidence indicates that distinct populations of iSCs control the division and differentiation of epithelial cells at different stages of development. They do this by producing factors such as wingless-related integration sites (WNTs) and bone morphogenic proteins (BMPs) (**Box 4**), whose concentrations vary along the crypt-villus axis[84,152,153], hence creating gradients[84,154] (**Figure 6**). In the small intestine, WNT proteins 3, 6, and 9b are produced in the stem cell niche by Paneth cells and act via the canonical Wnt signaling pathway, whereas iSCs produce WNTs 2b, 4, 5a, and 5b that act mainly via the non-canonical Wnt pathway[84] (**Box 4**). As there are no Paneth cells in the colon and no other epithelial cell subset has been found to express WNTs[133], iSCs may play the major role in epithelial cell renewal here. iSCs also express WNT-antagonists (Dickkops and secreted frizzled-related proteins) and factors stimulating WNT-signaling (e.g. R-spondins) at the base and top of colonic crypts, respectively, highlighting their crucial roles in both maintaining and restricting the epithelial stem cell niche[84,109,155]. Importantly, although the main functions of WNTs are thought to be restricted to the epithelial stem cell niche (**Box 4**), some WNTs are

expressed preferentially at the tip of villi/crypts and not around the base of the crypts[109,155], although the reason for this remains unknown.

Box 4: Stromal cell derived epithelial support factors and pathways

One of the most important pathways involved in the interaction between iMCs and epithelial cells is the WNT-pathway, a signaling pathway that can be divided into a canonical, β-catenin-dependent and a non-canonical, β-catenin-independent pathway[236]. Whereas the exact details of the non-canonical pathway remain to be elucidated, the canonical pathway is initiated by WNT binding and forming a complex with the two receptors Frizzled and Lipoprotein receptor-related protein 5/6, resulting in signaling events that prevent β-catenin breakdown and signaling[237].

WNT signaling is crucial for epithelial stem cell turnover and epithelial proliferation in general, with increased signaling and proliferation seen in epithelial cancers, whereas antagonizing WNT signaling leads to decreased proliferation and loss of crypts[236]. It is also important for cell-positioning by influencing the erythropoietin-producing human hepatocellular receptor (Eph)/Eph family receptor interacting proteins receptor/ligand pairs[238], and may itself be influenced by both agonists and antagonists[237].

Another major pathway involved in epithelial support is the BMP pathway, usually thought of as being antagonistic to WNT signaling, as it may reduce nuclear translocation of β-catenin in a phosphatase and tensin homolog dependent manner[84]. BMP signaling acts through a receptor complex of either one of three type I BMP-receptors (ALK2, BMPR1A, and BMPR1B) and the type II receptor BMPRII, and depending on the combination, this may lead to different kinds of responses to BMPs[239]. Such signaling induces differentiation and reduced proliferation, which is opposed in the epithelial stem cell niche by antagonists such as GREM1/2[238]. BMP signaling may be driven by Hedgehog (Hh) secreted by differentiated epithelial cells[84,164]. Here Hh binds the receptor Smoothened on subepithelial iMCs and induces nuclear translocation of GLI1, GLI2, and GLI3, leading to expression of BMPs, which in turn drives further differentiation of epithelial cells[84,164].

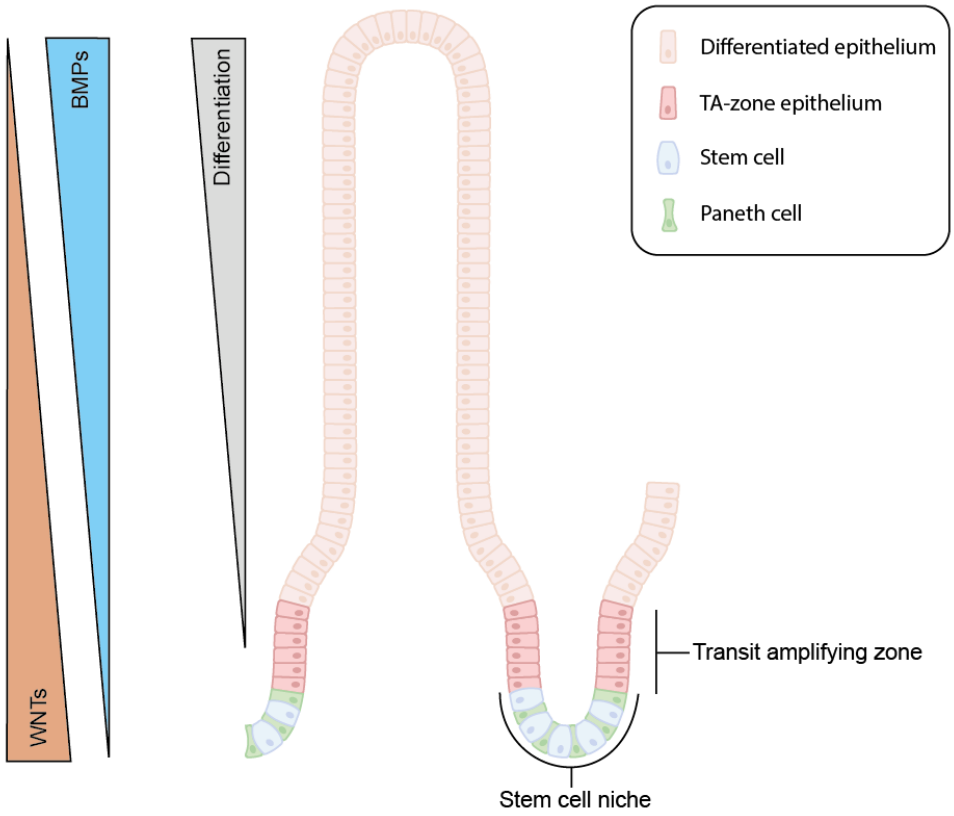

Figure 6 The human intestinal epithelial niche.

The epithelial niche of the human intestine is divided into distinct regions. The stem cells and Paneth cells occupy the stem cell niche at the bottom of the crypts, the former giving rise to transit amplifying cells that gradually move upwards as they divide. The transit amplifying cells in turn differentiate into mature, non-dividing epithelial cells that continue to differentiate as they move towards the tip of the crypt/villus. WNTs and BMPs form opposite gradients along the crypt-villus axis, with WNTs dominating at the bottom of crypts, and BMPs higher up in the crypt/villus.

2.4.1 Mesenchymal stromal cell subsets associated with epithelial support

As discussed above, the factors that iMCs produce to support epithelial function are known to be expressed in different anatomical locations in the intestinal mucosa. For example, fibronectin^{+}αSMA^{+} crypt iMCs and SMCs in the MM are known to produce BMP-antagonists like Gremlin 1/2 that inhibits BMP signaling in the stem cell niche[84,109]; conversely, αSMA$^{+/-}$ subepithelial FBs/MFBs/SMCs further up the crypt produce BMPs[84]. However, it has generally been difficult to pinpoint the exact phenotype and nature of the iMCs involved in epithelial cell support, due to a lack of knowledge of iSC heterogeneity and subset-specific markers/genes. Nevertheless, recent studies in mice are now beginning to address this issue. *FOXL1* is a gene expressed specifically by subepithelial iMCs and ablation of *FOXL1*$^{+}$ SCs severely impacts on crypt morphology in the colon and leads to reduced length of villi in the SI; selective deletion of functional WNTs in these cells leads to loss of epithelial proliferation and stem cells[132,156]. It has also been shown that the *FOXL1*$^{+}$ cells express mRNA for both repressors (e.g. BMPs) and activators (e.g. WNTs) of epithelial stem cell renewal, depending on their anatomical position[132,156], suggesting functional heterogeneity within this population. Others have found similar epithelial supporting iSCs located in the same subepithelial location using different markers. For instance, removing *WNT* expression from *GLI1*-expressing SCs results in the same loss of stem cells and proliferation in the colon as reported for deleting *FOXL1*$^{+}$ cells[133]. Single cell sequencing of *GLI1*-expressing cells showed that this population in fact contains eight different subsets, only some of which express the markers associated with location near the crypt base and they also express varying levels of *WNT* genes. Importantly however, the two main *WNT*-producing subsets were associated with the crypt and expressed CD34 but not *FOXL1*, the latter found in other iSC subsets[133]. Thus, the FOXL1-expressing iSCs that support epithelial proliferation may be located outside the stem cell niche, or the GLI1-expressing stem cell niche iSCs may be derived from a *FOXL1*-expressing precursor. In other work, it has been shown that the population of PDPN^{+}CD34^{+} iSCs found in the lower region of colonic crypts are enriched for factors supporting the epithelial stem cell niche, whereas PDPN^{+}CD34^{-} iSCs are found primarily at the top of the crypts and express differentiation factors such as BMPs[108].

Together these studies reveal that the subepithelial iSC compartment is essential for the maintenance and development of epithelial cells, with different subsets playing different roles depending on their anatomical position and factor secretion. In mice, the subepithelial iSCs found at the base of the crypts are presumably PDGFRα^{+}PDPN^{+}CD34^{+}, whereas those at the top of the colonic crypts or in the SI villus are PDGFRα^{+}PDPN^{+}CD34^{-}. Whether these phenotypes are similar in the human intestine remains to be seen.

2.4.2 Mesenchymal stromal cells and epithelial support during inflammation

iMCs play important roles in protecting the epithelial barrier during inflammation by supplying supportive factors. One candidate for providing such protection may be iMC-produced prostaglandin E, which is known to stimulate epithelial growth, inhibit epithelial apoptosis during insults and may induce canonical WNT-signaling. Production of prostaglandins by iMCs has been shown to maintain epithelial proliferation, but may also be a mediator of IBD depending on the dosage[157,158], with prostaglandin endoperoxide synthase-2 expressing iMCs potentially relocating towards the crypt under inflammatory conditions[159]. The protective effect of iMC-derived prostaglandins has been shown to be dependent on the IBD-associated gene *MAP3K8*, which is down-regulated in the intestinal mucosa of CD patients[160], suggesting that this pathway could be defective in IBD.

Tenascin-C is another protein capable of supporting epithelial barrier regeneration. It is upregulated during human IBD and Dextran sodium sulfate (DSS) colitis in mice[161], and its ablation exacerbates DSS-colitis[162]. in rats it is produced primarily by PDGFRα$^+$ subepithelial iMCs, with its production being induced by Platelet-derived growth factor (PDGF)-BB (the ligand for PDGFRβ) and TGFβ[162]. It is expressed by especially by SCs of SI villi in mice and at the top of colonic crypts in humans[161,163], suggesting that it exerts its protective abilities in these regions.

Hedgehog (Hh)-signaling from epithelial cells to underlying GLI1-expressing iMCs is important for epithelial differentiation and regulation of the epithelial stem cell niche under steady state conditions[164], but it is also important during inflammation and repair. Expression of *indian hedgehog* (*Ihh*) by mouse epithelial cells suppresses production of the chemokine CXCL12 by subepithelial PDGFRa$^+$Desmin$^+$αSMA$^+$ iMCs during homeostasis. When *Ihh* is ablated from epithelial cells, this leads to increased sensitivity to DSS colitis, possibly reflecting recruitment of inflammatory leukocytes due to increased production of CXCL12 by iMCs[165]. These results support other findings that CXCL12 is upregulated in both DSS colitis and IBD, and that antagonizing its receptor CXCR4 reduces inflammation in mouse models of colitis with accompanying downregulation of Hh target genes[165]. Hh-signaling induces IL-10 production by subepithelial iMCs and can protect against DSS colitis in mice[166], and has been shown to down-regulate a range of chemokines and cytokines in primary cultured human iSCs[167]. The number of *CD34*-expressing *GLI1*$^+$ iSCs has also been shown to increase after recovery from acute DSS colitis in mice[133], suggesting increased Hh-signaling in the iSC-compartment in the recovery phase, as GLI1 is a target gene of Hh-signaling[84,164]. Together these findings point towards a prominent role for an epithelial-iMC Hh signaling-axis in inhibiting inflammation. Finally, iMCs may also have a protective effect during inflammation by preferentially inducing the differentiation of goblet cells rather than absorptive epithelial cells, strengthening the mucus barrier, via the production of IL-33[168] or IL-24[169].

2.5 Modulation of immune responses by mesenchymal stromal cells

Studies mostly on cultured iSCs have revealed that they express and respond to various immune factors, support immune cell subsets, and directly or indirectly direct innate and adaptive immune responses. Although this is not addressed in detail in **Manuscript III**, the following pages will provide an overview of the immune functions associated with iSCs and hence suggest which avenues to pursue in future studies.

2.5.1 Microbial sensing

Sensing and responding to microbes is one of the main functions of the innate immune system and provides an important, early line of defense, as well as being important for initiating adaptive immune responses. Innate immune sensing relies on pathogen recognition receptors (PRRs) including surface bound and intracellular toll-like receptors (TLRs), intracellular nod-like receptors and other intracellular PRRs. Triggering of these receptors by their microbial ligands induces cascades of inflammatory mediators which recruit and activate immune cells. Cultured human iSCs have been shown to express *TLR1-9* and nucleotide-binding oligomerization domain-containing protein (*NOD)-1,2* mRNA, but only TLR2, TLR4 and NOD2 have been confirmed at the protein level[170,171], with their levels being lower than on epithelial cells[172]. Cultured iSCs also respond to TLR2, TLR4, TLR5 and NOD-2 ligands by producing cytokines such as ILs-1α/β,6,8,18, and 33[170,171]. Interestingly, besides their role in early warning and as mediators of inflammation, sensing of microbiota by iSCs after a breach in the epithelial barrier has also been suggested to induce expression of epithelial support-factors by the iSCs, enhancing barrier repair in epithelial cells[89]. iSCs may also respond to the microbiota-derived short chain fatty acids that are produced after metabolism of complex carbohydrates by intestinal anaerobes, and have been shown to attenuate IL-1β- and tumor-necrosis factor (TNF)α-induced pro-fibrotic matrix metalloproteinase expression in cultured human iSCs[173].

2.5.2 Recruitment and maintenance of immune cells

iSCs express a range of chemokines, cytokines and growth factors important for the recruitment and function of immune cells. One of the most characteristic products of iSC is IL-6, whose production is induced by pro-inflammatory cytokines such as IL1α, IL1β, and TNFα, as well as by PRR ligands[174]. iSC-derived IL-6 has been shown to support PCs in the LP[51,175] and can prevent apoptosis in mast cells *in vitro*[176], and coupled with the ability to express IL-15[177], M-CSF and GM-CSF[174,178], this suggests a prominent role for iSCs in forming leukocyte niches in the mucosa. iSC can also prevent T cell apoptosis through secretion of IL-10[179], and can recruit leukocytes via

production of the chemokines CXCL12[165], CCL2[180,181] (myeloid cells), CXCL1[182] (neutrophils) and CCL11 (eosinophils)[183]. Loss or reduction of pericytes or a relaxation (reduced contraction) of these cells increases vascular permeability and so may also impact on leukocyte recruitment[119].

2.5.3 Antigen presentation and tolerance induction

A small fraction of human colonic $CD90^{+}\alpha SMA^{+}$ iMCs expresses MHCII and the co-stimulatory molecules CD80/86, and these cells have been shown to drive allogeneic $CD4^{+}$ T cell proliferation *in vitro* in a MHCII dependent manner[184]. However, iSCs are not professional antigen presenting cells and express relatively low levels of CD80/86, leading to the hypothesis that their main role as antigen presenting cells under steady state conditions may be to inhibit T cell activation and induce tolerance[84,185]. Indeed, cultured human iSCs can induce the generation of $CD4^{+}$ T_{reg} from naïve allogeneic T cells via the production of prostaglandin E_2[129], and their ability to produce IL-10 could also be relevant here[84]. However, direct induction of tolerance in naïve T cells is only relevant for SCs of SLOs, as no naïve T cells are found in the LP.

Although thought to be MFBs of the LP[184] it is possible that the identified $MHCII^{+}$ cells represent iMCs from contaminating GALT, as iMCs of SLOs express MHCII at least in mice[186]. Furthermore, $CD90^{+}$ iSC have been shown to express programmed death ligand 1 (PD-L1) and PD-L2, enabling them to inhibit $CD4^{+}$ T cell activation[187]. As the expression of PD-L1 by iSCs can be upregulated by stimulation via TLR 1,2,4 or 5 and this enhances their suppressive effect on allogenic effector T cells, these findings suggest that iSCs may be capable of regulating inflammation[188]. iSCs may also inhibit T cell immune responses by indirect mechanisms. For instance, unknown factors derived from iSCs can prevent the activation of DCs *in vitro*[185], while mouse $PDPN^{+}CD31^{-}$ iSCs can induce the expression of the vitamin A metabolite RA by intestinal DCs *in vitro*, a property associated with the ability of DCs to induce tolerance in T cells[189]. However, it should be noted that these RA-inducing iSCs might not be iMCs, as cells with this phenotype are found in an interstitial location that is occupied by stellate shaped iSCs with RA-containing vesicles[111,189] that actually resemble EGCs[112]. This is an issue that needs to be clarified, but together it seems that iSCs may indeed be tolerogenic under homeostatic conditions[84]. Importantly, this property may be dysregulated during IBD, where the ability of iSCs to produce IL-10 and to induce T_{reg} cells is lost[129].

2.5.4 Inflammation and inflammatory bowel diseases

iSCs may also drive inflammation and adaptive immune responses and may even be responsible for the chronicity of IBD via the phenomenon known as "innate immune memory"[83], leading to a changed response to subsequent insults[190]. One way for iSCs to support active immunity is through their ability to respond to pro-inflammatory cytokines or PRR ligands by producing pro-inflammatory cytokines such as IL-1, IL-6 and IL-8, upregulating adhesion molecules such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM)[174,191], and producing extracellular matrix (ECM) proteins and ECM remodeling matrix metalloproteinases (MMPs)[85]. In addition, although IL-33 production is restricted to blood vessel associated iSCs in healthy human intestine, it can be produced by PDGFRβ$^+$ and αSMA$^+$ iSCs in UC, potentially induced by TGFβ and TLR3-signaling[192] or TNFα[193]. Oncostatin M (OSM) is a further mediator which may activate iSCs during inflammation. The expression of OSM and its receptor OSMR is increased in both CD and UC patients, with OSMR expression correlating closely with disease severity[194]. The OSMR is expressed almost exclusively by CD45$^-$EpCAM$^-$CD31$^-$ iSCs and stimulation of iSCs with OSM leads to production of pro-inflammatory molecules like IL-6, ICAM-1 and chemokines. Furthermore, blocking OSM significantly alleviated experimental colitis in a model of TNF-inhibitor resistant intestinal inflammation, and high OSM expression in patients prior to treatment was strongly associated with failure of anti-TNF-treatment[194]. Thus, iSCs could be an interesting target for the development of an effective treatment of these patients.

iSCs can also respond to cytokines released by T cells, suggesting that they may contribute to the effector phase of adaptive immune responses. Low levels of exogenous IFNγ induce MHCII expression and reduce αSMA expression in cultured human iSCs[184], whereas high levels reduce MHCII in cultured mesenchymal stem cells[84]. Pericytes can also express MHCII after stimulation with IFNγ *in vitro*[96], but whether this is also the case for LP pericytes remains unclear. Th-2-related IL-31 induces production of a range of chemokines, cytokines and MMPs by cultured human iSCs[195], whereas the Th-17 cytokines IL-17, especially IL-17A, and IL-22 upregulate IL-6, IL-8, LIF, MMP1, MMP3 and CCL2[168,178,196]. Interestingly, IL17A and IL-4 synergistically induce IL-6 mRNA in cultured human iSCs[89], and both Th2 and Th17 cytokines can have a pro-fibrotic effect on SCs[94], suggesting that iSCs can be increasingly activated, potentially leading to the induction of a pathological phenotype. Finally, as noted above, the ability of iSCs to produce IL-10 and to induce T_{reg} cells is lost in humans with IBD[129].

Mouse models have also suggested that iSCs may play a direct role in pathology during acute and chronic inflammation. TNF-overexpressing TNF$^{\Delta ARE}$ mice develop an ileitis similar to CD and the pathology is dependent on TNF-receptor-1 expressing SCs[197]. Specific overexpression of TNFα in epithelial cells also leads to local inflammation which is associated with upregulation of ICAM-1 and MMPs by CD90$^+$αSMA$^+$ iSCs, suggesting that epithelial-iSC interactions may drive pathology in chronic inflammation in the intestine[198]. Alterations of the

iSC compartment have also been identified during acute DSS colitis in mice, with activation of $PDPN^{+}CD34^{+}$ iSCs and upregulated expression of *VCAM1*, *ICAM1* and *lymphotoxin β-receptor*, as well as of growth factors, chemokines and pro-inflammatory cytokines[108]. As these mediators included *IL-7* and *CCL19*, it was suggested that at least some iSCs had acquired a phenotype resembling fibroblastic reticular cells of the GALT[108]. However, it should be noted that ILFs had not been removed from the tissues studied in these experiments and therefore it possible that the changes may have reflected an increase in GALT-iSCs expressing PDPN and CD34.

2.6 Summary and outlook – Chapter 2

The human intestinal LP contains a wide range of poorly characterized iSCs localizing to specific anatomical locations, from where they may have a range of functions important during homeostasis and disease. Such functions include the ability to respond to microbial products and cytokines/chemokines, recruiting leukocytes, supporting epithelia, and presenting antigens. However, ascribing these functions to specific subsets remains challenging, limiting our knowledge on the immunobiology of these cells. Various markers and uniquely expressed genes have been suggested to distinguish specific populations, but few have been verified, making it of paramount importance to identify such markers/genes and properly phenotype and localize the cells of the iSC compartment for the field to progress further. These issues where originally the main aims of my thesis and are tackled in **Manuscript III**, where we classify, phenotype and locate the various SC subsets, and provide transcriptional data suggesting specific functional roles for these subsets in human ileum and colon.

Aims of the thesis

This project is part of a bigger project termed "Regional Immune Modulation of Mucosal Inflammation" (RIMMI), the aims of which are to map the cells and pathways that regulate adaptive immune responses in different parts of the intestine during health and disease, with the long term goal of identifying novel targets for treating inflammatory bowel disease.

The overall aim of my project is to assess the heterogeneity of stromal cells along the length of intestine in health and disease. The secondary aim is to assess whether distinct stromal cell subsets play regionalized roles in innate and adaptive immune responses.

The thesis covers three manuscripts each of which had specific aims:

Manuscript I had the aim to identify and describe human GALT structures in terms of their overall structure and anatomy, their lymphocyte composition, and their contribution to site-specific IgA-responses.

Manuscript II had the specific aim to give a detailed description of a technique to identify and separate the GALT from LP along the length of the human intestine, as well as provide proof of principle that non T/B cells are also differentially distributed between these compartments and that the technique extends to tissue from patients with CD.

Manuscript III had the aim to give a detailed description of the complete iSC compartment in human ileum and colon, providing protein markers capable of separating individual SC subsets, and to outline potential roles for these in health and disease.

Summaries and discussions of manuscripts I-III

Manuscript I – Immune profiling of human gut associated lymphoid tissue identifies a role for isolated lymphoid follicles in regionalized adaptive immune responses

Summary

Here we developed a technique to identify and isolate human intestinal GALT from the LP, allowing us to distinguish between PP and M-ILF in the ileum and between SM-ILF and M-ILF in the LI. We then assessed how these structures were distributed in the ileum and along the length of the LI, before describing their structure and morphology, and demonstrating the presence of HEVs, lymphatics, and segregated B and T cell areas. The GALT was distinguished from LP by the absence of PCs, but it contained naïve T and B cells, together with GC B cells, all of which were lacking in LP. There were also significant differences between PPs and SM-ILFs, with PPs containing larger proportions of GC B cells and T_{EM} cells. We also performed CyTOF analysis of the T cell compartment of PPs, LP and colonic SM-ILFs, again showing substantial differences between GALT and LP, and highlighting significant heterogeneity in the effector T cell compartment. Finally, we performed IgA sequencing analysis of individual PP and SM-ILF follicles along with neighboring LP, finding that PP follicles and SM-ILFs are likely to seed ileal and colonic LP, respectively.

Discussion

One of the main findings of the manuscript was that there were substantial differences in the immune cell compartments of the intestinal LP and GALT; for example, PCs were restricted to the former and naïve T cells only found in the latter. Although previous studies would have predicted this observation, our finding that microscopic GALT structures are so numerous that they will inevitably contaminate normal "LP" tissue preparations will influence the way we interpret data derived from intestinal tissues not prepared using our new approach. Moreover, by providing novel ways of studying GALT directly and by showing that these tissues contain large numbers of CD4 T cells and naïve B cells, our study offers the opportunity to better understand the initiation of adaptive immune responses during intestinal infection and inflammation. This may eventually lead to new treatments.

It is important to realize that the tissues we examined were derived largely from cancer patients who have been noted previously to have higher amounts of colonic GALT[10] and altered tissue morphology even some

distance away from the tumor (>20 cm)[199]. We did compare colorectal cancer patients with non-cancer patients and detected no significant differences, but whether these patients can truly be considered healthy remains an open question, which we were unable to address due to sample-limitations. A further factor that could have influenced our results was the fact that our patients were also generally more than 60 years old and it is known that ageing affects the intestine by for example impairing nutritional absorption, immune cell function and microbial content[200]. The immune system as a whole undergoes profound changes during ageing, leading to a higher incidence of infection-related deaths and possibly contributing to other age-related diseases such as cancer and cardiovascular disease. The ageing immune system is also associated with a general increase in inflammation and upregulation of pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα, together with alterations in the distribution and function of individual leukocyte subsets including the loss of naïve lymphocytes[201]. Such effects may have influenced our results and for example, could help explain the large variations we observed in the proportions of naïve B cells between patients. In this respect, a recent study of the immune cell composition of the human intestine from young, middle-aged and old individuals by *Senda et al* observed that whereas SI GALT was gradually lost with age, colonic GALT numbers did not change[35]. However, it is important to note that the previous work did not distinguish between SM-ILF and M-ILF and had issues with contamination of their LP preparations by naïve T cells[24], making it unclear exactly which immune compartments were affected by ageing. It has been suggested that the different effects of ageing on GALT in the SI and LI could reflect higher exposure of the SI GALT to luminal contents and hence accelerated immunological senescence[24]. As we found SI GALT to be largely made up of the more exposed M-ILFs and PPs it would now be interesting to examine whether M-ILF, including colonic M-ILF, are relatively more frequent in young individuals, regressing more quickly with age. Applying our technique to a wider age range of individuals should improve our understanding of immunosenescence and age-associated intestinal pathology in general.

One observation we made, but were unable to explore in detail was the presence of large GALT structures with two or more follicles, especially in the proximal colon. Such structures have only been observed previously in the distal colon of young individuals[35], or as disease-associated "rectal tonsils"[30–34]. What these are remains to be investigated, although our preliminary studies suggested that their lymphocyte compartment was similar to that of SM-ILF (data not shown). This variation in size and complexity may simply reflect plasticity of the GALT compartment, increasing and decreasing according to the challenge at hand. Studying GALT in infected or inflamed tissues might help clarify this.

Our CyTOF analysis showed substantial heterogeneity within the T cell compartment of GALT, with T_{CM} cells exhibiting very little cytokine expression compared with T_{EM} cells and very different patterns of cytokine expression in LP and GALT. Although these topics have not been addressed previously in the gut, our results are

consistent with what is known about these cell types in other tissues[202]. Knowledge of the cytokine landscapes in different immunological compartments in health and disease could lead to a better understanding of what goes awry in diseases such as IBD.

Studies in mice have shown that while PPs generate IgA^+ B cells that seed the SI[27,51], those in colon are seeded by the cecal patch, with perhaps some contribution by SILT[67,11]. Our studies of human tissues are consistent with this anatomical pattern, as we found that PP and colonic SM-ILF contain B cell clones closely related to those in the respective segments of LP. Our methodology could also be exploited to explore the idea that some PCs in LP may be seeded by direct migration from neighboring GALT, as has been suggested previously (*Mucosal immunology textbook, 4th edition, 2015, chapter 31*). As well as providing a rapid and localized immune response to invading pathogens, such as process might also limit the spread of a potentially damaging immune reaction to areas where the microbe would be most beneficial.

The ability to analyze antibody producing B cells in individual follicles should also be of use for studying more basic biological questions such as how GC reactions develop in the gut. Although this has been well studied in other tissues[203], how it develops in the human gut is not well understood. The individual follicles we analyzed varied considerably in size and in proportions of GC B cells, suggesting each follicle was at a different stage of GC development. By analyzing several individual follicles of varying size using single cell sequencing, it would be possible to obtain individual snapshots of the GC reaction, its constituents and their interactions. It might even be possible to study cell-cell interactions by sorting T-B cell or B cell-FDC doublets for these analyses. Performing such studies on individual follicles might also help elucidate if T cell independent reactions occur in human GALT, a subject that is still debated[51]. Another question this could answer is the nature of factor(s) that drive immunoglobulin switching to specific isotypes such as IgA1 or IgA2[51,62]. Importantly, a larger proportion of intestinal B cells express IgG in IBD and it has been suggested that IgG may contribute to the pathology[51,60,61]. However what drives this change in Ig switching is unknown and the identification of the relevant factors could pave the way for new treatments of IBD.

One limitation of our analyses is that they were based on the relatively low numbers of suitable patients that became available over the time frame of our project. Future, larger scale studies should allow our results to be extended, providing further insights into whether multifollicular colonic patches, SM-ILFs and M-ILFs are different GALT structures or are simply different versions of the same tissue. Furthermore our CyTOF analysis was limited by expense and the need to optimize the phenotyping panels, meaning we were not able to exploit this approach to its fullest potential. Moreover, the *in vitro* stimulation protocol that was necessary for examining cytokine production in the CyTOF experiments led to down-regulation of CD4 and CD8 and imperfect separation

of some of the markers, making some subpopulations difficult to identify precisely. However the results we obtained and the novel techniques we described will provide an ideal basis for future, more detailed studies of this kind. Finally, if 10x single cell sequencing had been available to us early on in the project, this would have allowed much more comprehensive analysis of GALT, providing a truly unbiased approach to their cellular content and function.

Manuscript II – Identification, isolation and flow based immune profiling of human isolated lymphoid follicles

Summary

We describe a technique for separating the effector compartments of the intestinal LP and epithelium from the adaptive immune cell priming sites of the GALT. By physically peeling the LP away from the underlying SM, we show that SM-ILFs and M-ILFs can be isolated from the SM and LP, respectively. This can be done for both resected tissues and biopsies. We also noted tiny follicular-like structures in the peeled colonic LP, but we did not pursue this finding further. We further illustrate optimal ways of identifying complete and fractured PPs from elderly patients. Single cell suspensions can be obtained from LP and GALT compartments with high viability and even single follicles can be analyzed, the latter providing thousands of cells for flow cytometric analysis. Furthermore, we show that several distinct leukocyte subsets can be analyzed in each compartment by flow cytometry and that putative lymphoid tissue inducer cells are enriched in GALT. Finally, we show that this technique is also applicable to mildly inflamed tissues from patients with CD.

Discussion

The key finding of this manuscript is that the intestine contains two very different immunological compartments which can now be analyzed separately. Because they are not visible macroscopically, the smaller compartments of the GALT such as ILFs have often been ignored in the literature and it has been assumed erroneously that LP preparations only contain cells from the LP[24,51]. This can lead to inaccurate interpretation of data, exemplified by the reports of naïve T cells in the LP[204,205], a conclusion that we showed to be incorrect in **Manuscript I**. This is not the first attempt to isolate human ILFs from intestinal tissues, but previous methods have not allowed uncontaminated GALT to be isolated, or the smaller ILF structures to be identified and separated from LP preparations[25,40,59]. As IBD and other intestinal diseases are associated with increased numbers of GALT[10] and are usually assessed using whole biopsies, samples from such patients are likely to be subject to even greater levels of contamination. As a result, several findings of apparent disease-associated changes in gene/protein expression or cellular content may simply reflect a change in the ratio of GALT and LP-derived cells rather than processes of genuine pathogenic importance. Identifying exactly where these changes take place may provide novel insights into disease mechanisms and highlight new targets for treatment. It should also be noted that similar issues have compromised the study of GALT and LP in mice and in the future, it should be feasible to apply our approach to

the mouse intestine, although handling the thinner layers of SM and MM might prove difficult. Our technique may also be of relevance to other mucosal tissues such as the lung, stomach, nasal passages and urogenital tract, where secondary lymphoid organs are similarly embedded in the tissues and clearly separated layers can be observed. As well as providing new opportunities for studying these SLOs, this approach should allow mucosal cells to be studied in isolation.

One specific issue that our method may be particularly relevant for is the relative anatomical distribution of LTi cells and the other two subsets of human ILC3s[81]. Because these are phenotypically similar, it was not possible for us to determine whether the putative LTi we observed in GALT were genuine LTi or not. However, with techniques such as single cell sequencing, this would be possible and their currently unclear function in mature GALT could be explored[80,81]. Moreover, such investigations should reveal if the GALT-free LP contains any true LTi in addition to the other ILC3 subsets, a finding which could clarify whether the very small follicular-like structures we observed in peeled human colonic LP are the equivalent of the CPs observed in mouse intestine[206]. The presence of CPs in human intestine has previously been refuted based on the observation that only mature SLOs are found just few months after gestation[10,23]. However, these studies could have missed small LP-embedded structures. Using our technique on prenatal tissues combined with whole-mount staining for CP-specific markers similar to early studies on CPs/ILFs in mice[207,208], could resolve whether CPs are also found in humans. Finally, we observed a marked difference in the proportions of putative LTi between SM-ILFs and M-ILFs, but due to low numbers of samples it remains to be determined if this is a defining difference between these two compartments.

An additional lymphoid structure that could be present in human intestine, particularly during inflammation are tertiary lymphoid organs (TLOs). TLOs are postnatally induced organized lymphoid structures that in mice form independently of LTi and are associated with inflammatory disease including IBD[20]. Whether these structures have similar functions to GALT remains controversial[20,209,210]. In the intestine, they resemble PPs, comprising B cell follicles containing GCs, T cell zones, DCs and HEVs, and have no capsule or afferent lymphatics[209]. However, no consensus on how to distinguish TLOs from other GALT structures in humans has yet been reached[209], meaning that the intestinal TLOs that appear to have been identified previously might simply be naturally occurring GALT[211]. In mice, where TLOs can be induced in models of intestinal inflammation, it has also been questioned whether such structures are indeed formed *de novo*, or if they represent enlargement of pre-existing SILT[20]. As our technique allows for analysis of individual follicles in both healthy and inflamed tissues, these issues could potentially be solved phenotypically and functionally by comparing individual GALT with TLOs situated in deeper parts of the gut wall not normally containing lymphoid structures[211]. As TLOs are believed to play a direct role in disease pathology[20,209,210], discerning how they differ from normal GALT might help in the

understanding of how TLOs might influence disease initiation and development. In this regard, it has been noted that "true" TLOs might only be found in parts of the gut wall not occupied by GALT[211].

Another interesting observation we made here and in **Manuscript I** was the specific localization of M-ILFs to ileum and distal colon. These are also the preferred sites of IBD initiation, and as GALT are also suspected to be the initiation sites of IBD[212–214] and there is a direct relation between GALT numbers and IBD onset[26], this could suggest causality. One potential link could be the SED and the large number of M cells seen in these structures[10], allowing easier access for microbes, which could then initiate the inflammatory state that sets off these diseases. As our technique allows for the isolation of GALT from CD intestine, this could be investigated in future studies. Such studies may not have to be limited to CD, as pilot experiments have also shown this to be possible in inflamed tissues from UC intestine, although this still requires some optimization (not shown).

Manuscript III – Generation of a stromal cell atlas of the human intestinal lamina propria

Summary

We use our newly developed technique from **Manuscript II** together with single cell sequencing, immunohistochemistry and multicolor flow cytometry to phenotype, locate and describe the entire SC compartment in LP from human ileum and colon. By comparing with published datasets[102], we were able to generate combinations of markers that could identify all major SC subsets in both the ileum and colon. Of note, we find that FBs make up the two major subsets of SCs, with FB1 inhabiting the central (interstitial) area and FB2 occupying the subepithelial niche within the intestinal LP; each of these express different genes important for epithelial homeostasis. We also describe tissue specific differences in gene expression between individual subsets of the ileum and colon, and identify the cellular functions that may correlate with these differences. We further identity a putative mesenchymal precursor population and MFBs, which we verify by flow cytometry, and for the latter show to be enriched in inflamed tissues from patients with UC.

Discussion

Here we present a unique and comprehensive resource of information on the transcriptome, localization and phenotype of the iSC compartment of the human intestine, expanding greatly on our current knowledge (**Figure 7**). By exploiting these findings, it should now be possible to address outstanding questions on the nature and functions of individual iSC subsets and potentially identify novel ones.

One example of an unresolved issue is whether the ability of iSCs to express HLA-DR allows them to act as antigen presenting cells, as has been suggested previously[83,84,184]. Importantly, the identity of such HLA-DR$^+$ SCs has been unclear and by combining our cluster analysis with multicolor flow cytometry, we find considerable heterogeneity of HLA-DR expression levels amongst the different SC subsets (**Figure 8a-b**). Interestingly, the FBs express very low levels of HLA-DR, much lower than the highly expressing endothelial cells. This suggests that these studies may have had contaminating cells in their LP preparations similar to the HLA-DR antigen-associated invariant chain (CD74)-expressing FRCs identified by *Kinchen et al*. Furthermore, the differences in HLA-DR expression of specific subsets varied between the ileum and colon, with ileum subsets generally expressing higher levels (**Figure 8a-b**). Site-specific knowledge like this, along with the proportional and gene-expressional differences between SC populations of the ileum and colon that we observed might provide insights into why UC and CD show preference for specific regions of the gut[215], or why intestinal cancer is found primarily

in the LI[216]. Indeed colorectal cancer is thought to be promoted by a population of cancer associated FBs[92], suggesting that inherent differences between SI and LI FBs might be a contributing factor to the disease. This idea could be consistent with our finding that the most differentially upregulated gene in colon vs ileum FBs was periostin, a factor associated with cancer progression[217,218].

Additional insights that can come from our comprehensive analysis at the single cell level include identification of novel markers for the isolation of individual populations for deeper sequencing, proteomics analysis' and *in vitro* studies, as well as assessment of their localization *in vivo*. One of the most important aspects of SC function in complex tissues is to provide supportive niches for other cells and this has been difficult to study due to the lack of appropriate markers. One example of this in the intestine could be the PC (plasma cell) niche. These cells are thought to be located in the lower part of the interstitial (central) area of the intestinal LP, and require SC-derived IL-6 and CXCL12 for survival[51]. In this respect, we found IL-6 and CXCL12 expression to be highest in the interstitially located FB1 cells (**Figure 8c-d**), suggesting that these may be the SCs essential for PC survival. However, although we determined that FB1 and FB2 cells were located in different niches in the LP, we were unsuccessful in locating where individual subclusters of FB1 and FB2 were located. One way of doing this would be to search different parts of the villus-crypt unit for some of the differentially expressed epithelial support genes we identified by the single cell analysis, as many of these have been assigned a specific location along axis in the human colon[109]. Comparison of these published genes with the published single cell dataset from *Kinchen et al* shows that similar to what we showed in the manuscript FB1 generally have the highest expression of crypt-associated genes, whereas FB2 express the largest amount of tip-associated genes (**Figure 8e**). Moreover, an expressional gradient is observable within the FB1 clusters, suggesting that individual cell types amongst FB1 may locate to specific areas along the crypt-tip axis. This is complemented by a similar gradient in CXCL12 expression (**Figure 8d**, bottom), with $CXCL12^{hi}$ expressers presumably locating to the basal crypt, as its expression is suppressed by the tip/villus-associated epithelial-derived Hh[84,165]. However, this data will have to be verified using other methods such as immunohistochemistry and *in situ* hybridization.

The ability to localize individual FB subsets to specific regions along the crypt-tip/villus axis might help us better understand the underlying factors, mechanisms and cell-cell interactions important for epithelial homeostasis and disease. For example, barrier defects are a common feature of IBD[219–221] and this could be initiated or sustained by a shift in localization, proportions, and/or protein-expression of individual FB subsets. Importantly, such changes might be missed if locational context cannot be applied to the data, as individual factors can have profoundly different effects depending on their local environment. Furthermore, identifying where all SC subsets reside within the LP might allow future studies to construct a comprehensive overview of other immunologically

important niches *in silico*, as is being attempted for SLOs[222]. Work of this kind would provide novel insights with a broad impact on gut immunology and potentially identify subset-specific targets for therapy.

Many of our findings are in broad agreement with those of a recent paper showing single cell sequencing analysis of iSCs that was published after our study began[102], with the datasets giving rise to similar clusters with the exception of the small contaminating FRC (S4) population found in the data from *Kinchen et al.* Importantly, this study employed 10x sequencing, allowing for greater gene-expression counts per cell, and hence more information about individual cells and clusters. However, they only obtained data from two healthy and two UC patients giving them limited numbers of cells and increasing chances for patient specific data biasing their results. One point where our work differs is that we did not identify MFBs in our data from healthy intestine, instead identifying a cluster of SMCs. Employing their data along with multicolor flow cytometry revealed the presence of a small subset of contractile FBs distinct from SMCs, FB1 and FB2 seen only in very low numbers in healthy intestine. This indicates that the MFBs identified by *Kinchen et al* were likely SMCs, explaining why these were not observed to expand in patients with UC[102] as would have been expected[94]. In contrast, we found that our putative MFBs could only be identified in the single cell sequencing dataset from UC patients, and that these were enriched in mildly inflamed tissues from patients with UC. A similar issue of misidentification may explain the findings of a recent single cell sequencing study on mouse lung SCs that also identified αSMA-expressing putative MFBs that did not expand upon fibrosis, concluding instead that there was a general increase in αSMA expression by other FB subsets[223]. As in the work by *Kinchen et al*, these authors also did not identify a specific subset of SMCs, although such cells are known to be present in these tissues, suggesting that "true" MFBs are to be found within the αSMA-expressing FBs. Since other FB subsets such as FB2c did also express αSMA, it is also possible that MFBs are not a single defined population, but that several mesenchymal cells may conform to a more contractile phenotype upon stress. Addressing which of the contractile cell-types also have increased matrix-remodeling capabilities under inflammatory/fibrotic conditions might help resolve the exact phenotype(s) of MFBs. Precise identification of such pathogenic cell types is important for identifying new targets for the treatment of fibrosis and other MFB-related pathologies.

Translating our results to mice will be essential if we are to make substantial gains in our understanding of SCs in health and disease, as mouse models can help deconstruct the underlying mechanisms of SC biology to a much greater extent than in humans. Others in our lab are engaged in such studies and the necessity of comparing findings directly in the different species is illustrated by the work of *Kinchen et al*, which could not align individual FB1 and FB2 subsets in mouse and human intestine, despite identifying similar main subsets of SCs[102]. Indeed they found substantial discrepancies in the number and proportions of FB subsets between mice and humans, although this was not commented on. One example of this was the large discrepancy between human and mouse

intestine in the proportions of one of the FB subsets referred to by *Kinchen et al* as "S3". This may reflect the fact that the isolation method used was likely to result in cells derived from different anatomical compartments of the intestine in the two species, with mouse iSCs deriving from all tissues of the gut wall, whereas the human samples would be derived mainly from the LP. In agreement with this, one of the mouse S3-specific genes found by *Kinchen et al*. was ACKR4[102], a gene recently shown to be expressed only by murine submucosal adventitial cells[224], a finding we replicated in human tissues. Hence it is likely that the S3 population identified by *Kinchen et al* consists of a mix of "true" LP-derived FB1d/S3 and phenotypically similar adventitial cells from the SM. Such clarifications of the relationship between mouse and human iSC subsets should improve the translational value of future iSC studies in mice.

This study started with painstakingly testing various combinations of literature-derived protein markers by multicolor flow cytometry, to get an overview of the proportions and extent of human iSC heterogeneity. However, such an approach is limited in its capabilities and objectivity, hence we developed the droplet based single cell sequencing platform, providing the unbiased datasets most of this manuscript is based on. However, other superior single cell sequencing techniques were around at the same time, but were unavailable to us at the beginning of the PhD. In hindsight we should have established the collaboration for the employment of the 10x platform earlier. Although our technique provides many of the same insights as the 10x data provided by *Kinchen et al,* the sparse expression of all but the highest expressed markers makes drawing conclusions about cluster- or tissue-specific differences difficult. However, this technique had the benefit of being an order of magnitude cheaper and so enabled us to acquire more samples for analysis, and so limiting patient bias. In the mean time we have started to collect 10x datasets of healthy and inflamed intestinal tissues, but their complete processing and analysis could not be performed within the time-frame of this PhD. However, future work on these datasets should allow us to draw more firm conclusions about iSC function, ontogeny, and tissue-specific differences. Although the ambition was to map all SCs within the LP, doing so for very similar subsets, such as the FB1b and FB1c clusters proved beyond our present capabilities. This was in part due to the limitations with the techniques described above, but was also a consequence of the inability to translate gene-expression to protein expression, and protein-expression gradients seen by flow cytometry to immunohistochemistry. We will have to tackle these issues in the future unless the new datasets provide better discriminating markers. Another limitation of our studies was the lack of functional studies on individual subsets, potentially confirming or disproving functions suggested by the gene-expression data and in the literature. As mentioned, others in the group are translating some of our findings in humans to mice and investigating this in the setting of various mouse models, but we also tested *in vitro* cultures of these cells. Although pilot studies were successful in expanding iSCs, these studies also revealed a loss of heterogeneity, with most iSCs lost within a few days of culture and the remainder changing drastically compared to the their *ex vivo*

analyzed counterparts (not shown). Such issues will have to be resolved before functional studies can be taken further.

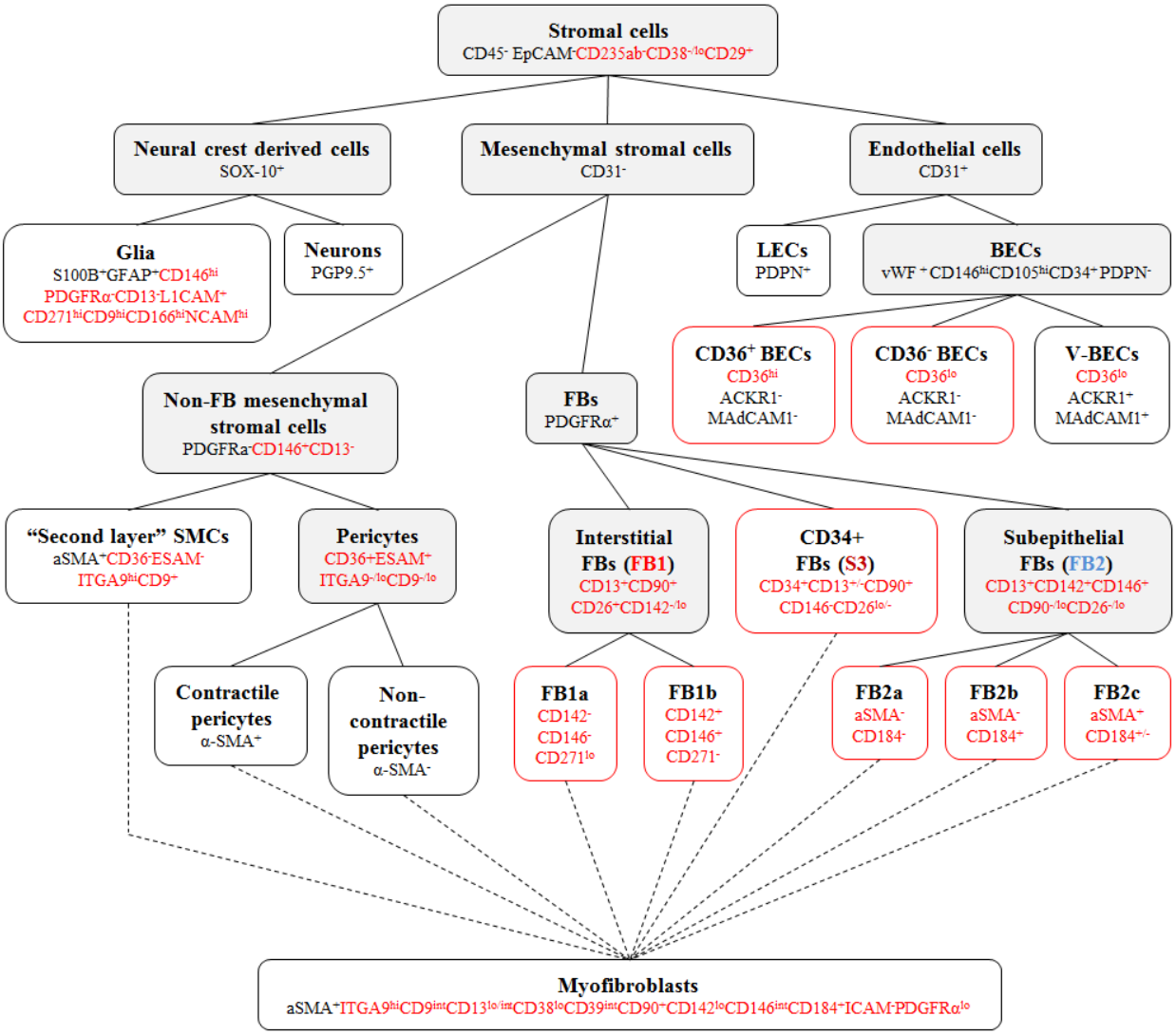

Figure 7. State of the art of human intestinal stromal cell heterogeneity at the end of the project.

Overview of the different populations of CD45-EpCAM- stromal cells (SCs) found in human intestine, showing independent lineages (grey boxes) and their subsets (white boxes), together with their expression of specific markers. Intestinal lamina propria (LP) SCs are found in three main linages, the neural crest-derived linage, the mesenchymal stromal cell linage, and the endothelial cell linage. These branch off into further subsets as shown in the figure. The identity of at least two types of interstitial fibroblasts (FBs) have now been established, along with a putative FB precursor, a third type of blood vessel endothelial cell (BEC), and two extra subsets of subepithelial FBs. The definition of an intestinal LP SC has now been expanded to (CD45-EpCAM-CD235ab-CD38-/lowCD29+). New markers discovered in this study are highlighted in red, whereas newly discovered subsets are outlined in red. Which mesenchymal stromal cells give rise to myofibroblasts still remains to be determined; abbreviations: V-BECs = venular BECs, LECs = lymphatic endothelial cells, SMCs = smooth muscle cells.

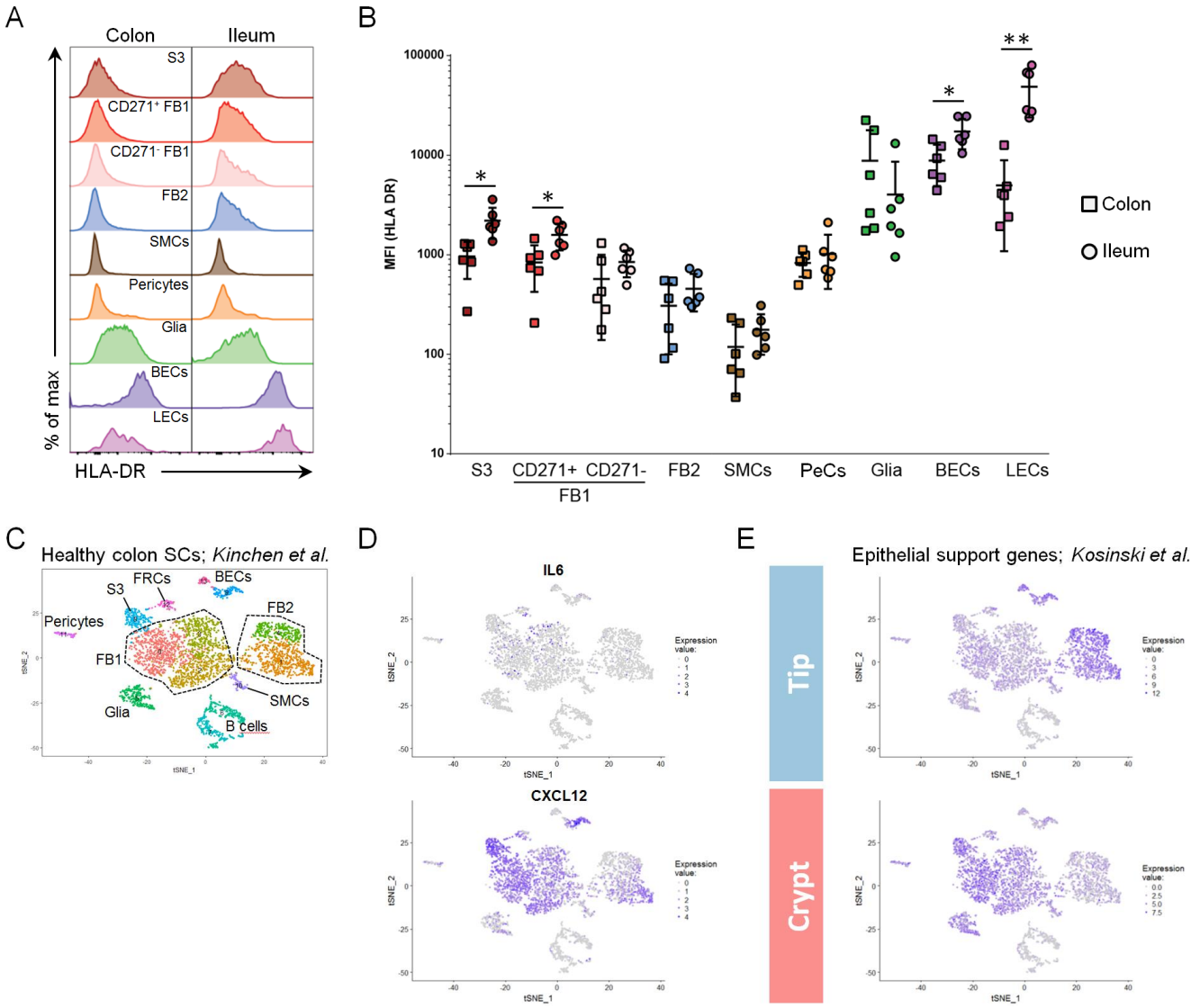

Figure 8. Additional analysis of human intestinal stromal cells.

(A) Representative histograms of HLA-DR expression by stromal cell 3 (S3), CD271^{+} and CD271^{-} subsets of fibroblast 1 (FB1), fibroblast 2 (FB2), smooth muscle cells (SMCs), pericytes (PeCs), glia, blood vessel endothelial cells (BECs) and lymphatic endothelial cells (LECs) in paired samples of human colon and ileum as assessed by flow cytometry. (B) Mean MFI of HLA-DR expression by SC subsets from paired samples of human ileum and colon. Results shown are Mean MFI on log10-scale; n = 6; * = $p < 0.05$, ** = $p < 0.01$, paired t-test. (C) SC clusters from healthy human colon from *Kinchen et al* as in *Manuscript III*, with subset names given in the figure; FRCs = fibroblastic reticular cells. (D) Overlay plots showing the expression of (top) IL-6 and (bottom) CXCL12 genes by the clusters of intestinal SC defined in (C); relative expression levels indicated in gradients of blue. (E) Combined expression of epithelial support genes expressed preferentially in (top) colonic tip and (bottom) crypt base SCs, as defined by *Kosinski et al*[109], by the individual clusters of intestinal SCs as defined in (C); relative expression levels indicated in gradients of blue. Tip genes = *BMP1*, *BMP2*, *BMP5*, *BMP7*, *BMPR2*, *SMAD7*, *JAG1*, *WNT5B*, *APC*, *TCF4*, *EFNA1*, *EFNB2*, *EPHA2*, *MAX*, *MXI1*, and *LAMA5*. Crypt genes = *NOTCH1*, *NOTCH2*, *NOTCH3*, *TLE2*, *FZD2*, *FZD3*, *FZD7*, *TCF3*, *DKK3*, *SFRP1*, *EPHA2*, *EPHA7*, *EPHB2*, *EPHB3*, *EPHB4*, *EPHB6*, *MYC*, and *LAMA2*.

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