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A TRIPARTITE OF IMMUNE-, EPITHELIAL-, AND NERVOUS-SYSTEMS IN THE HOMEOSTATIC REGULATION OF THE GUT

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A tripartite of immune-, epithelial-, and nervous-systems in the homeostatic regulation of the gut

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Various cell types in the intestinal mucosa are constantly exposed to complex signals emanating from the lumen, including the microbiota and its metabolites. How these bilateral interactions in turn influences intestinal homeostasis is an important question in order to understand microbiota-host interactions. This thesis has attempted to address this question in the following papers. Deletion of the diet- and microbiota-regulated aryl hydrocarbon receptor in CD11c+ cells was found to result in aberrant intestinal epithelium morphogenesis and increased susceptibility of these mice to chemically induced colitis (Paper I). Our data highlight a possible gateway of communication between the host and its environment, through the AhR in intestinal antigen presenting cells, consequently regulating intestinal epithelial cell biology and function.

In the second paper, we studied the impact of the microbiota on the development of the enteric nervous system (ENS). The ENS controls many aspects of gut physiology, including mucosal immunity. The major cellular component of the ENS is the enteric glia cell (EGC). Our data showcased that the migration and expansion of EGC networks in the lamina propria towards the lumen are under the influence of the microbiota. The postnatal expansion of mucosal EGC networks was found to coincide with the same period where the microbiota increases in number and diversity. Moreover, this microbiota-driven mechanism is an active process that can be impaired following the exposure to antibiotics, which abrogate signalling pathways mediating the host-microbe cross talk.

In the final manuscript, we developed a co-culture model system to study EGC functions further, in relation to intestinal epithelial barrier functions. Using genetic labeling techniques and live cell imaging, we observed close associations of EGCs with co-cultured intestinal epithelial organoids *ex vivo*, reminiscent of the contacts reported between these two cell types *in vivo*.

In conclusion, this thesis open more questions than answers especially as it addresses the issue of cross communication between different biological systems required for the development of complex organisms. The new player here is the microbiome and how it constantly affects the response of different cell types, including cell-to-cell communications, important for cellular adaptation to environmental cues. Future work will address the precise molecular and cellular mechanisms underlying the interplay between the microbiota and host-tissues to establish and maintain intestinal homeostasis.

LIST OF SCIENTIFIC PAPERS

I. **Chng SH**, Kundu P, Dominguez-Brauer C, Teo WL, Kawajiri K, Fujii-Kuriyama Y, Tak WM, Pettersson S. **Ablating the aryl hydrocarbon receptor (AhR) in CD11c+ cells perturbs intestinal epithelium development and intestinal immunity.** Sci. Rep. **6**, 23820; doi: 10.1038/srep23820 (2016).

II. Kabouridis PS, Lasrado R, McCallum S, **Chng SH**, Snippert HJ, Clevers H, Pettersson S, Pachnis V. **Microbiota controls the homeostasis of glial cells in the gut lamina propria.** Neuron. **85** (2): 289-95. (2015)

III. **Chng SH**, Bon-Frauches AC, Pettersson S and Pachnis V. **Establishing a co-culture system to study enteric glial cell functions.** *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
AhRR	AhR repressor
ALDH1a2	Aldehyde dehydrogenase 1 family member A2
APC	Antigen presenting cell
ARNT	AhR nuclear translocator
bHLH/PAS	basic helix loop helix/PER-ARNT-SIM
BMDC	Bone marrow derived dendritic cell
BMP	Bone morphogenetic protein
CD	Crohn's disease
CNS	Central nervous system
CONV	Conventionally raised animals
CSF-1	Colony stimulating factor 1
DC	Dendritic cell
DIV	Days *in vitro*
DRE	Dioxin response elements
DSS	Dextran sodium sulphate
EGC	Enteric glial cell
ENCC	Enteric neural crest cell
ENS	Enteric nervous system
FICZ	6-formylindolo[3,2-b]carbazole
GDNF	Glial cell derived neurotrophic factor
GF	Germ free
GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal
I3C	Indole-3-Carbinol
IBD	Inflammatory bowel disease
IEB	Intestinal epithelial barrier
IEC	Intestinal epithelial cell
ISC	Intestinal stem cell

KO	Knock-out
Kyn	Kynurenine
Lgr5	Leucine-rich repeat-containing G-protein coupled receptor 5
LMMP	Longitudinal muscle/myenteric plexus
LP	Lamina propria
mEGC	Mucosal EGC
MHC II	Major histocompatibility complex, class II
mLN	Mesenteric lymph node
MM	Muscularis macrophages
MP	Myenteric plexus
MPS	Mononuclear phagocyte system
Myd88	Myeloid differentiation primary response gene 88
POI	Postoperative ileus
PRR	Pathogen recognition receptor
SMP	Submucosal plexus
TLR	Toll-like receptor
Treg	Regulatory T cell
Trp	Tryptophan
VC	Villus-Crypt
VNS	Vagus nerve stimulation
XRE	Xenobiotic response elements

1 INTRODUCTION

1.1 OVERVIEW

The primary function of the gastrointestinal (GI) tract is to digest our daily food and for the absorption of nutrients. For this purpose, the GI tract has employed several biological systems to enable it to carry out these functions. First, the luminal surfaces are lined with a single layer of epithelial cells that constitutes the intestinal epithelial barrier (IEB), forming the major absorptive organ in the GI tract, in addition to separating us (the host) from its resident microbiota. Second, a huge proportion of our body's immune cells can be found underlying the mucosal surfaces of the intestines where they play crucial roles in driving oral tolerance and immunity against potential pathogens. Third, our intestine is immensely innervated with inputs from the central nervous system (CNS) in addition to having its own 'brain' - the Enteric Nervous System (ENS) that controls various aspects of GI functions, including peristalsis. Lastly, our GI tract is also the platform for exchange between the host and its external environment (the gut lumen) regardless of being inside of the body, as illustrated by our interaction with the resident microbiota. For reasons mentioned here, I have dedicated a huge part of my Ph.D. studies in trying to understand the interconnection between these different biological systems/platforms and how environmental factors can perturb the normal physiological interactions between them, leading to disease.

In order to respond to the dynamic environment, it is necessary to develop sensing mechanisms that upon exposure to extrinsic signals allow a cell to react and adapt quickly. To do this, cells need to regulate the expression of genes reciprocally to these signals in order to maintain their competitive advantage. Ligand-induced transcription factors are a class of structurally unrelated proteins that upon ligand activation, binds to the promoters and/or enhancer sequences of target genes to regulate transcription. In this manner, these factors are best-fit for acting as environmental sensors since they are able to modulate the expression of genes in a signal (ligand)-dependent way, thus supporting cells to respond to environmental cues rapidly. One such pathway of sensing and activation is the aryl hydrocarbon receptor (AhR) signalling pathway, which is studied in conjunction with intestinal antigen presenting cells (APCs) in the first part of this thesis.

The mucosal immune system is comprised of elements from the gut-associated lymphoid tissues (GALT) such as the Peyer's patches, isolated lymphoid follicles and mesenteric lymph nodes (mLN) while immune cells can also be found throughout the mucosa lamina propria. Intestinal APCs are seen as the sentinel cells that coordinate both innate and adaptive immune responses, important for tolerance and immunity through the use of receptors that identify pathogens, coined as pathogen recognition receptors (PRRs). Recently, the AhR (as mentioned above) has been proposed to be one of the PRRs[1], supporting a role for AhR in intestinal APCs and immunity.

To begin, I will first introduce the AhR, followed by overviews of the roles of different biological systems in GI tract functions, with references to AhR where applicable.

1.2 ARYL HYDROCARBON RECEPTOR BIOLOGY

The AhR is highly evolutionary conserved with homologues found in organisms for instance, in *C. elegans* and *D. melanogaster* apart from mammals[2]. This highlights the involvement of AhR signalling in fundamental biological processes critical for the well-being of complex organisms. We will introduce the canonical signalling pathway of the AhR.

1.2.1 Signalling pathways of the AhR

The AhR is a ligand-activated transcription factor and a member of the basic Helix-Loop-Helix/Per-Arnt-Sim (bHLH/PAS) family of proteins. It is expressed in many different cell types albeit at different levels. In the absence of a ligand, the inactive AhR is localised in the cytoplasm as a multi-protein complex. The complex consists of the AhR, heat shock protein 90 (Hsp90) dimer, co-chaperon p23 as well as the XAP2 protein[3-5]. Upon ligand binding, exposure of the N-terminal nuclear localization signal of AhR as a result of ligand binding-dependent conformational changes leads to the translocation of AhR and its cytosolic associated proteins into the nucleus. Once in the nucleus, the AhR nuclear translocator (ARNT) which is also a member of the bHLH/PAS family dimerizes with AhR and during that process uncouples AhR from its chaperons. The heterodimer of AhR and ARNT constitutes a functional transcription factor that binds to specific enhancer sequences commonly known as the xenobiotic response elements (XREs or dioxin response elements DREs) found upstream of AhR responsive genes. Mechanisms of transcriptional regulation are very similar to classical pathways given that AhR dependent signalling has been shown to rely on an array of common co-activators such as the p300 and the SRC protein[6,7]. The functional domains of the AhR protein with indicated regions showing their role, for example, in DNA binding or ligand binding are shown below (Figure 1).

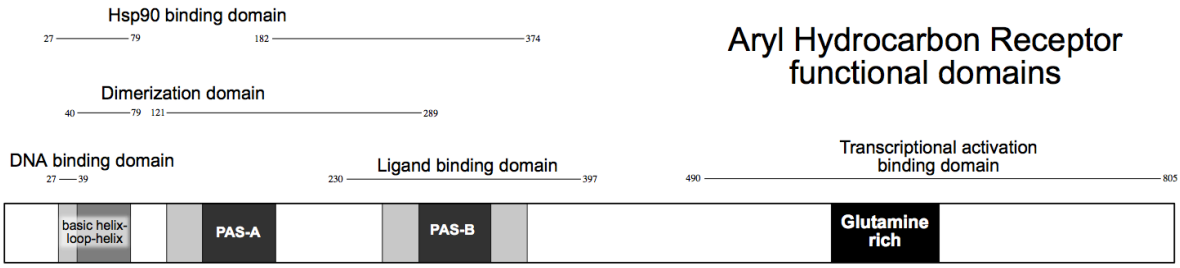

Figure 1. Functional domains of the AhR protein. By Jeff Dahl [CC BY-SA 4.0 (http://creativecommons.org/licenses/by-sa/4.0)], via Wikimedia Commons. https://upload.wikimedia.org/wikipedia/commons/e/e2/AHR_functional_domains.svg

As with all biological systems, negative feedback is essential and therefore the AhR signalling pathway is not an exception. Down-regulation of AhR signalling can be ascribed to two key mechanisms: (1) Degradation of AhR via the ubiquitin-proteosome pathway[8] upon export out of the nucleus; (2) Attenuation of signalling via AhR repressor (AhRR), another bHLH/PAS family member[9]. Being structurally related to the AhR, the AhRR competes

effectively against AhR for ARNT in the nucleus without ligand binding and thus exerts its repressive effects on AhR regulated genes. The AhRR has been shown to be expressed at high levels upon AhR activation[9] due to the binding of AhR:ARNT on XREs found upstream of AHRR promoter thereby provisioning the negative feedback loop.

The sequence of events upon ligand binding to AhR in the cytosol as illustrated is only one out of the many other possible signalling pathways that have been described in the literature. Of note, AhR signalling can be activated independently of a ligand, through phosphorylation mediated by a second messenger: cAMP[10]. In addition, direct cross talk with transcription factors such as NF-kb, retinoblastoma protein, estrogen receptor and protein kinase pathways have also been reported[11]. In turn, these interactions afforded AhR the ability to influence a myriad of cellular processes such as cell proliferation and differentiation, vascular development and more, in a cell/tissue type specific manner.

More recently, the AhR pathway has been implicated in development, tissue regeneration and cancer, through its interaction with β catenin/Wnt signalling pathway[12-14]. Corroboratively, the activation of Wnt signalling was found to increase the transcription of *Ahr* in at least three different cellular contexts[15-17]. Nonetheless, the exact mechanisms linking the two pathways are currently unclear as AhR activation could modulate Wnt signalling in both directions (up or down) as summarised in a recent review[12]. For this thesis the scope of the investigation is not on the various pathways/effects mentioned, excellent reviews for further analysis of the diversity in AhR signalling can be found in the literature[11,18-20].

1.2.2 Toxicity, Xenobiotics and Natural Ligands

In the early days, AhR activation is thought to be largely dependent on the binding of environmental contaminants. For example non-halogenated polycyclic aromatic hydrocarbons and halogenated aromatic hydrocarbons are the most common, thus giving the receptor its name. Among these, the most well-studied environmental pollutant that elicits AhR-mediated toxicity is the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin). Since the 1980s, dioxin toxicity in animals has been widely documented which include but not limited to wasting, lymphoid involution, hepatotoxicity, epidermal changes (chloracne), gastric lesions, teratogenicity and endocrine effects[21].

The AhR activating potential of ligands can be easily examined by studying for the up-regulation of well characterized AhR responsive genes such as xenobiotic metabolizing enzymes of the cytochrome P450 family: CYP1A1, CYP1A2 and CYP1B1 as well as its repressor AhRR. For this reason, many believed initially that the putative function of the AhR was to sense xenobiotics and subsequently up-regulate the expression of phase I and II enzymes to facilitate the excretion of these foreign chemicals from the body. However, the hypothesis was not sufficient in explaining the toxic effects caused by dioxin exposure. The half-life of dioxin in rodents is around 2 weeks, while in humans is estimated to be about 7 years[22]. Thus, the persistence of dioxin within the body could theoretically result in the over-activation of AhR, leading to either the up or down regulation of AhR responsive genes over

long periods of time. In this aspect, it is plausible to think that dioxin-mediated toxicity was due to the over or under activity of AhR responsive genes *per se*. Interestingly, sensitivity to dioxins and its toxic effects were found to be variable between species or even within the same species[23]. Taken together, it is unlikely for organisms to retain the *Ahr* gene through evolution just for the sensing of xenobiotics in the environment. A more logical hypothesis would be that lessons learned from toxicological studies were a reflection of normal physiological responses becoming disorganised as a consequence of persistent activation of the AhR by environmental pollutants. In other words, independent of mediating dioxin-related toxicity effects, the AhR has in its own, biological relevance in the proper functioning of whole organisms.

Only recently, investigators embarked on the search for promising endogenous and environmentally-derived AhR ligands[24,25], including polycyclic compounds commonly found in our diet[26,27]. Examples include but not restricted to tryptophan (Trp) metabolites such as kynurenine (Kyn) and 6-formylindolo [3,2-b] carbazole (FICZ); indoles such as indole-3-carbinol (I3C) found in cruciferous vegetables; Indigoids as well as arachidonic acid metabolites. Of interest, bacteria themselves (*Lactobacillus bulgaricus* OLL1181) can act as activators of the AhR pathway[28]. However, whether the strain of bacteria involved secretes enzymes that convert intestinal tryptophan to AhR ligands or it secretes AhR ligands directly remain to be elucidated. In support of the former, *Lactobacillus reuteri* in the presence of high Trp but low levels of carbohydrates has been shown to express high levels of ArAT-related aminotransferase, which is involved in the production of indole-3-aldehyde, a reported AhR ligand[29]. Taken together, it is conceivable that high concentrations of AhR activating ligands exists in the GI tract (Figure 2).

With the identification of putative endogenous AhR ligands in addition to the knowledge gained from studying the toxicity effects of AhR over-activation, evidence supporting its physiological functions became notable. Following, efforts in understanding the role of AhR signalling in immunology gained great momentum in recent years[30,31]. This is not surprising given that some of the most evident toxic effects of dioxin in animals were thymus involution and immuno-suppression in general. In the following chapter, I will provide some recent evidences supporting the critical role played by AhR, mainly in mucosal immunity.

1.2.3 AhR in intestinal immune homeostasis

Apart from well-recognized immune-toxic effects caused by dioxin exposure, numerous high profile reports confirmed the involvement of the AhR in regulating immune functions. One of the key discoveries was based on human hematopoietic stem cells where treatment with AhR antagonists promoted their expansion *ex vivo*[32]. In support for the role of AhR in the immune system, cell types from both the innate and adaptive divisions of the immune system were discovered to express high levels of AhR[33]. However, the exact mechanisms behind AhR-dependent modulation of the immune system and its constituents (various immune cell types) remain to be elucidated.

One interesting paradigm presented in the current literature suggests that the specificity of AhR ligands can lead to contrasting immunological outcomes. For instance, using an autoimmune disease mouse model, dioxin treatment was shown to be protective by promoting the generation of regulatory T cells (T_{reg}) while FICZ treatment worsens disease scores by enhancing Th17 responses instead[34,35]. It is still unclear why dichotomy exists during AhR activation, mediating opposing T helper cell subset responses *in vivo*.

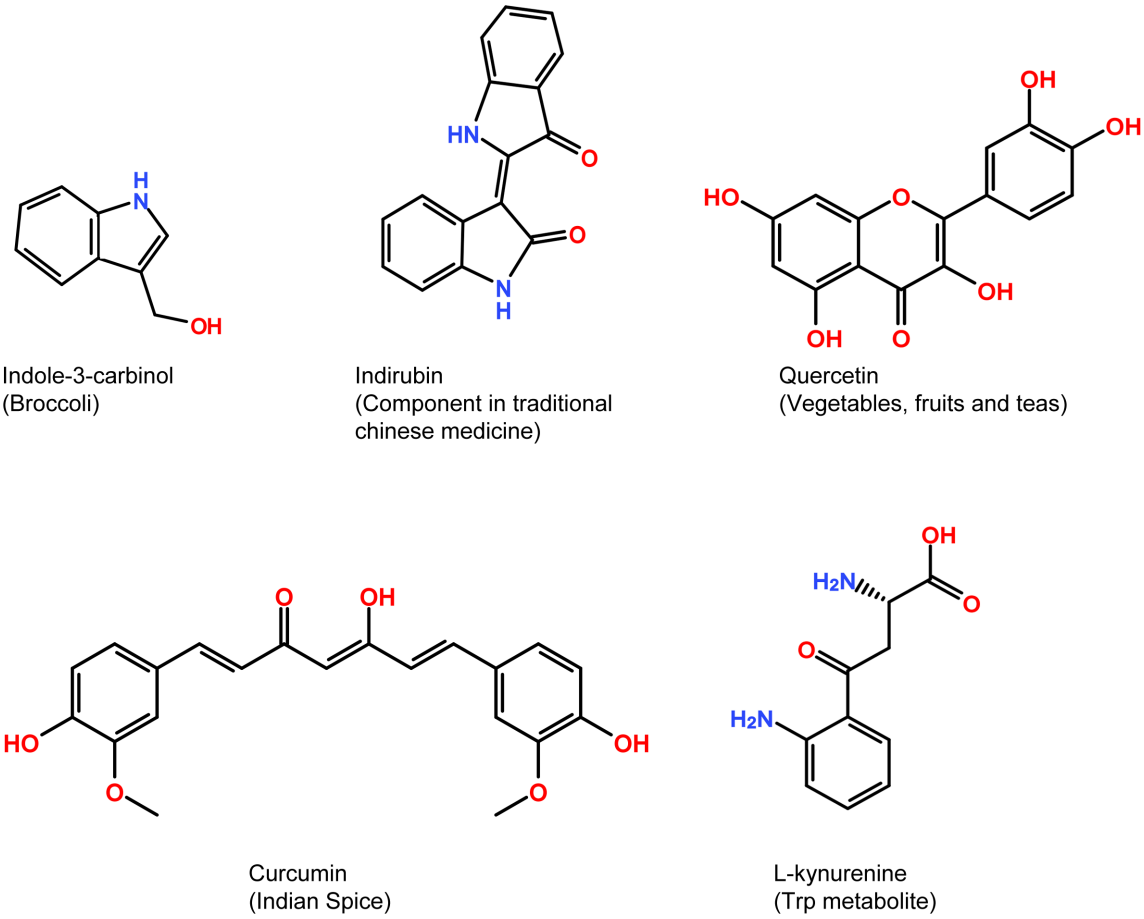

Figure 2. Some examples of AhR activating ligands found within the intestinal lumen.

Numerous members of the bHLH/PAS protein family such as the hypoxia-inducible factors can function as environmental sensors. As such, cell types localized to mucosal layers may utilize the AhR as a means to sense changes in the external environment across the epithelium, eliciting an appropriate response in return. An excellent illustration to support the above hypothesis was shown by Li and colleagues[36], where a diet devoid of AhR ligands was found to reduce the number of intraepithelial lymphocytes in the mouse intestinal lamina propria (LP). In parallel, it was demonstrated that adult AhR knockout (KO) mice lacked intraepithelial lymphocytes in both the skin as well as the intestinal LP, exhibiting a functional requirement for AhR to promote the survival and function of these lymphocytes[36,37]. This phenomenon was also associated with a weakened mucosal barrier, increased bacteria load and heightened susceptibility to chemically induced colitis in AhR KO mice[36]. More recently, pigmented virulence factors such as pyocyanin or naphthoquinone

phthiocol derived from pulmonary pathogens like *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* respectively have been shown to activate the AhR[1]. The activation of AhR by these factors was demonstrated to drive cytokine and chemokine production that are thought to protect the host against bacterial insults[1]. The authors then conclude that the inability of AhR KO mice to defend against *P. aeruginosa* infection resulting in the histopathology detected within the lungs was attributed to the loss of a pathogen recognition receptor- the AhR[1]. Interestingly, the photoproduct of intracellular tryptophan (or FICZ) has been recently demonstrated to suppress inflammatory responses and ameliorate disease severity in a mouse model of Psoriasis, which was abrogated when AhR was absent[38]. Taken together, these findings suggest a strong association of AhR signalling in maintaining the homeostasis of mucosal surfaces, acting potentially as a sensor for changes in the external environment important for host defense and repair mechanisms.

In further support of AhR's participation in maintaining intestinal health, AhR signalling has been shown to be essential for the maintenance and function of IL-22 producing innate lymphoid cells in the LP[39,40] as well as driving the postnatal development of isolated intestinal lymphoid follicles[39,41]. As expected, AhR KO animals were found to be highly susceptible to *Citrobacter rodentium* infection[40], a likely consequence of the absence of innate lymphoid cells and IL-22 secretion by these cells. Notably, factors that negatively regulate the AhR pathway have been shown to down-regulate IL-10[42] or IL-22[43] production in T cells, a scenario that could help explain the pathogenesis of certain forms of inflammatory bowel diseases in human patients where AhR protein levels were found to be down-regulated[44]. Conversely, experimental colitis in animals was shown to be attenuated when AhR ligands were administered prior to the induction of colitis, due to the selective differentiation of T_{reg}[45] or as a result of heightened production of prostaglandin E2 in the colon upon AhR activation[46]. Interestingly, evidence from a recent study which has suggested that the human *AHR* gene may have gained a unique feature by selecting for microbial-derived indoles as potent AhR agonists[47], further bolsters the perception that AhR is central to promoting host-microbe commensalism and sustain intestinal immunity.

Intestinal mucosal dendritic cells (DCs) and macrophages, which collectively are the major subsets of APCs present in the LP play a crucial role in both tolerance mechanisms as well as immunity against pathogens[48,49]. Intestinal APCs are specialized cell types that could sample luminal contents in a collaborative process, leading to the induction of tolerance toward fed antigens[50]. While AhR expression is detected in APCs, its function in mucosal APCs and how the receptor and these sentinel cells could cooperatively sense the microenvironment to maintain intestinal homeostasis remains unexplored. In the following chapter, I will introduce the major APC subsets found in the small intestinal LP, discuss their functional properties in addition to exploring the available literature on the role of AhR in APCs.

1.3 MONONUCLEAR PHAGOCYTES IN THE GUT

Phagocytic cells are abundant in the GI tract, poised to perform essential biological processes such as the clearance of apoptotic cells or for the sampling of antigens in the gut lumen. The

mononuclear phagocyte system (MPS), consisting of monocytes, macrophages and DCs contribute to a large proportion of these phagocytic cells found within the gut. Traditionally, these three groups of cells are classified under a single system under the notion that both macrophages and DCs were derived from a common monocytic precursor[51]. However, with recent evidences stemming from lineage tracing studies, the classification of these cells within the original MPS scheme requires some restructuring[52]. For example, it has been shown recently via genetic fate mapping or 'cellular barcoding' strategies that classical DCs are derived from a pool of common DC precursors of adult hematopoietic stem cell origins instead of monocytes as thought previously[53,54]. Further, CD11c integrin and MHCII complex, used as general markers for DCs are also found to be expressed by intestinal macrophages, rendering the use of these surface markers to identify functionally distinct APC subsets a serious challenge[55,56]. Following, efforts in classifying what is a DC or a macrophage by drawing inference from the literature is near impossible as investigators often categorize the cells of interest based on their perceived function, leading to further confusion among researchers. For instance, some may view $CX_3CR1^+CD11c^+$ cells as DCs since they were found to extend transepithelial dendrites[57,58] while others would consider them as intestinal macrophages due to their co-expression of macrophage markers such as CD64[59]. Nonetheless, the birth of a new classification method focusing on the developmental origins of these cells in combination with the identification of unique molecular factors that are crucial for the differentiation and/or maturation of specific subtypes may prove to be a step in the right direction[52].

For simplicity, I will classify the *bona fide* DCs as those that are dependent on Flt3L/Flt3R signalling that could either be CD11b positive or negative found within the small intestinal LP. Within the small intestinal LP DC populations, around 30% are known to express the gut-epithelial homing CD103 integrin in combination with CD11c and MHCII, as seen in close proximity with the intestinal epithelial barrier (Figure 3). Other DCs subsets in the SI LP include $CD103^-CX_3CR1^+CD11b^+$ DCs that can be further subdivided into CCR2 expressing or non-expressing DCs[60]. Conversely, intestinal macrophages are largely derived from blood $Ly6C^{high}$ monocytes that extravasate into the tissue, differentiating into $CD64^+CX_3CR1^{high}F4/80^{high}$ expressing tissue-resident macrophages[61-63]. A summary to show the various cell surface markers expression by monocytes, macrophages and DCs from the MPS system in the intestinal LP is presented in Table 1.

Functionally, DCs from the small intestinal LP but not macrophages are known to be able to migrate to the regional lymph nodes, in this case, the mLNs via the up-regulation of CCR7 expression to stimulate naïve T cells[60,64]. Interestingly, it has been shown that intestinal macrophages (CX_3CR1^{high}) are also capable of trafficking antigen from the LP into the mLN, albeit only during dysbiosis induced by antibiotics treatment[65]. The restricted migration of intestinal macrophages into the mLN was found to be myeloid differentiation primary response gene 88 (Myd88) signalling dependent[65]. Myd88 is an adapter protein involved in the downstream response of activated receptors of the toll-like receptor family, which recognizes a variety of bacterial-derived products (also known as pathogen associated

molecular patterns). The Myd88-dependent inhibition on antigen trafficking via intestinal macrophages therefore emphasizes the interaction between the resident microbes and its host to control unintended inflammation as a result of increased antigen presentation under steady-state conditions[65].

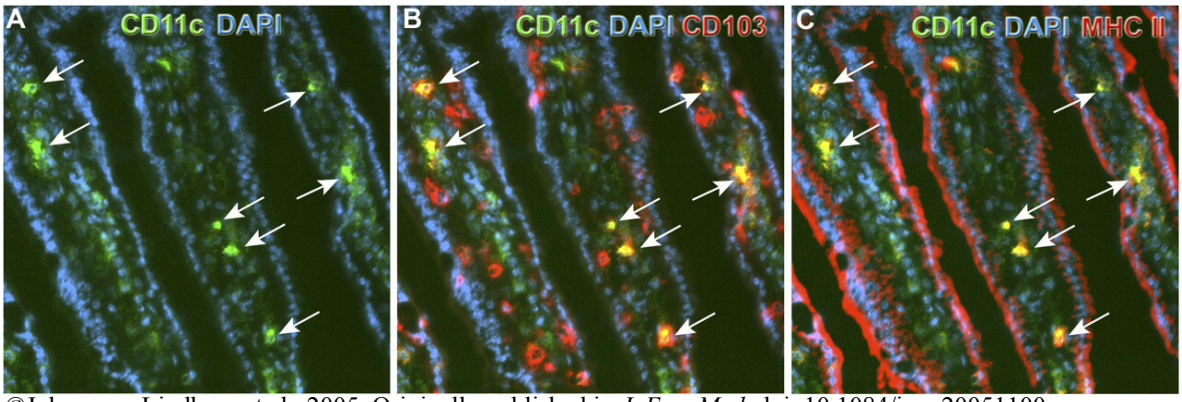

Figure 3. Antigen presenting cells are found in the lamina propria and are close to the intestinal epithelial cells. Intestinal villus sections co-stained for CD11c in green (A), CD103 in red (B) and MHC II in red (C), showing the presence of both $CD103^+$ antigen presenting cells (arrows pointing to $CD11c^+CD103^+MHCII^+$ marked cells) within the lamina propria of the villus.

The etiology of inflammatory bowel diseases (IBDs) has long been thought as a consequence of overt immune responses toward the otherwise harmless microbiota and food antigens. In turn, mechanisms that are tailored to promote tolerogenic environment in the intestinal mucosa are highly desired in order for the GI tract to optimally perform its intended physiological functions. For example, $CD103^+$ DCs isolated from both the LP and mLNs were found to be highly specialized in priming tolerogenic T cell responses via a retinoic acid-dependent manner to generate $Foxp3^+$ T_{reg}[66,67]. This functional specialization requires the expression of the aldehyde dehydrogenase 1a2 enzyme (ALDH1a2), which is involved in the conversion of dietary vitamin A to retinoic acid by $CD103^+$ DCs[67]. $CD103^+$ DCs in the LP and the mLN consists of either the $CD11b^+$ or $CD11b^-$ populations as described earlier. Interestingly, both subsets when singly targeted for deletion were found to be redundant for both mLN and LP T_{reg} populations[68]. A defect in the LP T_{reg} numbers was only revealed when both subsets of $CD103^+$ DCs were ablated simultaneously, possibly as a consequence of reduced 'gut-tissue imprinting' by these DCs, resulting in lowered expression of the gut-homing receptor CCR9 on mLN T_{reg} cells[68]. Taken together, these data highlight the increased propensity of both $CD103^+$ DCs in generating T_{reg} over other DC populations and suggests redundancies in gut imprinting of newly generated T_{reg}, possibly as a contingency plan to maintain tolerance in the LP.

Even though the preferential generation of T_{reg} is known to be driven by $CD103^+$ DCs, the maintenance of regulatory T cell signature/Foxp3 expression in T_{reg} was surprisingly dependent on the paracrine release of IL-10 from $CD11b^+$ myeloid cells[69]. Correlating with

an earlier study[70], these $CD11b^+$ myeloid cells were postulated to be intestinal macrophages[69]. Following, another study suggested that oral tolerance induction and T_{reg} expansion in the LP requires IL-10 secretion from intestinal CX_3CR1^+ macrophages[71]. Together, it seemed plausible that intestinal macrophages, similar to LP $CD103^+$ DCs, adopt an immune-tolerant phenotype in the gut microenvironment and secrete high levels of IL-10 to fortify the immune tolerant setting.

Surface marker expression by monocytes, macrophages and dendritic cells in the intestinal mucosa.

	Newly extravasated monocytes	Mature macrophages	Dendritic cells
CD11b	+	+	+/–*
CD11c	–	++	+++
CD14	+	++	–
CD64	Low	+++	–
CD103	–	–	+/–*
CD172a	+	+	+/–*
F4/80	Low	+++	–
MHCII	–	+++	+++
Ly6C	+++	–	–
CX3CR1	++	+++	+/–*

* These markers define functionally distinct dendritic cell subsets with specific transcription factor requirements.

Table 1. Cell surface expression levels of various known markers by different subsets of intestinal LP APCs. *Table from "The monocyte-macrophage axis in the intestine" by Calum C. Bain and Allan McI Mowat is licensed under CC BY 3.0*

Of interest, a recent report has shown that CX_3CR1^+ macrophages in an IL-10 deficient environment acquired a pro-inflammatory phenotype and were found in high numbers in the mLN[72], similar to what has been reported in animals with dysbiosis induced by antibiotics treatment[65]. When challenged with an intestinal pathogen (*C. rodentium*), $CX3CR1^+$ macrophages in an IL-10 deficient background produced elevated levels of IL-23, which was positively correlated to increased mortality of infected mice[73]. It was later confirmed in both studies that intrinsic/autocrine IL-10R signalling in intestinal macrophages was crucial for the prevention of spontaneous and infectious colitis respectively but not IL-10 *per se* in the IL-10 deficient background[72,73].

Through a concerted effort, intestinal DCs and macrophages play distinctive roles that shape the unique immune tolerant environment present in the intestines, important for oral tolerance induction and intestinal homeostasis. In addition, a recent study has provided novel evidence that oral tolerance induction requires intestinal DCs and macrophages to work cooperatively[50]. CX_3CR1^+ macrophages were demonstrated to send trans-epithelial dendrites

to first collect luminal antigens and then deliver these antigens through gap junctions to $CD103^+$ DCs that subsequently migrate to the mLN to prime responding T cells[50]. Of note, these specialized functions of intestinal DCs and macrophages resulting in differential T helper cell responses were discovered to be dependent on mouse strain/housing conditions as well as gut regional localizations, highlighting the influence of extrinsic factors on APC functions[74]. In support of this, intestinal macrophages were found to interact with their immediate microenvironment, for example, with enteric neurons within the muscular layers and to acquire preferential tissue protective characteristics compared to their counterparts found within the LP[75].

The prospect of having the AhR, acting as an environmental sensor in intestinal APCs, fine-tuning their effector functions in response to the dynamic microenvironment is therefore a likely event and a key interest of this thesis's work. In the following sub-chapter, I will introduce some of the published findings on the role of AhR in DCs and macrophages.

1.3.1 AhR in Antigen Presenting Cells

1.3.1.1 AhR and Dendritic Cells

Dendritic cells, given their ability to integrate signals from the environment via an array of innate PRRs and subsequent efficient activation of T cells, they are specialised to direct and orchestrate both innate and adaptive immune responses. DCs are a heterogeneous population of immune cells found in both lymphoid and non-lymphoid organs. Importantly, increased frequencies of non-lymphoid organ DCs are commonly found at host-environmental interfaces (the mucosal tissues) such as in the lungs, skin and gut surfaces[76]. Consequently, they are indispensable players in the maintenance of mucosal immune homeostasis[77] and also in the establishment of oral tolerance as mentioned earlier[78]. A recent work targeting the deletion of MHCII specifically in classical DCs further supported the central role of these cells in maintaining homeostasis[79]. The lack of antigen presentation by classical DCs was found to induce chronic inflammation in the gut, which could be alleviated by antibiotics treatment or completely abolished in animals raised in germ-free conditions[79], emphasising their role in maintaining host-microbe mutualism. Interestingly, the ablation of $CD103^+CD11b^-$ but not $CD103^+CD11b^+$ DCs in the colon was shown to worsen the severity of experimental colitis, revealing functional differences between different DC subtypes during inflammation. With the identification of functional AhR in DCs[80,81] and the reported roles of AhR functions in lymphocytes, we and others aimed to understand the role of AhR in DCs given their superior ability to modulate immune homeostasis.

Notably, AhR activation via treatment with a non-toxic ligand has been shown to give rise to tolerogenic DCs that preferentially drive the differentiation of $Foxp3^+$ T_{reg} *in vivo*[82] or inhibit Th17 expansion *in vitro*[83]. Accordingly, in the absence of AhR activation, the tendency of bone marrow derived DCs (BMDCs) in promoting T_{reg} formation in co-culture experiments was significantly reduced as shown in a kynurenine-dependent pathway[84]. Also, BMDCs treated with dietary AhR ligands; I3C or Indirubin-3'-Oxime were found to alter their cell

surface marker expression levels and cytokine secretion profile[85], a phenomenon that could be driven by the cross-talk between AhR and NF-κB signalling pathways[86]. In parallel, treatment of human monocyte-derived DCs with AhR ligands such as 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester or ITE was found to deliver similar effects[87]. However, the results presented by various groups were not completely consistent. Maturation markers, for example, CD86 expression by BMDCs were increased in some studies[86,88,89], but not in others upon AhR activation[87,90]. These discrepancies could be easily explained due to the use of different AhR ligands and hence we should exercise caution when interpreting the role of AhR in these cells when different types of ligands were used in experiments. Nonetheless, these studies highlighted the heterogeneity of AhR activation in DCs, dependent on the type of AhR ligands used and the source/subtype of DCs tested.

An alternative way to study the role of AhR in DCs was to perform loss of function studies. Two independent groups had noted that DC-specific deletion of AhR partially abrogated the well-established immuno-suppressive effects of TCDD administration in animal models for lung infection[91] and multiple sclerosis[92]. Taken together, the current literature supports the perception that AhR activation in DCs, *in vitro* and *in vivo* model systems, is needed for preventing hyper-reactive immune responses.

1.3.1.2 AhR and Macrophages

Macrophages are widely viewed as highly phagocytic cells that are important for clearing cell debris, engulfing bacteria and participating in tissue repair and remodeling mechanisms among other diverse functions. Investigations on the role of AhR in macrophage functions were relatively fewer however; data supporting its role in macrophages, important for host defense had already emerged. Using THP-1 (human monocytic cell line) cells and bone marrow-derived macrophages, the authors of one study demonstrated the need for AhR in macrophages to facilitate its activation upon exposure to IFNγ and *M.* tuberculosis[1]. The loss of this activation step was believed to be the cause of increased susceptibility to *M. tuberculosis* infection of AhR KO mice in the same study[1]. Subsequently, the discovery of pigments from *M. tuberculosis* (eg. naphthoquinone phthiocol) that could bind and activate the AhR led the authors to propose adding the AhR to the list of PRRs. Corroboratively, another study has reported the functional requirement for intrinsic AhR activity to promote the survival of macrophages, in addition to the production of reactive oxygen species by these cells to clear an intracellular bacteria- *L. monocytogenes* infection[93]. Interestingly, *L. monocytogenes* infection was found to induce higher levels of IL-6 and TNFα production by AhR-deficient macrophages, which correlates with the increased mortality of AhR KO animals upon the same infection[93]. The authors then concluded that AhR is essential for the suppression of pro-inflammatory cytokines secretion by macrophages while enhancing their survival and ability to kill bacteria[93]. In support of this, an overexpression of a pre-microRNA species that blocks the translation of ARNT (AhR's dimerization partner) was found to reduce the suppression of pro-inflammatory cytokines by AhR activating ligands in lipopolysaccharide (LPS)-activated macrophages[94]. Together, it appears that AhR activating

signals restricts pro-inflammatory circuits in macrophages, similar to what has been reported thus far for AhR-mediated effects in DCs.

Given the close proximity of LP APCs to the intestinal barrier, we became interested in the interactions between APCs and intestinal epithelial cells (IECs) in the context of maintaining mucosal homeostasis under steady-state conditions. Intestinal APC subsets were recently shown to participate in β-catenin/Wnt signalling pathways[95], which is a major signalling pathway involved in regulating intestinal epithelial cell development and function. With evidences of cross talk between AhR and β-catenin/Wnt signalling pathways in various cellular contexts, we postulated that by deleting AhR specifically in intestinal APCs, we might reveal defects in epithelial barrier functions, as a consequence of Wnt signalling perturbations in APCs. In the next chapter, I will first introduce the role of IECs in innate immunity, followed by the reported interactions between IECs and intestinal APCs. A summary on intestinal epithelium differentiation and renewal with reference to AhR and/or APC mediated effects will also be presented.

1.4 INNATE IMMUNITY: ROLE OF INTESTINAL EPITHELIAL CELLS

The intestinal epithelial barrier (IEB), represented by only a single layer of cells has provided a niche for the exchange of ions, metabolites and dietary components among others across the epithelium. Hence, the IEB acts as the gateway for the myriad of signals originating from the lumen, which targets various aspects of host physiology, including critical biological processes such as shaping the mucosal immune system. The breakdown of the IEB results in unresolved inflammation over time and is one of the most prevalent causes of chronic IBDs, signifying the undisputed role of the IEB in regulating immune responses of the gut[96].

Apart from forming a physical barrier, the IEB constantly secrete factors to help keep the microbiota in check. The IEB is made up of mainly absorptive cells (enterocytes) but also contains in a salt-and-pepper fashion, numerous secretory cell types (Goblet cells, Paneth cells, Enteroendocrine cells and Tuft cells). Mucin-2 (Muc-2), a heavily glycosylated protein secreted predominately by goblet cells in both the colon and the small intestines forms an important extracellular matrix structure that prevents the invasion of microbes. Particularly in the colon, Muc2 participates in the formation of an inner denser mucus (devoid of microbes) and an outer domain, which is loose and allows the seeding of microbes[97]. It is believed that the outer layer mucus provides an ecosphere, with attachment sites for the microbiota, which consequently facilitate the selection of certain species of bacteria that could colonize the gut[98]. Genetic ablation of Muc2 expression in mice was shown to cause spontaneous colitis in addition to increasing the chance of these animals to develop tumors[99]. This underscores the protective role of epithelial-derived mucin against uncontrolled inflammation and tumorigenesis. Other factors such as defensins and a C-type lectin (regenerating islet-derived protein III γ- Reg3γ) that provide bactericidal activities are mainly produced and secreted by the Paneth cells found at the base of small intestinal crypts. These Paneth-cell derived factors control the numbers as well as the composition of microbes by targeting conserved moieties of their outer membrane structural proteins. Of note, the production of these antimicrobial

peptides can be increased via IL-22 or IL-17 signalling in IECs[100]. These cytokines are secreted by various mucosal LP immune cell subsets[101] and shown to be important for the defense against pathogenic bacteria[102].

Recently, intrinsic activation via microbiota-derived signals in IECs were reported to be crucial for promoting IEC survival via the production of epidermal growth factor receptor ligands that may act in an autocrine loop[103] or enhance IEC function by promoting the expression of tight junction proteins to maintain the IEB[104]. Of note, many of these responses are downstream of the Toll-like receptor (TLR) signalling pathways and together with other microbial recognition pathways, converge to enhance NF-κb activity in IECs[105]. Moreover, a breakdown interactions between these pathways were found to induce spontaneous colitis[106] or increase the susceptibility of mice to chemically induced colitis[107]. In summary, these findings underscore the significance of the interrelationships between the resident-microbes and IECs, critical for IEB function, homeostasis and repair.

Apart from microbial-recognition pathways, recent findings have provided insights about normal physiological processes (in this case ER stress response), which when they are perturbed in IECs can lead to spontaneous enteritis[108]. The induction of ER stress intrinsically in IECs via the ablation of X-box-binding protein 1 (Xbp1) was shown to deplete differentiated Paneth cells and to a lesser extent, Goblet cells in the small intestines[108]. In general, it was noted that Xbp1 deletion in IECs increased their production of pro-inflammatory cytokines and chemokines in response to TNFα or TLR5 agonist stimulation[95]. Of interest, hypomorphic variants of the *XBP1* gene was found to confer a genetic risk for developing IBDs[107]. Additionally, in a follow up study, specific deletion of Xbp1 in Paneth cells was sufficient to induce spontaneous enteritis, leading the authors of the study to conclude that Paneth cells could serve as the initiation site for intestinal inflammation, implying a possible mechanism behind Crohn's disease (CD)[109]. Surprisingly, the authors did not find any changes to IEB permeability in IEC-specific Xbp1 KO animals, suggesting that permeability changes is not a prelude to inflammation in this context[108]. Nonetheless, these findings indicate that a defective response of the IECs to stimuli from the local environment can result in disease.

In support of the above, molecular factors targeting the AhR signalling pathway in IECs were recently shown to modulate intestinal inflammation[110,111]. In one of the two studies conducted, an inverse relationship between microRNA-124 and AhR protein levels was noted when analyzing colonic samples from active CD patients[110]. Subsequently, miRNA-124 was found to negatively regulate AhR protein translation and its overexpression could exacerbate experimental colitis[110]. Conversely, in another study, AhR activation in IECs via FICZ treatment was found to reduce IEC-derived IL-7, ameliorating inflammation induced by dextran sodium sulphate (DSS)[111]. Taken together, it appears that AhR functions as an environmental sensor in IECs, where its activation limits IEC response to inflammatory signals, promoting tolerance.

1.4.1 Intestinal Epithelium and Mucosal Antigen Presenting Cells Cross Talk

Besides creating separate domains (inside versus outside), the intestinal epithelium also participates in generating efficient immune responses at highly specialized sites such as the Peyer's patches (PP) or isolated lymphoid follicles. Consequently, the epithelium at those sites is commonly referred to as follicular-associated epithelium (FAE). Under steady state conditions, Microfold (M) cells represents approximately 10% of all epithelial cells found within the FAE, a specialized cell type for the phagocytosis and transcytosis of luminal antigens across the epithelium to be captured by APCs in the underlying lymphoid follicle[112]. This process of antigen sampling, through M cells has been shown to be critical for generating immune-tolerant IgA responses toward the commensal microbiota[113]. The antigen sampling by the intestinal epithelial has long thought to be restricted to M cells however, it has been shown recently that small intestinal Goblet cells could also assist in antigen sampling by transferring soluble antigens to $CD103^+$ DCs directly[114]. Although the functional significance of these two distinct routes of antigen entry mediated by the epithelium remains unclear, it is evident that the IEB cooperates with sub-epithelial APCs to prime adaptive immune responses, implying a key role for their interaction in intestinal immune homeostasis.

In the presence of commensal microbiota, the intestinal epithelium plays an important role in inducing tolerance. Epithelial-derived factors such as TGF-β1, TSLP and retinoic acid (RA) are secreted in response to microbiota-derived signals and were found to imprint dendritic cells in the intestinal lamina propria, instructing them to drive T_{reg} responses over other T helper cell subsets in the draining lymph nodes[105,115]. Corroboratively, migratory DCs isolated from the mLNs were found to induce higher levels of T_{reg} compared to DCs isolated from the spleen in an RA and TGF-β1 dependent manner as mentioned earlier[66,67]. Importantly, the activation of retinoic acid receptors on T cells up-regulates their expression of CCR9, allowing them to migrate towards the gut upon leaving the lymph nodes. Thus, the conditioning of DCs via IEC-secreted factors, which indirectly instructs the gut homing of activated T cells, is essential for inducing tolerance in gut mucosal tissues.

While these studies highlight an active interaction between mucosal DCs and the intestinal epithelium in response to environmental factors, DC-dependent effects acting on the intestinal epithelium is much less understood.

1.4.2 Intestinal Epithelium Differentiation and Renewal

The intestinal epithelium is one of the most regenerative organ of the body, requiring only an estimated three to five days for the whole epithelium to be replaced with fresh cells orientating from the crypt bottom, in mice (Figure 4). This remarkable feat is accomplished through the presence of a stem cell niche at the base of the crypt where intestinal stem cells (ISCs) expressing the Leu-rich repeat-containing G protein-coupled receptor 5 (Lgr5) can be found sandwiched by Paneth cells, in the small intestines. These $Lgr5^+$ ISCs (also known as crypt base columnar cells) can undergo continuous cell cycling for self-renewal and to give rise to daughter cells, which go on to repopulate the gut epithelium at regular intervals. This

rapid turnover program is usually tightly regulated, but can be disrupted during pathological conditions where signalling pathways governing ISC proliferation and differentiation are perturbed, leading to inflammation[109,116] and/or tumorigenesis[108,117-119]. Of note, energy deprivation as a result of calorie restriction has been shown to enhance ISC function, emphasizing the responsiveness of the ISC niche to the dynamic microenvironment[120]. Taken together, the intestinal epithelium and its characteristic high turnover rate are well suited to respond swiftly to a wide array of insult and also changes in physiological requirements.

Broadly categorized into absorptive and secretory cell types, IECs can be further subdivided into five different mature subtypes. The over-arching signalling mechanisms that regulate $Lgr5^+$ ISC maintenance versus proliferation and/or differentiation into different subtypes rely mostly on two signalling pathways: Wnt signalling and Notch signalling[119]. While activation of both signalling cascades are crucial in maintaining the 'stem-ness' of a $Lgr5^+$ ISC, the differentiation programs of progenitors may reflect a disparity in preference for the two pathways. For instance, a Notch target gene, Hes-1, activates genes involved in the differentiation of precursors into the absorptive lineage while suppressing secretory cell-type specification by down-regulating Math-1[121]. In contrast, Wnt signals activates Math-1 expression, and in conjunction with other transcription factors, contribute to the differentiation of progenitors into one of the four known secretory lineages[122,123]. Interestingly, a recent study found that by suppressing Wnt signalling simultaneously, one could block the significant expansion of Math-1^+ secretory cells caused by Notch signalling blockage[124]. These data suggests that a constant re-balancing of morphogenic pathways is necessary for the full functioning of the gut epithelium. Of note, the reduction of Dkk1 (a Wnt antagonist) expression was found to increase IEC proliferation, mainly in the colon and enhanced recovery upon DSS induced colitis[125]. However, these mice also developed abnormal crypt architecture coupled with hyper proliferation of IECs during epithelial restitution in response to the injury[125]. This emphasizes the need for a delicate fine-tuning of well-balanced signals for intestinal epithelial homeostasis and repair.

Not until recently, most *in vitro* studies involving IECs were performed on transformed cell lines or cells derived from human colorectal adenocarcinomas. In 2009, Sato T., et al showcased a robust method to culture primary intestinal epithelium in a three-dimensional matrix derived from a single FACS sorted $Lgr5^+$ ISC[126]. That was just two years after the discovery of *Lgr5* as a marker for ISCs in both the small intestines and the colon[127]. The elegance of this *ex vivo* system is that one could observe, in real time, the transformation of a single ISC into an organoid containing numerous budding structures that resemble individual crypts *in vivo*. In essence, it is now possible to study the differentiation of IECs, from committed precursors into mature differentiated cell types. Targeting various signalling pathways or genetically ablate or overexpress genes, techniques that were technically challenging and time consuming to perform *in vivo* could now be simplified. Following we harnessed the benefits of this technique to establish a co-culture system to study the

interaction of mucosal DCs (with or without AhR signalling) with the intestinal epithelium (Paper I).

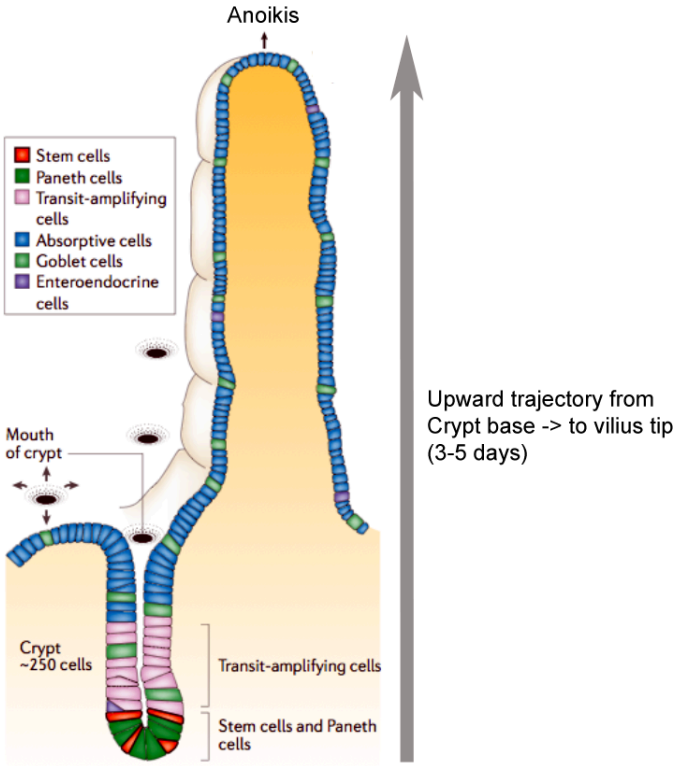

Figure 4. Movement of progenitor cells up the crypt-villi axis is a continual process. Adapted by permission from Macmillan Publishers Ltd: *Nature Reviews Genetics* **7**, 349-359, copyright (May 2006)

Non-cell autonomous effects of Paneth cells was recently demonstrated to influence ISC biology[120], suggesting that cell types that are in close association with the intestinal epithelium may similarly respond to signals from the environment, in turn affecting IEC function. Apart from immune cells and mesenchyme cells, cellular components of the enteric nervous system (ENS) were found to be in close association with the intestinal epithelium (in the villi and areas surrounding the crypt). In the next chapter, I will introduce the ENS briefly, followed by how the ENS contributes to intestinal barrier functions and immunity.

1.5 THE ENTERIC NERVOUS SYSTEM

The ENS is known to have extensive control over physiological processes such as GI motility, fluid secretions, the local control of blood flow as well as mucosal immune system regulation. Primarily, the ENS is arranged into two concentric plexuses namely the myenteric plexus (MP) and the submucosa plexus (SMP). The MP is 'sandwiched' between the longitudinal muscle and circular muscle layers while the SP is found within the submucosa layer as shown (Figure 5). The intrinsic nervous system of the gut (ENS) also receives inputs from the CNS by both sympathetic (via celiac ganglion) and parasympathetic pathways (vagus nerve), hence may also act as the gateway for the brain-gut axis.

Being the largest component of the peripheral nervous system with neuronal numbers matching those that are found within the spinal cord, the ENS earns itself the title of the 'second brain'[128]. The ENS is composed of both neurons and glial where glial cells were reported to be outnumbering the enteric neurons at a ratio of 4:1 or higher[129]. A tissue preparation of the longitudinal muscle and myenteric plexus (LMMP) revealed multiple ganglia consisting of neuronal cell bodies in close contact with enteric glial cells (EGCs). Each ganglion could also be seen interconnected by inter-ganglionic connectives (Figure 6 and Figure 7). In addition to their primary locations within the plexuses, nerve fibres and glial cell bodies can be found extending deeper into the mucosal layers and within the villus[130,131].

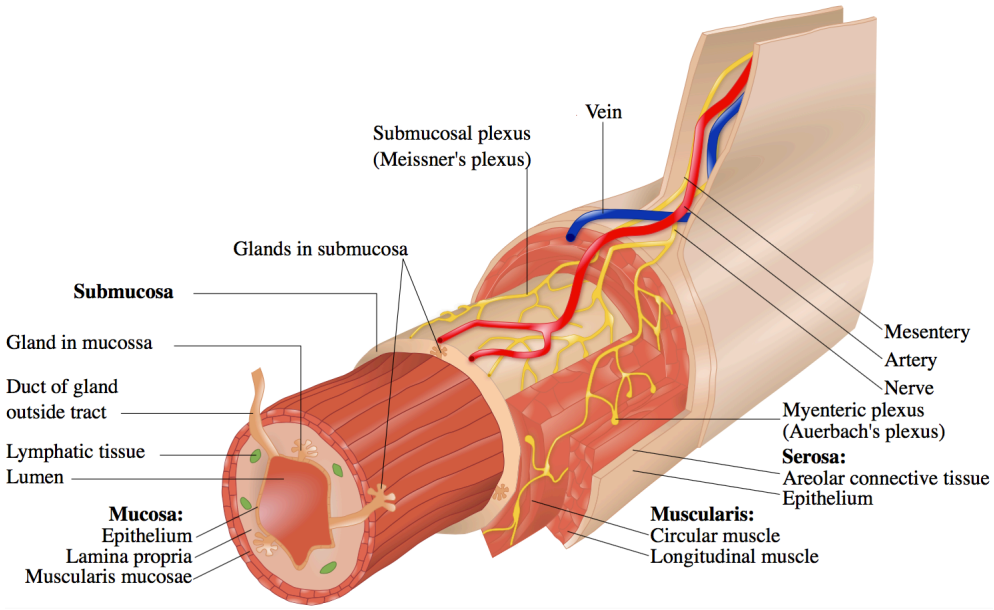

Figure 5. Locations of the myenteric- and the submucosa plexus found within the gut wall. *By Goran tek-en [CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0)], via Wikimedia Commons. https://upload.wikimedia.org/wikipedia/commons/3/31/Layers_of_the_GI_Tract_english.svg*

The development of the ENS (in *mus musculus*) starts with the delamination of a subset of vagal neural crest cells from the neural tube at around E8.5, which later invade the foregut mesenchyme at around E9.0-E9.5. Upon entry, these cells now designated enteric neural crest cells (ENCCs) migrate in a rostro-caudal direction to colonise the whole gut, a process which is completed by around E15-E15.5[132]. Of note, sacral neural crest cells also contribute, albeit at lower levels compared to vagal neural crest contributions. Sacral neural crest cells colonise the hindgut in a caudal to rostral direction starting from the colon at around E13.5[133]. Of note, the survival, proliferation and migration of these ENCCs are essential for the completion of the developing ENS. Molecularly, the expression of receptor tyrosine kinase RET, SRY-box 10 (Sox10) transcription factor and G protein coupled receptor- endothelin receptor B (Ednrb) in migrating ENCCs were discovered to be vital for their survival and timely migration throughout the GI tract during embryogenesis[134]. As such, genetic perturbations in these genes and their associated signalling pathway components can lead to varying degrees of aganglionosis, which is a failure of ENCCs to completely colonise the whole GI tract, resulting in distal regions of the gut wall being deprived of neurons and glial cells[134].

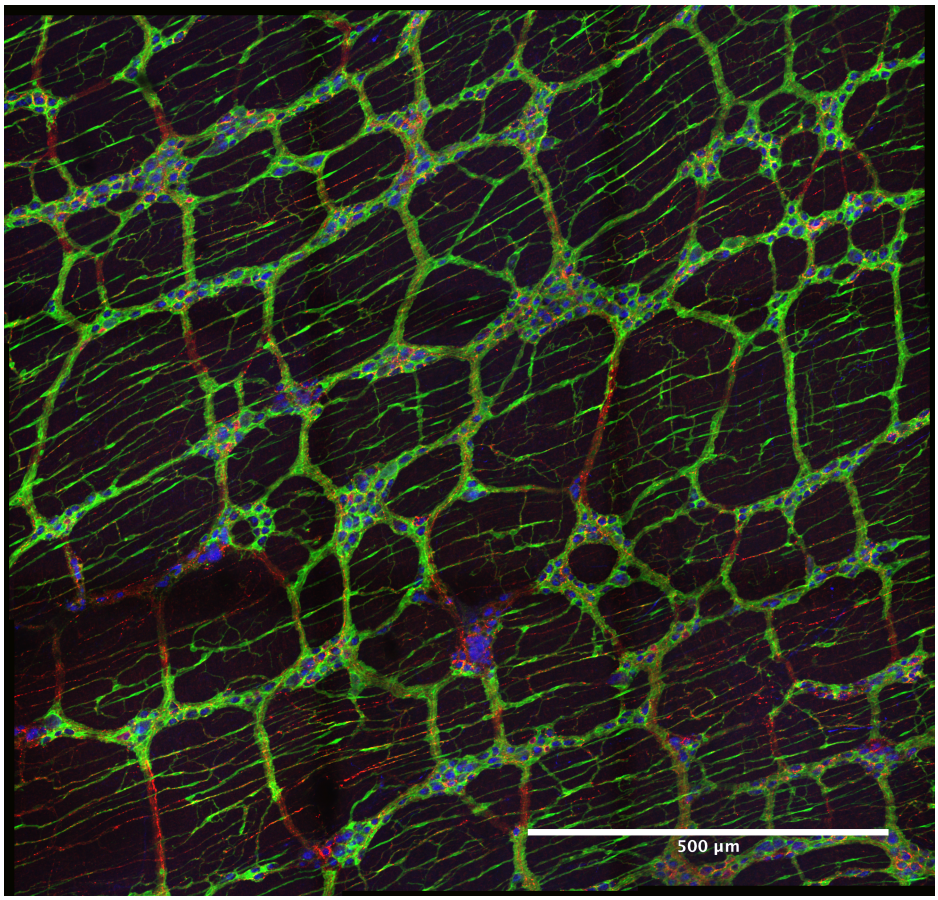

Figure 6. The Myenteric Plexus. Neuronal cell bodies and choline acetyltransferase (ChAT) positive fibres are labelled in blue (HuC/D) and red ($ChAT^{+}$) respectively while EGCs are labelled in green ($S100\beta^{+}$) via fluorescence immuno-staining.

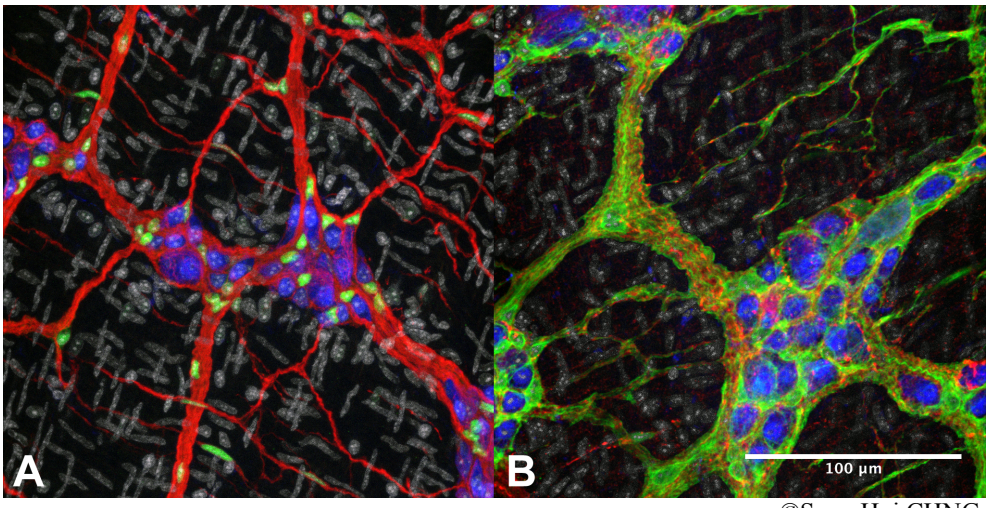

Figure 7. A closer look into individual ganglia of the myenteric plexus. Neuronal cell bodies are labelled in blue (HuC/D). Neuronal fibres stained in red are visualised with antibodies against Tuj1 (A) or ChAT (B). EGCs are labelled in green via Sox10 nuclear staining (A) or S100β immuno-staining revealing glial fibers (B).

In human patients, aganglionosis often occurs in the distal region of the gut and can be easily identified at birth due to the presence of congenital megacolon, commonly known as the Hirschsprung's disease. Other intestinal motility disorders such as slow transit constipation and intestinal pseudo-obstruction where subtle defects in ENS functionality were found [135,136] also underscores the importance of having a fully functional ENS for intestinal health.

Recently, the roles of the ENS in controlling intestinal inflammation have been described, providing evidences for bidirectional communication between the intrinsic nervous system of the gut and intestinal immune cells, which is in the interest of this thesis. In the subsequent chapters, I will cover some of the recent advances in our understanding of how the ENS can modulate intestinal immune functions. Topics on how our resident microbiota influences the function of the ENS will also be discussed.

1.5.1 Role of ENS in mucosal immunity

In recent years, our understanding of how the gut-brain axis can modulate inflammatory responses has only begun to take flight. The seminar study conducted by Tracey and colleagues[137] had demonstrated the capacity of vagus nerve stimulation (VNS) on dampening immune responses upon LPS challenge *in vivo*, paving the way for many studies thereafter. Since then, several studies have demonstrated an increased sensitivity of vagotomised animals to models of intestinal inflammation, such as those induced by DSS [138-140]. Notably, a recent paper has provided the mechanistic evidence of how VNS could control post-surgery intestinal inflammation (post-operative ileus - POI), independent from VNS effects on the spleen or T cells.[141] The authors showed that the activation of α7 nicotinic acetylcholine receptor (α7nAChR) expressed by intestinal resident macrophages mediated via VNS could reduce the levels of pro-inflammatory cytokines such as IL-6 and IL-1β detected in the muscularis externa after POI induction[141]. The same study concluded by proposing that the anti-inflammatory effects of VNS on surgery-induced intestinal inflammation was an indirect consequence through the actions of cholinergic neurons of the myenteric plexus acting on resident macrophages[141]. In parallel, an earlier study showed that the activation of cholinergic myenteric neurons by 5-HT_4R agonists could protect against POI by inducing the release of acetylcholine from these neurons that acts on α7nAChR expressed by macrophages to inhibit pro-inflammatory responses[142]. Conversely, intestinal inflammation was found to be associated with changes to the chemical nature of myenteric neurons[143] or increased activation of enteric glial cells[144]. Taken together, these findings highlight the constant dialogue between the ENS with inputs from the central nervous system among others to regulate immune functions of the gut.

An increasingly important aspect in understanding human physiology is the study of the less human, our resident microbiota. Recent advances in the field of host-microbe interactions have uncovered many unanticipated properties of microbes for example, the putative ability of the microbiota in regulating anxiety-like behaviour of experimental organisms[145,146]. Whether these observations are linked to direct or indirect mechanisms originating from the luminal microbiota remains to be determined. An interesting feature of the ENS and its

constituents is that, PRRs such as TLRs commonly expressed by immune cells are also expressed by enteric neurons and EGCs[147]. Therefore, it is plausible that the ENS, with its vast organisation of neurons and glia cells covering areas including the mucosal layers, could respond directly to microbiota-derived signals. Indeed, some evidences in support of the above have been reported in the literature. In particular, TLR4 KO mice or mice with specific deletion of Myd88 in neural crest cell derivatives were shown to harbor reduced nitrergic neurons and exhibit delayed GI motility[148]. The authors also note that mice housed in germ-free conditions presents similar phenotypes when compared to TLR4 KO mice, implying a role for the microbiota in regulating GI motility through TLR4 signalling[148]. A plethora of studies lending their support for the above findings where changes related to the microbiota and/or diet, consequently modulating intestinal transit have been reported[149-153]. Although the exact mechanisms on how the diet-microbial axis could perform this intricate act remains inconclusive, some evidence points towards the involvement of the TLR signalling[148] and/or GDNF/RET[151,154] signalling pathways within the ENS in addition to energy availability derived from the diet[149,152]. Interestingly, the literature also provides some evidence where the effects of microbiota on ENS development was shown to present itself as early as 3 days postnatally[155]. Together, it seems that the ENS is highly plastic and matures alongside environmental cues from the microbiota and/or diet-derived signals after birth.

1.5.1.1 Bidirectional communication between the ENS and the immune system

Intestinal muscularis macrophages (MM), located within the smooth muscle layers were first described more than 20 years ago[156] and subsequently thought to interact with neurons at the layer of the MP to modulate immune responses in the gut[156,157]. Indeed, two recent findings have confirmed the interactions between enteric neurons and MM where their crosstalk was found to be important for controlling intestinal motility[158] or immune protection of the ENS in the context of an infection[75]. In the first study, the authors identified enteric neurons as the source for colony stimulating factor 1 (CSF-1), a factor crucial for the development of MM while bone morphogenetic protein 2 (BMP2), which was shown to activate the BMPRII signalling pathway in enteric neurons was found to be expressed by MM reciprocally[158]. In addition, BMP2 supplementation was shown to rescue the hyper-contractility reported in MM depleted mice, confirming the role of BMP2 produced by MM in regulating intestinal motility[158]. Interestingly, the reported communications between the two cell types (neuronal and immune cell) in controlling intestinal motility was also found to be microbiota-dependent[158]. Specifically, in antibiotics treated mice and in LPS-stimulated primary enteric neurons, BMP2 expression by MM was reduced and CSF-1 production from cultured primary enteric neurons was increased respectively[158], highlighting the previously unreported interaction between the two cell types driven by the resident microbiota. In the second study, it was revealed that upon bacterial infection, extrinsic sympathetic fibers innervating the gut muscularis were rapidly activated[75]. This swift neuronal response was coupled with the activation of β2-adrenergic receptors expressed by MMs that further reinforced its steady state tissue-protective phenotype, a phenomenon that was identified when the authors compared the gene-expression profiles of isolated MMs with LP macrophages[75]. The authors

then conclude that the autonomic response towards an intestinal pathogen may be important for the preservation of the integrity of the ENS against any inflammation-induced tissue damage[75]. Of note, the exact mechanisms activating the enteric neurons (intrinsic or extrinsic) by bacteria in these contexts remain unclear.

Analogous to microbiota driven effects on host responses as illustrated above, recent findings also shed light on the consequences of ENS defects on microbiota diversity and composition [159,160]. These studies were based on a mouse model of Hirschsprung's disease where the *Ednrb* gene was mutated in both alleles, leading to colonic aganglionosis at birth[161,162]. In addition to the skewed microbiota diversity in mutant animals, the authors of one of the two studies reported an increased risk for enteroinvasion by *E.coli* and impaired mucosal defence [159]. Of interest, the defective control of mucosal inflammation in TLR-2 KO mice was found to correlate with impaired Ret-GFRα1 signalling, which is important for maintaining the neuro-chemical coding and architecture of the ENS. Following, administration of exogenous glial cell-derived neurotrophic factor or GDNF (the ligand for Ret-GFRα1 signalling) was shown to restore the ENS defects and ameliorate chemically induced colitis in these TLR-2 KO mice when compared to untreated controls. Taken together, these findings underscores the inter-dependencies between the microbiota, the ENS and the immune system.

1.5.1.2 Interplay between the Enteric Glia Cells and the Intestinal Epithelium in Intestinal Inflammation

A relatively new functional unit coined as the gut-vascular unit, critical for inhibiting the systemic dissemination of unwanted materials such as microbes has been recently described [163]. In that report, it was postulated that the EGCs, together with pericytes that surrounds the endothelial cells form the gut-vascular unit, a similar cellular setup compared to the blood brain barrier in the CNS[163]. Increasingly, EGCs have been at the focus of understanding the role of ENS components in regulating intestinal immunity in addition to maintaining the integrity of the intestinal epithelial barrier[131,164,165]. Based on transcriptomic analysis[166], co-culturing of IECs with EGCs was found to induce the expression of genes related to differentiation and cell adhesion but repressed the expression of genes related to immunity and cell proliferation in IECs. At present, at least four different EGC-derived factors that could regulate IEC biology (differentiation and proliferation among others), consequently regulating intestinal barrier functions have been identified[167-170]. Of interest, S-nitrosoglutathione, one of the four glial-derived factors mentioned has been reported to block the down-regulation of ZO-1 expression by IECs during *Shigella flexneri* infection, reducing bacteria entry and lessening the lesions caused by the infection. In summary, these findings emphasize the role of EGCs in reinforcing the intestinal epithelial barrier, thus participating in the maintenance of intestinal homeostasis.

As mentioned earlier, the expression of various TLRs have been confirmed in EGCs, suggesting that these cells could respond directly to microbiota-derived signals[147,171]. For example, in a Myd88-dependent fashion, EGCs were found to induce NO production by secreting S100β, contributing to inflammation[171,172]. These results indicate a role for the

microbiota in directly regulating immune functions of EGCs. However, under steady conditions, it is not clear whether the microbiota has a role in the homeostatic regulation of EGCs (Paper II).

Of interest, EGCs were found to express the major histocompatibility class II (MHC II) molecule, albeit only in inflamed areas of CD patients and barely detectable in healthy controls[173]. Following, as several lines of evidence suggest, EGCs can 'turn on' their antigen presentation potential at the onset of inflammation or during infections[171,174,175], indicative of a role for EGCs in controlling inflammation. In support, two independent studies have shown that targeted deletion of EGCs can lead to spontaneous fulminant jejuno-ileitis[176] or enterocolitis analogous to human necrotizing enterocolitis[177], highlighting the remarkable immune-protective function of EGCs in the intestinal mucosa. Additionally, similar to astrocytes in the CNS, EGCs can be identified via their expression of the glial fibrillary acidic protein (GFAP)[178], where increased GFAP expression is associated with EGC activation[144]. Interestingly, the levels of GFAP in addition to GDNF were found to be differentially regulated when comparing patients suffering from ulcerative colitis to patients with CD[179]. The disparity between the reported levels of GFAP and GDNF in different IBD types may provide insights into the pathogenesis of chronic inflammatory disorders where EGCs plays a central role.

Using the expression of GFAP and the S100-calcium binding protein[180] to identify EGCs, a huge quantity of these cells can be found forming dense glial networks at the base of the crypts, including the mucosa, in both the small intestines and the colon[130,181] (also in paper III). Of interest, recent work by Bohorquez, D. V. and colleagues has demonstrated a direct physical association between EGCs with enteroendocrine cells (which are rich in vesicles containing hormones that regulate appetite and consumption), suggesting a role for EGCs in hormonal control[182]. Of interest, microbiota derived metabolites were recently shown to regulate serotonin biosynthesis in enteroendocrine cells[183,184], raising the possibility of an indirect effect of microbes acting on EGCs via epithelial cell types[185]. In summary, these discoveries uncover the existence of intricate relationships between EGCs and the intestinal epithelium still largely unexplored. For example, what is the physiological importance of EGCs found at the base of the crypts compared to those found deep into the mucosa (within the villi LP)?

In this thesis, we explored the highly dynamic interactions of various biological systems, with each other and/or with the external environment, using specific aims as presented on the next page.

2 AIMS

The overall aim of this thesis was to examine the interrelationships of different biological systems when interacting with the external environment, including the resident microbiota to maintain intestinal homeostasis. The GI tract was the organ of choice for our studies since multiple biological systems are in close association with one another, in addition to being constantly challenged with the myriad of signals emanating from the intestinal lumen.

Specific aims for each paper:

- Paper I – To study the role of an environmental sensor, the AhR in antigen presenting cells in the context of maintaining intestinal homeostasis and controlling inflammation.

- Paper II – To investigate the role of the microbiota in the homeostatic regulation of enteric glial cell networks.

- Paper III – To establish a stable co-culture system to uncover the significance of enteric glial cell interactions with intestinal epithelial cells.

3 METHODOLOGICAL HIGHLIGHTS

3.1 MICROFLUIDICS BASED GENE EXPRESSION PROFILING

During the course of our work, FACS sorted intestinal APC subsets (per mouse) yielded insufficient numbers for a microarray type study. Fortunately, we came across a new 'chip', which allowed us to perform qRT-PCR based assays with a pre-selected set of genes using much less starting material. The chip required minute volumes per sample, at 5µl, due to its architecture that operates using microfluidics-based technologies[186]. A total of 48 or 96 samples can be loaded onto each chip (depending on the type of chip used). These samples will then be individually mixed with 48 or 96 gene-expression assays of choice in segregated micro-chambers on the chip. This means that one could compare the relative expression of at least 48 genes from one sample to the other 47 samples tested in one go. We managed to interrogate the relative expressions of 48 genes of interest among distinct intestinal APC subsets and across biological groups using this method. Of note, introducing air bubbles while manually loading samples and/or assay reagents could destroy the whole setup process. The priming (mixing of samples and assay reagents, guiding them into their respective micro-chambers) of the chip is done automatically via a machine. Importantly, there are two issues to note when using this technique. First, the approach is biased and one might miss interesting differences due to the pre-selection criteria. Second, a pre-amplification step for each cDNA sample, using the primers designed for all 48 genes of interest needs to be performed. Assuming that amplification efficiencies for each specific amplicon are different, one should not compare the relative expression of selected genes within the same cell. Nonetheless, it is still a powerful technique for comparing between groups, when sample volumes are scarce.

3.2 GERM-FREE ANIMALS

The germ-free (GF) model is currently one of the most powerful techniques employed to study the interrelationships between microbes and its host. The title of 'germ-free' refers to animals that are raised in a completely sterile environment, devoid of all other microorganisms such as bacteria, viruses and fungi. The concept of a GF model is attributed to Louis Pasteur more than a century ago, although he believes that life cannot flourish without the presence of microorganisms. Indeed, it took a number of years before germ-free colonies (rats) could be setup, due to the lack of knowledge on diet supplementation. Vitamins B and K needs to be added in the diet for example, as they will be lost in the absence of microbes. Apart from nutritional supplements, another major challenge was to keep animals GF, without any contamination. Current technologies utilize clear plastic isolators, where in-flow of air is sterile filtered and all materials such as bedding and cages were autoclaved before use. Additionally, frequent tests for bacteria growth in fecal homogenates are done to ensure that colonies remain GF. 16s PCR testing are also occasionally carried out to identify any non-cultivable bacteria that were present.

The derivation of germ-free mice starts with performing cesarean sections on full term mothers and deliver the pups, still in the uterine sac, into the plastic isolators after passing

through antiseptics to be resuscitated and cared for by GF foster mothers. An advantage of this method is that it allows researchers to derive his/her genetically modified animals as GF, to study the interactions between the microbiome and the gene of interest. Alternatively, researchers can also mono-colonize or introduce a group of known microbes into GF animals, giving rise to gnotobiotic ('known life' in Greek) animals to question the role of a particular species and/or composition of bacteria. Importantly, experiments should not start right after derivation into GF conditions as the first litter came from a mother that was not GF and hence were all exposed to microbial influence during *in utero* development.

Anatomically, GF mice are very different from conventionally raised animals. Most notably, the caecum of GF mice can be as big as 4-8 times compared to their conventionally raised counterparts, due to the accumulation of undigested fibers. In addition to anatomical differences, various defects in the development, metabolism and the immune system of GF animals have been reported[187]. Hence, disease phenotypes associated with GF conditions could also be seen as secondary consequences due to the absence of microbes. In such cases, wide-spectrum antibiotics are often used to decipher the direct roles of the microbiota. Still, the GF model remains a key tool in demonstrating the intricate involvement of our resident microbiota in our daily lives, spurring further on-going mechanistic studies.

3.3 TISSUE-SPECIFIC REPORTER MOUSE LINES

To perform tissue-specific ablation of a gene of interest, most researchers rely on the Cre-lox system. The Cre enzyme is capable of performing site-specific recombination by recognizing short target sequences known as loxP sites. Depending on the orientation of loxP sequences, the Cre enzyme can either excise the sequence sandwiched between two loxP sequences in tandem (Figure 8A) or catalyse the inversion of sequences flanked by two loxP sites ('flox-ed') that are in opposite orientations (Figure 8B). Cre expression can be restricted to specific tissues (or cell types) by the use of tissue-specific promoters/enhancer elements.

For temporal control of Cre activity (especially useful if a gene of interest was found to be embryonically lethal when deleted constitutively), the Cre enzyme was 're-engineered' to fuse with a mutant form of the human estrogen receptor (also known as the $CreER^{T2}$ transgene), keeping the recombinase from entering the nucleus upon its translation in the cytoplasm. Only when tamoxifen is administrated, the engineered protein would be able to translocate into the nucleus to perform its intended function in a timely fashion.

Apart from deleting genes temporally via scheduled injections of tamoxifen, reporter constructs can be introduced instead to perform lineage-tracing studies at specific time points. The *R26R-Confetti* multi-colored construct is one such example (Figure 9). The *R26R-Confetti* multi-colored reporter was first used in conjunction with a heterozygous mouse carrying the $CreER^{T2}$ transgene, under the control of *Lgr5* promoter/enhancer elements, to study the dynamics of ISC function in maintaining the intestinal epithelium[188]. The design of the construct was adapted from an earlier work (Brainbow 2.1), which encodes for four fluorescent proteins derived from jellyfish[189]. The process of excision and/or inversion of the

floxed reporter alleles are stochastic and random, where labeling efficiencies are dependent on the dose of tamoxifen given.

During our studies, we combined the *R26R-Confetti* allele with mice heterozygous for Sox10::CreER^{T2}, allowing us to stochastically label peripheral glial cells with one of the 4 possible colors. Using this strategy, we studied the dynamics of adult EGC networks.

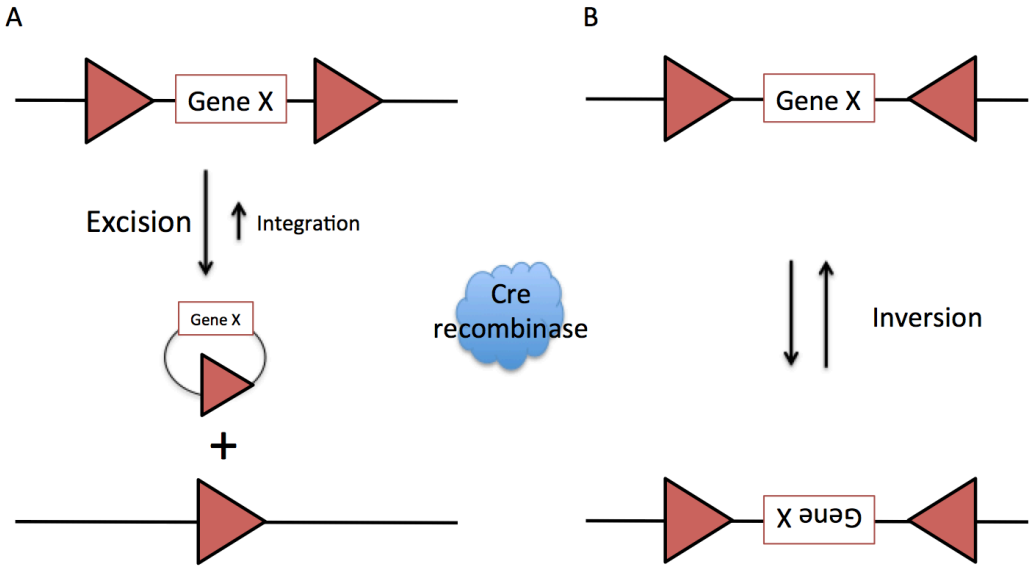

Figure 8. Cre mediated excision or inversion of 'floxed' genes/sequences

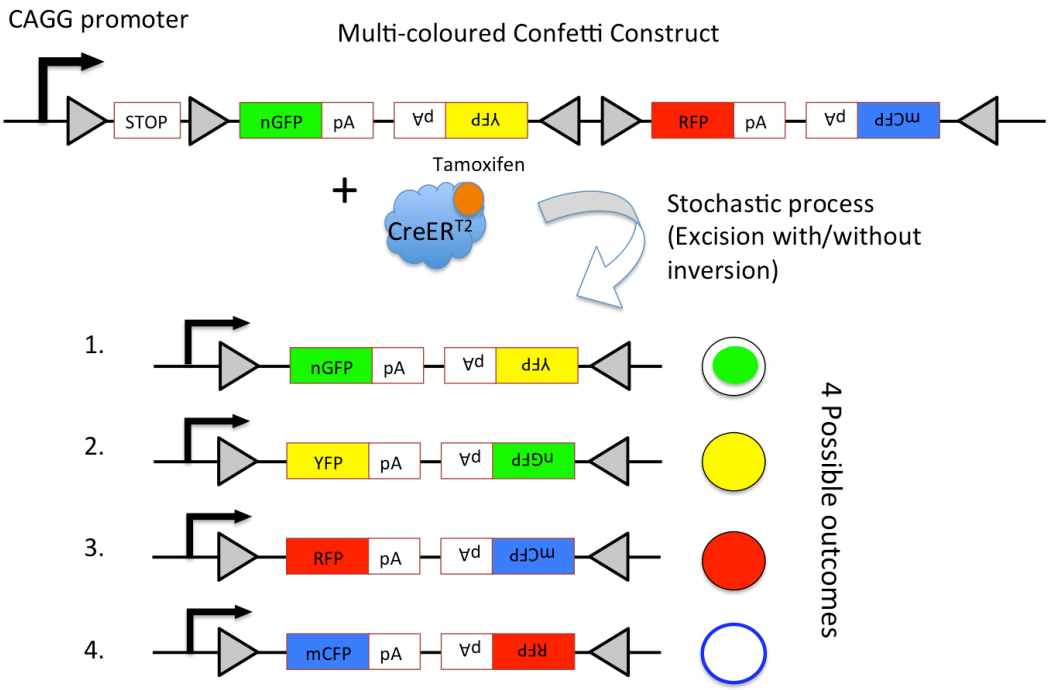

Figure 9. *R26R-Confetti* transgene. Stochastic action by the CreER^{T2} enzyme dependent on tamoxifen induction can generate 4 possible colours from the multi-coloured reporter. nGFP- nuclear GFP. mCFP- membrane-tethered CFP. The expression of RFP or YFP is cytosolic as shown. pA- poly-A tail.

4 RESULTS AND DISCUSSION

This thesis represents a compilation of three papers that in a concerted manner, examines the dynamic interactions between different biological systems of the host with the external environment. The first paper focuses on an environmental sensor and its function in sentinel cells such as the mucosal APCs, important for intestinal innate immunity and homeostasis. The second paper investigates the enteric glial cell network post-natally and how this process is regulated by the presence of microbiota. Finally in the third paper, we proceeded to set up a co-culture system to study the interaction between intestinal epithelial cells and enteric glial cells in a three dimensional matrix.

4.1 PAPER I: ABLATING THE ARYL HYDROCARBON RECEPTOR (AHR) IN CD11C+ CELLS PERTURBS INTESTINAL EPITHELIUM DEVELOPMENT AND INTESTINAL IMMUNITY

The involvement of AhR signalling in various autoimmune and chronic inflammatory diseases has been shown in the context of AhR KO mice[34,40,45]. We were interested to examine if the loss of AhR specifically in intestinal APCs could modulate the disease progression of a well-established acute colitis model. To achieve this, we proceeded to obtain a mouse line with specific deletion of AhR in the intestinal APCs. We crossed the $AhR^{fl/fl}$ mice generated by the laboratory of Dr. Christopher A. Bradfield[190], which is available at Jacksons' Laboratory with another mouse line expressing Cre-recombinase under the control of the CD11c promoter. The CD11c driver for Cre-recombinase was selected since both intestinal DCs and macrophages constitutively express CD11c, allowing us to delete AhR in both populations simultaneously. After a few rounds of crossings, we maintained our intestinal APC-specific AhR KO mouse line by crossing $AhR^{fl/fl}$; $CD11c::Cre^{+/-}$ with $AhR^{fl/fl}$; $CD11c::Cre^{-/-}$ animals to generate equal proportions of experimental ($11c^{AhR-/-}$) and control ($11c^{AhR+/+}$) mice. RT-PCR data generated from FACS sorted populations of various DC and macrophage subsets from the LP showed efficient depletion of AhR expression only in Cre^{+} ($11c^{AhR-/-}$) animals.

Exposed to 2% DSS in their drinking water, we found that the experimental group ($11c^{AhR-/-}$) was more susceptible to the chemically induced colitis, losing significantly more bodyweight and displayed enhanced colon shortening compared to the control group. In addition, we found increased levels of acute phase proteins expression in the liver of the $11c^{AhR-/-}$ group when compared to the control, suggesting an inadequate resolution of inflammation locally that resulted in systemic effects. Interestingly, we did not find any significant difference when we compared the T-cell specific AhR KO mice with controls for bodyweight loss and colon shortening at end point, suggesting that the increased sensitivity of AhR KO mice is mainly due to the loss of AhR signalling in intestinal APCs.

Epithelial injury is believed to be the initiation step to colitis induction in the DSS model. Following, we suspected that in the absence of AhR in APCs, abnormalities in the epithelium might already be present under steady state conditions. Intestinal APCs were known to participate in Wnt/β-catenin signalling pathway for tolerance induction[95] and hence we

decided to screen the intestinal epithelium for changes, in the Wnt/β-catenin signalling pathway in hopes to reveal any defects in intestinal epithelium morphogenesis. Motivated by our findings, where increased Wnt target genes expression was found in the ileal epithelium of $11c^{AhR-/-}$ animals, we went on further to perform more sensitive assays such as *in situ* hybridization with specific probes to detect possible differences in the composition of cell types in the intestinal epithelium. Our data revealed that in the absence of AhR in $CD11c^{+}$ cells, the number of $Olfm4^{+}$ ISCs and Goblet cells were increased but $Cryptdin4^{+}$ Paneth cells were reduced in these mice. These findings were of huge interest to us for three reasons. (1) These changes raises the possibility that intestinal APCs could participate in lineage decisions of differentiating IECs, possibly as another example for non-cell autonomous effects acting on ISC function reported earlier[120]. (2) Our data presented here are in line with previous publications where ISC numbers and Wnt target genes expression were often up regulated when Paneth cells were lost or defective[117,118]. (3) Were these effects observed a direct or an in direct consequence of the loss of AhR signalling in APCs?

To test for direct effects, we pooled FACS sorted intestinal migratory APCs from the mLN of $11c^{AhR-/-}$ or $11c^{AhR+/+}$ mice and co-cultured these cells with isolated small intestinal crypts from $AhR^{fl/fl}$ mice for a period of 5 days *in vitro*. The APCs from the mLN were chosen as isolating adequate numbers of APCs from the lamina propria was technically challenging and we also reasoned that the majority of $CD11c+MHCII^{high}$ cells in the mLN were $CD103^{+}$ DCs that migrated into the lymph nodes from the LP under steady state conditions. We found that organoids co-cultured in the presence of AhR-deficient DCs ($DC^{AhR-/-}$) when compared to those grown in the presence of AhR-sufficient DCs ($DC^{AhR-/-}$) expressed reduced levels of all secretory cell type markers, including the master transcription factor for secretory cell type specification: Math-1. Interestingly when we increased the ratio of DC: Crypts seeded at the start of the co-culture from 1:1 to 5:1, we found that the expression of Sox9, a transcription factor important for Paneth cells was reduced while the marker for goblet cells (Muc2) was increased in the $DC^{AhR-/-}$ group compared to the 'no' DC group (organoids without the addition of DCs). These results were in agreement with the *in vivo* data obtained earlier whereby reduced numbers of Paneth cells but increased numbers of goblet cells were observed in the $11c^{AhR-/-}$ mice. Notably, in all scenarios tested, we did not find any changes in the levels of Lgr5 expression, suggesting that the AhR-DC axis-dependent effects did not affect the ISC population. Also worth mentioning, the 'no' DC group control revealed DC specific effects, for example, organoids grown in the presence of DCs were found to express higher levels of ChgA, a marker for enteroendocrine cells. Taken together, our data indicate a role for AhR signalling in guiding IEC lineage specification via intestinal APCs.

Next, we questioned if there were any intrinsic defects in intestinal APCs due to the loss of AhR signalling, resulting in alterations of cell signalling pathways that could affect IEC specification. From the literature, intestinal DCs and macrophages can be further subdivided into various groups as discussed in the introduction of this thesis. In this paper, we tried to sort out different subtypes as best as we could, grouping intestinal APCs broadly into three groups namely, $CD103^{+}CD11b^{-}$ DCs, $CD103^{+}CD11b^{+}$ DCs and $CD103^{-}CD11b^{+}F4/80^{+}$

macrophages. Similar to earlier studies where activation of AhR signalling in BMDCs was shown to modulate cell surface expression of conventional markers[88-90], the loss of function of AhR in our study was associated with a general decrease in all three conventional markers analyzed (CD103, CD11b and CD11c). These alterations in cell surface markers expression suggest functional changes in AhR-deficient DCs, prompting us to investigate the gene expression profiles of the three major APC subsets identified here. Indeed, with a selection of genes that we analyzed, Wnt7a and Dkk3 were amongst the list of genes that were differentially regulated when AhR was absent in intestinal APCs. Although we found that AhR-deficient APCs also expressed lower levels of tolerance-related genes such as TGF-β1, ALDH1a2 and IL-10, we did not detect any significant differences in inflammatory T cells in the LP of $11c^{AhR-/-}$ mice compared to the controls. However we did find a significant increase, at two-fold of $CD4^{+}IFN\gamma^{+}$ T cells in the mLN of $11c^{AhR-/-}$ mice. Future work on the effects of AhR activation or inhibition in distinct APC subsets, paying particular attention to the regulation of the Wnt/β- catenin pathway, which consequently affects IEC biology, may be of great interest.

In summary, we uncovered an unexpected role for AhR in regulating intestinal epithelium morphogenesis through the deletion of AhR in intestinal APCs. The well-described environmental sensor, the AhR, recently coined as a pathogen recognition receptor was befitting[1], given its expression in sentinel cells of the mucosa (the intestinal APCs) that in turn coordinates innate immune responses required to maintain intestinal homeostasis.

4.2 PAPER II: MICROBIOTA CONTROLS THE HOMEOSTASIS OF GLIAL CELLS IN THE GUT LAMINA PROPRIA

As the general interest of our lab was to study host-microbe interactions, we became interested to find out if the nervous system of the gut responds to microbiota-derived signals. In the second paper, we studied the relationship between a subpopulation of EGCs that is found within the mucosa, with the intestinal microbiota. EGCs are a heterogeneous group of cells, with at least four distinct subtypes based on their morphological appearances[131,191]. Mucosal EGCs (mEGCs) can be found within the LP, forming extensive networks starting from the level of the crypts and all the way up into the villus-tips[130,181], making several contacts with mucosal tissues such as the epithelium, neurites and endothelial cells[181,182]. We began by tracing the formation of these intricate networks starting from embryonic stage (E) 16.5. To our surprise, immunostaining of intestinal sections at E16.5 revealed that $S100\beta^{+}$ EGCs were not detected in almost all the villi examined. In contrast, among all the villi that we studied, approximately 50% of them were positive for neurites that arises from the enteric ganglia. This trend was found to be similar at postnatal day (P) 0 however, the number of EGCs found in the LP along the villus-crypt (VC) unit increased significantly to ~32% from near zero by P10. The percentage of EGC^{+} VC units, along with the appearance of two or more EGC per VC unit was found to increase further until adult stages (8-12 weeks). To investigate the potential effects of weaning on the homeostasis of EGCs, we setup two groups of animals (of various ages) for comparisons. The first group of animals was weaned for 7

days prior to sample collection, with ages P27, P32 and P38 (post-weaning group) while the second group consists of those that stayed with their mothers till ages P18, P21 and P27 (pre-weaning group). We found that the number of mEGCs quantified within the pre-weaning group was significantly lower when compared to the post-weaning group. Interestingly, we also noted that the numbers from the pre-weaning group and the post-weaning group were comparable to those recorded for P10 and adult animals respectively. Furthermore, when we compared the two animals of the same age (P27) derived from the two different groups, their numbers was found to be significantly different, which suggests that the increase of mEGCs detected over time were not subjected to 'age' *per se*, but a factor of the weaning process. Taken together, our results indicate that the development of the mEGC network is not complete at birth and responds to signals associated with the postnatal environment and changes in nutrition.

Given the dynamic nature of the mEGC network, we reasoned that what we had observed might represent only a snapshot of a constant flow of mEGCs, possibly derived from sources such as the MP and/or the SMP within the intestinal wall. To test this hypothesis, we combined the *Sox10::CreER*^{T2} transgene, which allows for the specific expression of Cre recombinase in peripheral glial cells upon tamoxifen induction[192], with a mouse carrying the Cre-dependent multicolor *R26R-Confetti* reporter[188] to perform lineage tracing studies to track EGCs at stipulated time points. Four days after tamoxifen induction (T0), we analyzed the tissues to determine the baseline labeling efficiency of EGCs in adult mice. At T0, most confetti^{+} EGCs were located in the ganglionic plexi of the MP and the SMP while only ~18% of VC units analyzed contained mostly single color labeled EGCs. Next, we analyzed the tissues derived from T15 and T90, which is 15 days and 90 days post induction to follow the fate of labeled EGCs. Although the labeling intensity of EGCs within the MP and SMP remained largely similar at T15 and T90, huge numbers of labeled EGCs were detected within the LP of the VC unit, at ~72% and ~65% of all VC units examined respectively. Interestingly, at both time points (T15 and T90), labeled EGCs detected within the VC units were represented by different confetti colors compared to the single colored EGCs seen at T0, suggesting that EGCs in the VC units were derived from multiple linearly unrelated EGC sources coming from the ganglionic plexi. Further, when we used an alternative driver (*hGFAP::CreER*^{T2}) for inducible *Cre* expression in a relatively smaller subset of EGCs[192], similar data were obtained. Together, our lineage-tracing strategy has revealed a constant replenishment of mEGCs, possibly originating from plexuses of the outer intestinal wall, contributed by Sox10^{+} and GFAP^{+} glial cells.

Given that the mEGC network develops substantially upon weaning, which coincides with the period where the complexity of intestinal microbiota increases and matures[193], we went on to investigate the prospective connection between these two events by quantifying the number of EGCs in GF mice and compare that to their conventionally raised (CONV) counterparts. Although we did not find any significant difference between the EGC numbers within the two major plexuses when comparing between the two groups, the mean and density of mEGCs detected in GF mice was found to be significantly lower than their CONV

controls. The reduction in numbers was seemingly restricted to EGCs within the villi, and less so for EGCs that were at the level of the crypts. To probe if the microbiota driven development of the mEGC network had critical developmental windows, we conventionalized GF animals (CONV-D) at 4 weeks of age and harvested the intestinal tissues at 8 weeks of age for comparisons. When compared to the GF mice, the mean and density of EGCs in the villi of CONV-D animals were found to be at levels usually seen for CONV animals, suggesting that the microbiota controls EGC migration and this process is not restricted to early postnatal developmental periods.

Given that EGCs are plastic by nature[191] and possess the ability to respond to the presence of the microbiota, we questioned if the maintenance of EGCs within the villi is dependent on functional host-microbe interactions. Adult mice aged 8-12 weeks were treated with antibiotics for a period of 3 weeks and thereafter the intestinal tissues were collected and immunostained for the S100β marker. Our results revealed a significant reduction of $S100\beta^{+}$ cells within the villi of mice treated with antibiotics compared to untreated controls. Similarly, using the inducible lineage tracing protocol described earlier, we found that antibiotics treatment interfered with the homeostatic migration of EGCs into the villi, significantly reducing the number of multi-colored EGCs in the villi at T15 compared to untreated controls. Taken together, our experiments indicate that signals originating from the microbiota are crucial in initiating, in addition to sustaining the centripetal flow of EGCs from the peripheral plexi into the LP.

In conclusion, we have disclosed a previously unappreciated centripetal movement of EGCs into the mucosa from the peripheral plexi, initiated and controlled by microbiota-derived signals. Of interest, EGCs are known to express receptors that recognize bacterial products such as LPS or are in close contact with the intestinal epithelium. The exact mechanisms of how the microbes dictate the out-ward movement of EGCs and the functional significance of such a migration during health and disease remains to be investigated.

4.3 PAPER III: ESTABLISHING A CO-CULTURE SYSTEM TO STUDY ENTERIC GLIAL CELL FUNCTIONS.

We hypothesized that EGCs are responding to extrinsic signals emerging from the resident microbiota via various direct or in-direct mechanisms[185]. For example, the intestinal epithelium may secrete chemo-attractants upon activation by microbiota-derived signals, leading to the migration of EGCs into the villi. In order to examine the interactions of EGCs with the intestinal epithelium further, we began by setting up *in vitro* cultures of primary EGCs, to be used later for co-cultures with IECs.

As described in the Introduction section of this thesis, the MP is sandwiched between the outer longitudinal muscle layer and the inner circular muscle layer. Thus, upon enzymatic digestion of the longitudinal muscle layer with the MP attached underneath, we were able to grow EGCs in culture using previously published methods[144,191]. Capitalizing on a genetic labeling technique (by combining Sox10::$CreER^{T2}$ transgene with R26R-tdTomato reporter),

EGCs can be visualized directly in culture, simplifying efforts to compare different culture conditions to define an optimal protocol to expand and maintain EGCs. In both culture conditions that we had tested (serum-free versus serum-positive culture conditions), tdTomato$^+$ EGCs were readily identified, which were also positive for other known EGC markers such as GFAP[178], S100β[194] and Foxd3[195]. Surprisingly, when we analyzed the number of tdTomato$^+$ cells that were labeled with 5-ethynyl-2'-deoxyuridine (EdU) after a short exposure, we found that serum-free conditions gave rise to a higher proportion of proliferating (EdU$^+$tdTomato$^+$) cells at 3 days *in vitro* (DIV3). Further, serum-free conditions also gave rise to higher proportions of tdTomato$^+$ cells that co-expressed Sox2, a marker commonly associated with stem cell-like characteristics. Taken together, it appeared that primary EGCs grown in serum-free conditions exhibited a more proliferative potential at DIV3.

Subsequently, we followed our cultures, comparing conditions with or without the use of serum as before till DIV6. We found reciprocal effects on the expression of Sox2 and S100β in tdTomato$^+$ cells, dependent on the culture conditions used. The proportion of tdTomato$^+$ cells that were Sox2$^+$ and S100β$^+$ in serum-positive conditions was significantly lower and higher compared to serum-free cultures respectively. Additionally, we also noted a significant number of DAPI$^+$ cells that were positive for α-smooth muscle actin (α-SMA) in serum-positive cultures while much less were detected in serum-free cultures. Of interest, S100β expression has been associated with the loss of progenitor-like characteristics of GFAP$^+$ cells within the CNS[196]. Taken together, our data suggests that serum free conditions favor the maintenance of progenitor-like characteristics and were less permissive for the growth of αSMA$^+$ fibroblasts.

Apart from molecular differences detected in differentially cultured EGCs, their morphological appearances were also found to be largely different. GFAP immunostaining revealed smaller cell sizes in serum-free cultures compared to serum positive conditions. Moreover, EGC colonies in serum-free conditions were more widespread (individual cells were further apart) while EGCs in serum-positive conditions formed tight colonies. Importantly, these phenotypic morphologies and cell 'placements' were found to be reversible, by switching culture conditions from serum-free to serum-positive and *vice versa*. Our data are in line with previous reports where EGCs were found to be highly plastic and exhibit heterogeneity in their morphologies *in vivo*[191]. Interestingly, we found that passaging cells grown in serum-free conditions resulted in the formation of neurosphere-like bodies (a ball of cells made up of progenitors, neurons and glia cells), suggesting that such culture conditions may favor the maintenance of progenitor cells *in vitro*.

As serum-free conditions gave rise to highly proliferative EGCs with less contamination from other cell-types in culture, we followed this protocol for the expansion of primary ECGs in subsequent co-culture experiments. Given that the established protocol for culturing intestinal organoids differs from that of EGCs, we first tested if EGCs can be maintained in organoid-culture conditions. We found that primary EGCs derived from our initial expansion protocol

required the addition of soluble factors from a commercially available basement membrane extract (Matrigel©) when cultured in organoid base-media, which includes growth factors specific for intestinal organoids. These observations raises interesting questions on the effects of factors commonly found in Matrigel© and/or growth factors that were added specifically for organoid growth, which may modulate EGC functions.

Finally, in three-dimensional co-cultures where intestinal organoids and EGCs were embedded in the basement membrane matrix, we observed close associations of EGCs with intestinal organoids, indicative of an active communication between the two cell types. We also noted different types of associations, for example, EGCs could be seen wrapping around organoid structures and cell debris or appeared to be in contact with a single cell on the organoid surface. With recent evidences describing physical contacts between specific subtypes of IECs (enteroendocrine cells) with EGCs, it remains to be investigated if what we had observed is reminiscent of their interactions *in vivo*.

In summary, we have established a workflow for the development of an *ex vivo* system to study the interaction of EGCs with the intestinal epithelium at near physiological conditions. We believe that the benefit of such a system lies in the possibility of including other cell types such as immune cells and/or myofibroblast commonly found in the same anatomical location, allowing more physiologically-relevant experiments to be conducted.

5 CONCLUDING REMARKS AND PERSPECTIVES

In the first paper, we studied the functions of AhR specifically in intestinal APCs. Arguably, the AhR itself has been a topic of interest, given its highly evolutionary conserved nature. Homologues of the mouse/human AhR can be found in much simpler multicellular organisms such as *C.elegans* where a role for AhR in neuronal development has been described[197]. Following, it is thought that the AhR acquired the ability to respond to environmental factors as a result of adaption to participate in the modulation of different aspects of organismal biology as we have discussed here. Apart from AhR's well-known functions in xenobiotic metabolism, its involvement in immune system regulation has garnered a lot of attention lately. The great interest in this area could be related to the ascribed functions of the intestinal microbiota in shaping our mucosal immune system's development and function. Microbial-derived metabolites were reported to be putative ligands for the AhR and the survivability of certain innate lymphoid cells, among others, dependent on intrinsic AhR signalling supports this hypothesis. Also, various planar polycyclic hydrocarbons can bind the AhR, and the downstream effects may reflect subtle differences in the conformational changes induced by the binding of different ligand types. Perhaps it is due to the fact that AhR appears to have differing functions in different cell types and/or contexts depending on the type and availability of ligands. My 'gut' feeling about the importance of AhR in biology is that it is not involved in 'all or none' responses but rather, a factor that tilts the tide of a battle between two or more activating signalling pathways. This is not hard to accept as the complete AhR KO mice are viable and breeds normally in general, albeit with some developmental defects as reported previously. An example could be taken from the response of AhR-deficient BMDCs to LPS stimulation. Without any challenge, both WT and AhR-deficient BMDCs did not secrete detectable levels of IL-10. However, when stimulated (with LPS), AhR-deficient BMDCs produced significantly less IL-10 compared to WT controls[84]. This may be an explanation as to why defects in $AhR^{-/-}$ mice were very subtle and only surfaces when challenged with insults. Similarly, in our study, phenotypic changes detected in AhR-deficient DCs and the associated abnormalities in the intestinal epithelium of $11c^{AhR-/-}$ mice were not immediately apparent (no spontaneous colitis detected) but these data gave us a strong indication of perturbations in the absence of AhR in CD11c-expressing cells. Moreover, our results suggest that intestinal APCs could modulate IEC differentiation program via AhR signalling, which is an interesting topic to be studied further in detail. Of note, AhR signalling in IECs was recently reported to be protective against chemically induced colitis[42], suggesting a role for intrinsic AhR activation in IECs during an inflammatory insult. Taken together, it seems that the AhR functions in concert with many other signalling pathways, providing an informed-input to assist the host/cell in deciding which biological stance, for example, to activate or remain dormant in response to environmental cues.

Every organism has to interact closely and adapt to changes within its environment. The functional significance of such interactions can be exemplified by the development of chronic inflammatory disorders in the presence of 'friendly' gut microbiota or diet-related compounds

such as gluten in genetically susceptible individuals. The ENS is well known for controlling various aspects of GI functions, including the modulation of mucosal immunity and IEB permeability. In our second study, the centripetal flow of a cellular component of the ENS, the EGCs were found to be both initiated and maintained by the resident microbiota. Our findings highlight the plasticity of the ENS in responding to signals originating from the lumen. Questions remain on the functional requirements for the migration and maturation of EGC networks deeper into the mucosa where abundant microbes are found immediately across the epithelium. Of interest, mEGCs were reported to be in close contact with several mucosa tissue types in the same anatomical location, such as endothelia cells, IECs and possibly immune cells. Drawing parallels from recent findings that the microbiota play a central role in controlling blood brain barrier permeability after birth[198], EGCs may respond to the presence of microbes and assist in the maintenance of the gut-vascular barrier[163] to prevent the entry of microbes and/or antigens into the blood stream. In addition, heterogeneity exists among the pool of EGCs in the gut as mentioned, where at least four different EGC subtypes were classified through their distinct morphological phenotypes. Interestingly, most of the 'microbiota sensitive' EGCs were largely Type III mEGCs[131,191] (based on their morphologies), suggesting a specific role for this EGC subtype in responding to luminal bacteria. However, whether these differences translate into discrete functions for various EGC subtypes remain to be investigated. Moving forward, advanced methodologies based on single-cell expression data and bioinformatics might provide the missing link to understanding the functions of distinct EGCs subtypes, in relation to gut homeostasis.

The intestinal epithelium represents a vital barrier to safeguard the host by forming a physical and biochemical separation away from the external environment. We have found that an environmental sensor, in combination with sentinels of the mucosal immune system (intestinal APCs), can modulate the composition of IEC subtypes under steady state conditions. The dynamic nature of the intestinal epithelium is well poised to adjust to changes in the microenvironment as a consequence of, for instance, a change in diet or dysbiosis caused by a recent infection and/or lifestyle–related alterations. The constant replenishment program of the intestinal epithelium depends heavily on the highly proliferative ISCs found at the bottom of each crypt. The area immediately around the ISCs constitutes the intestinal stem cell niche. Besides Paneth cells, immune cells and myofibroblasts, ENS components were also found in close association with intestinal crypt structures, possibly playing a role in the maintenance of the stem cell niche and consequently ISC function. The continuous interplay between different biological systems indicates that not one system interacts with the environment alone. Each system is under the influence of one another, dependent on the wide-array of activating environmental cues. Looking forward, there is a need to develop methods that could study the complex interrelationships between different systems at near-physiological states to understand the combinatorial outcomes upon exposure to environmental signals. Intestinal epithelial organoid co-cultures with other cell types of interest, in combination with the use of microfluidics based-organ chips[199] may open up new avenues toward that goal.

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