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**

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Chng Song Hui Confidential Page 1 of 92

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TABLE OF CONTENTS

ABBREVIATIONS.....9

PUBLICATION LIST 11

SUMMARY.....12

1. INTRODUCTION.....14

1.1 Overview 14

1.2 Aryl hydrocarbon receptor biology 16

1.2.1 Signalling pathways of the AhR 16

1.2.2 Toxicity, xenobiotics and natural ligands 18

1.2.3 AhR in intestinal immune homeostasis..... 21

1.3 Mononuclear phagocytes in the gut 24

1.3.1 AhR in antigen presenting cells 30

1.3.1.1 AhR and dendritic cells 30

1.3.1.2 AhR and macrophages 32

1.4 Innate immunity: Role of intestinal epithelial cells..... 33

1.4.1 Intestinal epithelium and mucosal antigen presenting cells cross talk 36

1.4.2 Intestinal epithelium differentiation and renewal 38

1.5 The enteric nervous system 41

1.5.1 Role of ENS in mucosal immunity 45

1.5.1.1 Bidirectional communication between the ENS and the immune system 47

1.5.1.2 Interplay between the enteric glia cells and the intestinal epithelium in intestinal inflammation 49

2. AIMS 52

3. METHODOLOGICAL HIGHLIGHTS.....53

3.1 Microfluidics based gene expression profiling.....53

3.2 Germ-free animals.....54

3.3 Tissue-specific reporter mouse lines 55

4. RESULTS AND DISCUSSION.....58

4.1 Paper I: Ablating the aryl hydrocarbon receptor (AhR) in CD11c+ cells perturbs intestinal epithelium development and intestinal immunity 58

4.2 Paper II: Microbiota Controls the Homeostasis of Glial Cells in the Gut Lamina Propria 62

4.3 Paper III: Establishing a co-culture system to study enteric glial cell functions. 66

5. CONCLUDING REMARKS AND PERSPECTIVES 70

6. REFERENCES 74

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 <i>in vitro</i>
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

PUBLICATION LIST

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1. **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).
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SUMMARY

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 signaling 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.

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 PRRs¹, 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.

It is now clear that cancer is no longer a singular disease but a collective syndrome characterized by six unique cancer hallmarks: sustained proliferative signaling, evasion of growth suppressors, replicative immortality, resistance to cell death, induction of angiogenesis, and initiation of invasion and metastasis (Hanahan and Weinberg, 2011; Figure 1).

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 mammals². 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 protein³⁻⁵. 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-proteasome pathway⁸ upon export out of the nucleus; (2) Attenuation of signalling via AhR repressor (AhRR), another bHLH/PAS family member⁹. 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 activation⁹ 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: cAMP¹⁰. In addition, direct cross talk with transcription factors such as NF- κ b, retinoblastoma protein, estrogen receptor and protein kinase pathways have also been reported¹¹. 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 pathway¹²⁻¹⁴. Corroboratively, the activation of Wnt signalling was found to increase the transcription of *Ahr* in at least three different cellular contexts¹⁵⁻¹⁷. 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 review¹². 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 effects²¹.

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 years²². 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 species²³. 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 pathway²⁸. 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 ligand²⁹. 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 AhR³³. However, the exact mechanisms behind AhR-dependent modulation of the immune system and its constituents (various immune cell types) remain to be elucidated.

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

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*.

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 colleagues³⁶, 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 mice³⁶. 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 AhR¹. The activation of AhR by these factors was demonstrated to drive cytokine and chemokine production that are thought to protect the host against bacterial insults¹. 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 AhR¹. 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 absent³⁸. 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* infection⁴⁰, 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-10⁴² or IL-22⁴³ 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-regulated⁴⁴. 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 activation⁴⁶. 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 agonists⁴⁷, 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 antigens⁵⁰. 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 precursor⁵¹. However, with recent evidences stemming from lineage tracing studies, the classification of these cells within the original MPS scheme requires some restructuring⁵². 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 CX₃CR1⁺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 CD64⁵⁹. 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 direction⁵².

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⁻CX₃CR1⁺CD11b⁺ DCs that can be further subdivided into CCR2 expressing or non-expressing DCs⁶⁰. 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 macrophages⁶¹⁻⁶³. 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.

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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.

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 treatment⁶⁵. The restricted migration of intestinal macrophages into the mLN was found to be myeloid differentiation primary response gene 88 (Myd88) signalling dependent⁶⁵. 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 conditions⁶⁵.

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

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

* 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

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⁺ DCs⁶⁷. 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} populations⁶⁸. 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} cells⁶⁸. 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 cells⁶⁹. Correlating with an earlier study⁷⁰, these CD11b⁺ myeloid cells were postulated to be intestinal macrophages⁶⁹. Following, another study suggested that oral tolerance induction and T_{reg} expansion in the LP requires IL-10 secretion from intestinal CX₃CR1⁺ macrophages⁷¹. 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.

Of interest, a recent report has shown that CX₃CR1⁺ macrophages in an IL-10 deficient environment acquired a pro-inflammatory phenotype and were found in high numbers in the mLN⁷², similar to what has been reported in animals with dysbiosis induced by antibiotics treatment⁶⁵. 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 mice⁷³. 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 cooperatively⁵⁰. CX₃CR1⁺ 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 cells⁵⁰. 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 functions⁷⁴. 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 LP⁷⁵.

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 surfaces⁷⁶. Consequently, they are indispensable players in the maintenance of mucosal immune homeostasis⁷⁷ and also in the establishment of oral tolerance as mentioned earlier⁷⁸. A recent work targeting the deletion of MHCII specifically in classical DCs further supported the central role of these cells in maintaining homeostasis⁷⁹. 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 conditions⁷⁹, 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 pathway⁸⁴. 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 profile⁸⁵, a phenomenon that could be driven by the cross-talk between AhR and NF- κ B signalling pathways⁸⁶. 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 effects⁸⁷. 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 infection⁹¹ and multiple sclerosis⁹². 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 IFN γ 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 study¹. 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* infection⁹³. Interestingly, *L. monocytogenes* infection was found to induce higher levels of IL-6 and TNF α production by AhR-deficient macrophages, which correlates with the increased mortality of AhR KO animals upon the same infection⁹³. 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 bacteria⁹³. 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 macrophages⁹⁴. 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 pathways⁹⁵, 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 gut⁹⁶.

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 microbes⁹⁷. 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 gut⁹⁸. Genetic ablation of Muc2 expression in mice was shown to cause spontaneous colitis in addition to increasing the chance of these animals to develop tumors⁹⁹. 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 - Reg3 γ) 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 IECs¹⁰⁰. These cytokines are secreted by

various mucosal LP immune cell subsets¹⁰¹ and shown to be important for the defense against pathogenic bacteria¹⁰².

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 loop¹⁰³ or enhance IEC function by promoting the expression of tight junction proteins to maintain the IEB¹⁰⁴. 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- κ B activity in IECs¹⁰⁵. Moreover, a breakdown interactions between these pathways were found to induce spontaneous colitis¹⁰⁶ or increase the susceptibility of mice to chemically induced colitis¹⁰⁷. 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 enteritis¹⁰⁸. 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 intestines¹⁰⁸. In general, it was noted that Xbp1 deletion in IECs increased their production of pro-inflammatory cytokines and chemokines in response to TNF α or TLR5 agonist stimulation⁹⁵. Of interest, hypomorphic variants of the *XBPI* gene was found to confer a genetic risk for developing IBDs¹⁰⁷. 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 context¹⁰⁸. 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 patients¹¹⁰. Subsequently, miRNA-124 was found to negatively regulate AhR protein translation and its overexpression could exacerbate experimental colitis¹¹⁰. 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 follicle¹¹². This process of antigen sampling, through M cells has been shown to be critical for generating immune-tolerant IgA responses toward the commensal microbiota¹¹³. The antigen sampling by the intestinal epithelium 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 directly¹¹⁴. 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- β 1, 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- β 1 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 microenvironment¹²⁰. 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 signalling¹¹⁹. 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 blockage¹²⁴. 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 colitis¹²⁵. However, these mice also developed abnormal crypt architecture coupled with hyper proliferation of IECs during epithelial restitution in response to the injury¹²⁵. 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 $Lgr5^+$ ISC¹²⁶. That was just two years after the discovery of *Lgr5* as a marker for ISCs in both the small intestines and the colon¹²⁷. 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.
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Non-cell autonomous effects of Paneth cells was recently demonstrated to influence ISC biology¹²⁰, 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 higher¹²⁹. 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-E15.5¹³². 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 E13.5¹³³. 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 embryogenesis¹³⁴. 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 cells¹³⁴.

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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 (S100β⁺) via fluorescence immuno-staining.

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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 S100 β 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 colleagues¹³⁷ 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 DSS¹³⁸⁻¹⁴⁰. 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 $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) expressed by intestinal resident macrophages mediated via VNS could reduce the levels of pro-inflammatory cytokines such as IL-6 and IL-1 β detected in the muscularis externa after POI induction¹⁴¹. 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 macrophages¹⁴¹. In parallel, an earlier study showed that the activation of cholinergic myenteric neurons by 5-HT₄R agonists could protect against POI by inducing the release of acetylcholine from these neurons that acts on $\alpha 7$ nAChR expressed by macrophages to inhibit pro-inflammatory responses¹⁴². Conversely, intestinal inflammation was found to be associated with changes to the chemical nature of myenteric neurons¹⁴³ or increased

activation of enteric glial cells¹⁴⁴. 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 EGCs¹⁴⁷. 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 motility¹⁴⁸. 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 signalling¹⁴⁸. 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 reported¹⁴⁹⁻¹⁵³. 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 signalling¹⁴⁸ 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 postnatally¹⁵⁵. 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 ago¹⁵⁶ 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 motility¹⁵⁸ or immune protection of the ENS in the context of an infection⁷⁵. 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 reciprocally¹⁵⁸. 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 motility¹⁵⁸. Interestingly, the reported communications between the two cell types (neuronal and immune cell) in controlling intestinal motility was also found to be microbiota-dependent¹⁵⁸. 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 respectively¹⁵⁸, 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 activated⁷⁵. This swift neuronal response was coupled with the activation of β 2-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 macrophages⁷⁵. 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 damage⁷⁵. 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 defence¹⁵⁹. Of interest, the defective control of mucosal inflammation in TLR-2 KO mice was found to correlate with impaired Ret-GFR α 1 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-GFR α 1 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 described¹⁶³. 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 CNS¹⁶³. 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 analysis¹⁶⁶, 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 identified¹⁶⁷⁻¹⁷⁰. 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 S100 β , 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 controls¹⁷³. 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-ileitis¹⁷⁶ or enterocolitis analogous to human necrotizing enterocolitis¹⁷⁷, 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 activation¹⁴⁴. 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 CD¹⁷⁹. 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 protein¹⁸⁰ 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 control¹⁸². 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 types¹⁸⁵. 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 technologies¹⁸⁶. 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 reported¹⁸⁷. 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 administered, 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 epithelium¹⁸⁸. The design of the construct was adapted from an earlier work (Brainbow 2.1), which encodes for four fluorescent proteins derived from jellyfish¹⁸⁹. 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. Bradfield¹⁹⁰, 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 induction⁹⁵ 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⁺ ISC 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 earlier¹²⁰. (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 indirect 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 markers⁸⁸⁻⁹⁰, 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- β 1, 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 γ ⁺ 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 befitting¹, 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 β ⁺ 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 induction¹⁹², with a mouse carrying the Cre-dependent multicolor *R26R-Confetti* reporter¹⁸⁸ 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 EGCs¹⁹², 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 matures¹⁹³, 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 nature¹⁹¹ 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 S100 β marker. Our results revealed a significant reduction of S100 β ⁺ 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 mechanisms¹⁸⁵. 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 GFAP¹⁷⁸, S100 β ¹⁹⁴ and Foxd3¹⁹⁵. 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 S100 β in tdTomato⁺ cells, dependent on the culture conditions used. The proportion of tdTomato⁺ cells that were Sox2⁺ and S100 β ⁺ 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, S100 β expression has been associated with the loss of progenitor-like characteristics of GFAP⁺ cells within the CNS¹⁹⁶. Taken together, our data suggests that serum free conditions favor the maintenance of progenitor-like characteristics and were less permissive for the growth of α SMA⁺ 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 EGCs 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 described¹⁹⁷. 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 controls⁸⁴. 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 colitis⁴², 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 endothelial 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 birth¹⁹⁸, EGCs may respond to the presence of microbes and assist in the maintenance of the gut-vascular barrier¹⁶³ 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 chips¹⁹⁹ may open up new avenues toward that goal.

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SCIENTIFIC REPORTS

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Ablating the aryl hydrocarbon receptor (AhR) in CD11c+ cells perturbs intestinal epithelium development and intestinal immunity

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Diet and microbiome derived indole derivatives are known to activate the ligand induced transcription factor, the Aryl hydrocarbon Receptor (AhR). While the current understanding of AhR biology has confirmed its role in mucosal lymphocytes, its function in intestinal antigen presenting cells (APCs) is poorly understood. Here, we report that *Cre*-mediated deletion of AhR in CD11c-expressing cells in C57/BL6 mice is associated with altered intestinal epithelial morphogenesis *in vivo*. Moreover, when co-cultured with AhR-deficient DCs *ex vivo*, intestinal organoids showed reduced SRY (sex determining region Y)-box 9 and increased Mucin 2 expression, which correlates with reduced Paneth cells and increased goblet cell differentiation, similar to the data obtained *in vivo*. Further, characterization of intestinal APC subsets, devoid of AhR, revealed an expression pattern associated with aberrant intrinsic Wnt pathway regulation. At a functional level, the loss of AhR in APCs resulted in a dysfunctional epithelial barrier, associated with a more aggressive chemically induced colitis compared to wild type animals. Our results are consistent with a model whereby the AhR signalling pathway may participate in the regulation of innate immunity through intestinal epithelium development and mucosal immunity.

The gastrointestinal tract is constantly exposed to myriads of signals coming from our diet, the commensal bacteria and xenobiotics which all have an impact on host physiology. It is therefore interesting to think that a single transcription factor could bring together various information, be it endogenously or exogenously derived, to modulate gene expression in response to these signals. One potential candidate is the aryl hydrocarbon receptor (AhR), a ligand induced transcription factor. Besides industrial by-products¹, planar indoles consisting of mainly tryptophan metabolites derived from the microbiome or from our diet (cabbage, broccoli and cauliflower) have been shown recently to activate the AhR^{2,3}. Moreover, recent publications have placed AhR as a critical player in mucosal barrier defence, most notably for its role in IL-22 production in lymphocytes⁴. The current view supports the notion where the AhR functions as a sensor^{5,6}, consistent with other members of the basic Helix-Loop-Helix/Per-Arnt-Sim (bHLH/PAS) family of transcription factors such as HIF-1 α , which senses oxygen levels within a cell.

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The mucosal immune system is tasked with maintaining the integrity of the epithelial barrier that provides both physical and biochemical restriction to unwanted luminal components from entering the lamina propria. Mucosal dendritic cells (DCs), armed with diverse signalling machineries are specialised to maintain intestinal homeostasis and are key to preserving a tolerant environment^{7,8}. In addition, intestinal DCs and/or macrophages residing in the lamina propria are known to sample antigens found in the intestinal lumen via dendrites extending through inter-epithelial spaces^{9,10} or from goblet cells¹¹ directly. Interestingly, DCs and macrophages have been reported to express the AhR protein, at levels similar to those found in lymphocytes¹². Although the role of AhR in lymphocyte differentiation and function has been well described⁶, its function in intestinal antigen presenting cells (APCs) remains unclear.

The intestinal epithelial lining is highly dynamic and replenishes itself completely every three to five days¹³. Leu-rich repeat-containing G protein-coupled receptor 5 (Lgr5) –positive, crypt base columnar cells are stem cells that can undergo asymmetric division for self-renewal and to give rise to daughter cells that go on to repopulate the gut epithelium at regular intervals. This replacement program is constitutive, but sensitive to changes within the microenvironment. For example, restriction of calorie intake in mice was found to boost the size and activity of the stem cell pool via nutrient-sensing mechanisms within the stem cell niche¹⁴. The over-arching signalling mechanisms that regulate Lgr5+ stem cell biology and epithelium homeostasis rely mostly on two signalling pathways; Wnt signalling and Notch signalling¹⁵. While activation of both signalling cascades are necessary in maintaining the stem cell pool, the differentiation program of progenitors may reflect a disparity in preference, often in an opposing fashion¹⁶. In general, Notch activation pushes the precursors toward an absorptive phenotype while Wnt activation drives precursors toward a secretory cell lineage. Fate decisions in this scenario resemble a fine equilibrium that can be tipped to either side, where non-cell-autonomous signals coming from neighboring cells could also participate in. Consequently, cells located within the intestinal stem cell niche, which includes epithelial, stromal and hematopoietic lineages are key contributors to the combinatorial instructions regulating stem cell maintenance and differentiation. Hence, as Wnt signalling components are expressed by mucosal DCs^{17,18} and macrophages¹⁸, it is possible that these Wnt molecules may participate in the lineage decisions of intestinal precursors and/or stem cell maintenance. Moreover, the current literature provides evidences for the existence of cross-talk between the AhR and Wnt/ β -catenin pathways in liver progenitors¹⁹, prostate cancer cells²⁰ and colon cancer cells²¹. Thus, it is likely that AhR could also interact with Wnt/ β -catenin pathways in the context of intestinal APCs. However, such a connection has not been made and whether the interplay between these two pathways within intestinal APC subsets could modulate the development of intestinal epithelium in the same microenvironment remains largely unexplored.

Here, we assessed whether intestinal DCs and macrophages could sense the external environment through AhR and thereby influence intestinal homeostasis. In CD11c-specific AhR knockout (KO) mice ($11c^{AhR-/-}$), deleting AhR preferentially in CD11c-expressing intestinal mucosal DCs and certain macrophage subsets, we demonstrate a moderate but specific increase in small intestinal epithelial stem cell numbers and atypical differentiation of epithelial precursors *in vivo*. These changes are associated with increased susceptibility of the $11c^{AhR-/-}$ mice to chemically induced epithelial injury and colitis. Further, intestinal organoids exposed to AhR deficient DCs in co-cultures were found to express reduced levels of mature secretory cell type markers, which was dependent on the ratio of co-cultured DCs to isolated crypts *ex vivo*. Our data underscores the unique role of intestinal DCs and macrophages in maintaining the integrity of the intestinal epithelium via sensing of the external environment through AhR.

Results

$11c^{AhR-/-}$ mice are highly sensitive to DSS-induced colitis. The involvement of intestinal APC subsets in modulating inflammatory responses as well as immune tolerance is undisputed. Here, we were interested in the function of AhR in these cells given that AhR KO mice were reported to be more susceptible to a plethora of intestinal challengers^{22,23}. We ablated the environmental sensor and recently coined pathogen recognition receptor⁵, the AhR, specifically in both DCs and macrophages found in the intestinal lamina propria (LP). This was done by crossing a mouse where the *Ahr* gene is floxed in both alleles²⁴ with another that expressed the *Cre* recombinase driven by the promoter of *Itgax* or CD11c to generate AhR-sufficient ($11c^{AhR+/+}$) or AhR-deficient ($11c^{AhR-/-}$) littermates for comparisons^{25–27}. FACS sorted populations corresponding to DCs (R1 and R2) and macrophages (R3) showed efficient *Cre*-mediated deletion of *Ahr* expression but not in CD11c+MHCII- cells within the lymphocyte gate, demonstrating specificity of the mouse line (Supplementary Figure S1). PCR reactions designed previously²⁴ to detect the excised regions in targeted cells also showed clear excised bands only when *Cre* was present in FACS sorted DCs from the mesenteric lymph nodes (MLN) and the small intestinal LP (Supplementary Figure S1). Next, to challenge the $11c^{AhR-/-}$ mice, we supplied 2% dextran sodium sulphate (DSS) in the drinking water *ad libitum*, to induce acute inflammatory colitis. Interestingly, the $11c^{AhR-/-}$ mice showed considerably more weight loss and increased colon shortening compared to their controls, similar to the phenotype previously reported for AhR KO mice²² (Fig. 1a,b). Correlating with the heightened sensitivity to the acute inflammatory model, we also noted an elevation of acute phase protein genes expressions in the liver, suggesting systemic engagement of inflammatory processes in the $11c^{AhR-/-}$ mice compared to the control mice. Serum amyloid A1, A2 and A3 were increased by ~10, ~18 and ~233 fold respectively and haptoglobin, lipopolysaccharide-binding protein and lipocalin-2 expression were also massively increased in DSS treated $11c^{AhR-/-}$ mice compared with the controls (Fig. 1c). To assess whether these perturbed responses to DSS colitis were cell type specific, we generated T cell specific AhR KO mice ($Lck^{AhR-/-}$) and exposed them to the 2% DSS challenge. No noticeable difference in the severity of DSS induced colitis between the $Lck^{AhR-/-}$ mice and the $11c^{AhR+/+}$ controls was observed as shown in Fig. 1a–c and Supplementary Figure S2. No difference in the body weight of the $11c^{AhR-/-}$ animals versus littermate controls was detected (Supplementary Figure S2) at steady state. These data suggests that AhR in APCs plays a protective role in DSS-induced colitis.

Figure 1. 11c^{AhR-/-} mice are more susceptible to DSS-induced colitis. (a) The body weight of each mouse within various groups of mice were recorded daily and expressed as percentage of initial weight in grams over the course of the 2% DSS induced acute colitis. Data points are shown as mean ± SEM (n ≥ 8 per group). (b) Colon lengths of mice from various groups were measured in centimeters at end-point. Each symbol represents an individual mouse and horizontal lines show the mean ± SEM (n ≥ 8 per group), Student's t test: **P < 0.01; ***P < 0.001. (c) Quantitative RT-PCR analysis of acute phase proteins genes expression in the liver post-DSS treatment. Bar graphs depict mean ± SEM (n ≥ 8 per group), Mann-Whitney test: *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

11c^{AhR-/-} mice exhibited deregulated small intestinal epithelial cell development. DSS has been widely used as an agent to disrupt intestinal barrier integrity, leading to inflammation. The increased susceptibility of 11c^{AhR-/-} mice to DSS induced epithelial damage suggests an altered intestinal epithelial landscape. Intestinal epithelial cells (IECs) take part in intestinal tolerance by secreting factors that crosstalk with the APCs⁷. This interaction is bi-directional where APC derived signals, in turn, influence intestinal epithelial cell biology and function. Together with the knowledge that Wnt pathway members are expressed by intestinal APC subsets¹⁷ and the increased sensitivity of 11c^{AhR-/-} mice to DSS challenge, we ensued to perform a pilot study on intestinal epithelial scrapings to investigate for perturbations in the Wnt pathway in IECs in the absence of AhR in intestinal APC subsets *in vivo*. Here, we noted a general increase in the expression of Wnt target genes such as axin inhibition protein-2 (Axin2), bone morphogenetic protein-4 (Bmp4), leucine-rich

Figure 2. Altered intestinal epithelium morphogenesis in adult $11c^{AhR-/-}$ mice. (a) Quantitative RT-PCR analysis on Wnt-target genes expression from ileum epithelial scrapings. Data were pooled from 3 independent experiments and presented as mean $\pm$ SEM. Each symbol represents a single mouse. (b) *In situ* hybridization (ISH) and Periodic acid-Schiff (PAS) staining performed on paraffin-embedded sections of the ileum. Arrows point at stained goblet cells in the villus. (c) Quantification of intestinal stem cell and Paneth cell numbers. Graphs depict mean $\pm$ SEM of counted cells per crypt. More than 30 crypts were counted per animal ($n = 4$). (d) Quantification of goblet cells and villus length. Goblet cell numbers were counted and presented as a function of its respective villus length. Graphs show mean $\pm$ SEM ($n = 3$). Student's t-test: * $P < 0.05$; **** $P < 0.0001$.

repeat-containing G-protein coupled receptor-5 (Lgr5) and cellular myelocytomatosis oncogene (c-Myc) via RT-PCR assays conducted on IEC scrapings isolated from the $11c^{AhR-/-}$ mice (Fig. 2a). Motivated by these findings that indicated aberrant Wnt signalling activities in IECs, we performed *in-situ* hybridisations using probes that specifically label intestinal stem cells (olfactomedin 4 or Olfm4) and Paneth cells (Cryptdin-4) in addition to carrying out PAS staining, which labelled mainly goblet cells (Fig. 2b and Supplementary Figure S3). We found a slight increase in both intestinal stem cell and goblet cell populations while Paneth cell numbers were reduced in the ileal epithelium of $11c^{AhR-/-}$ mice (Fig. 2c,d). Of note, the average villus length measured was shorter in the mutant mice compared to the control group (Fig. 2d).

Attenuated *ex vivo* differentiation of secretory cell types in organoids exposed to AhR-deficient DCs.

The results we obtained raised an important question of whether the differences observed were a direct or indirect effect of AhR deficiency in intestinal APC subsets. To address this question, we first capitalized on a recently established protocol²⁸ that facilitated the growth of isolated intestinal crypts, which contained stem cells that can indefinitely self-renew, proliferate and differentiate into all known epithelial lineages *in vitro*. Using the aforementioned protocol, we established co-culture systems involving isolated intestinal DCs and intestinal organoids in hopes to answer the above question. In this scenario, the ideal experiment was to isolate LP DC and

macrophage subsets at numbers reasonable for co-culture experiments. However, due to technical reasons, we chose to work with CD11c+MHCII^{high} DCs isolated from the MLNs as they were more readily accessible and believed to have migrated from the LP²⁹, making them the next best cells to test in our assay. Thus, we conducted the following experiments using MLN DCs harvested from either the 11c^{AhR+/+} or the 11c^{AhR-/-} littermates and co-cultured these cells with small intestinal crypts derived from AhR^{fl/fl} mice. The co-cultures were kept for a period of five days to allow epithelial precursors to differentiate into mature cell types over the course of the experiment. The start date of co-cultures was denoted as Day *in vitro* (DIV) 1 and co-cultures were stopped on DIV 5 accordingly. At DIV1, 3 and 5, we fixed some of the co-cultures and visualised for the presence of DCs embedded in the Matrigel via immunofluorescence staining. As shown in Supplementary Figure S4, DCs (red and arrowheads) counterstained with DAPI for nuclei were detected. Of note, images from DIV5 show DC with condensed nucleus, indicating a dead or unhealthy cell compared to cultures fixed at earlier time points, consistent with the short half-life of primary DCs. Following, we studied the markers for differentiated epithelial cell types and stem cells in organoids harvested at DIV 5. Markers used included intestinal alkaline phosphatase (IAP) for absorptive enterocytes, lysozyme 1 (Lyz1) for Paneth cells, mucin 2 (Muc2) for goblet cells, chromogranin A (ChgA) for enteroendocrine cells and lastly, Lgr5 for stem cells. Interestingly, while Lgr5 expression levels were similar comparing the two groups, we found that all markers for secretory cell types were significantly down regulated in organoids co-cultured with AhR-deficient DCs but not WT DCs (Fig. 3a). In agreement, the master transcription factor *Math1* required for the differentiation of all secretory cell-types was similarly down regulated, but not *Hes1* that supports enterocyte differentiation (Fig. 3a). Furthermore, SRY (sex determining region Y)-box 9 (Sox9), a transcription factor important for the differentiation of Paneth cells¹⁵ and a Wnt target gene³⁰ was also found to be down-regulated, albeit only in one out of two independent experiments conducted (Fig. 3a). Expression levels of cell-cycle genes *CyclinD1* and *PCNA* did not reveal any statistically significant differences in proliferation (Fig. 3a), but the average size of the organoids cultured with AhR-deficient DCs at end point were found to be smaller (Fig. 3b–d). In addition, toward a more physiologically relevant level and for equitable comparisons, we went on to setup co-cultures with increased number of DCs to Crypt ratio at 5:1, plus a control group where organoids were grown without DCs. The results obtained were in a similar trend as to those reported differences at 1:1 ratio presented in Fig. 3a, as shown in Supplementary Figure S4. Accordingly, for Muc2 (goblet cell marker) and Sox9 (Paneth cell differentiation marker), one-way ANOVA with Dunnett follow up tests revealed statistically significant differences between the control and the DC^{AhR-/-} group but not with the wild-type DC^{AhR+/+} group (Supplementary Figure S4). Of note, relative expression of PCNA was also reduced in the DC^{AhR-/-} group compared to the DC^{AhR+/+} group in the same experiment (Supplementary Figure S4). Taken together, our data suggests a shortfall in Wnt signalling dependent differentiation and/or proliferation of IECs when AhR was absent in co-cultured DCs.

AhR deficiency perturbs intestinal APCs homeostasis. Prompted by these findings, we then focused on identifying aberrations, if any, in intrinsic Wnt signalling components of various intestinal APC subsets *ex vivo*. To begin, we first attempted to categorise the different subtypes of major APCs found within the LP where CD103 (integrin α E or *ITGAE*) positive APCs are the *bona fide* DCs while CD103 negative, F4/80 (*EMRI*) positive and CX₃CR1 chemokine receptor high expressing cells are the macrophages in the LP at steady state³¹. Using three different gating strategies, we could differentiate the macrophages from the DC pool and further characterize the DCs into two distinct groups (Fig. 4a). Thus, the cells gated in R1, R2 and R3 represents CD103+CD11b–DCs, CD103+CD11b+DCs and CD103–F4/80+ macrophages respectively (Fig. 4a). From our FACS analysis data, we learned that in the absence of AhR, the expression of known cell-surface markers as mentioned above were largely irregular where all three subsets of APCs were affected (Fig. 4b,e). In addition, the percentage of R2 DCs was reduced; concomitant with a slight increase in the percentage of R1 DCs without significant changes in R3 gated macrophages detected (Fig. 4c). Since intestinal CD103+ LP DCs (R1 and R2 cells) are known to migrate into the MLNs at steady state, we analysed and found that the percentage of CD103+ DCs in the MLNs was decreased compared to control animals (Fig. 4d). Increased CD103 expression was shown previously via AhR activation in glioma cells³² and its down-regulation here in both CD103 expressing DC subsets (Fig. 4e) supports the deletion of functional AhR in the 11c^{AhR-/-} mice. Collectively, these results may reflect functional consequences of the loss of AhR in these cells at steady state. Subsequently, we FACS sorted the LP APCs into three distinct groups as described from the 11c^{AhR-/-} mice and compared their gene expression profile with those of their littermate controls. We performed these experiments using a micro-fluidics based RT-PCR system³³ and a pre-selected list of genes (Supplementary Table 1), paying particular interest to those in the Wnt pathway. Interestingly, we found that the expression of Wnt7a was down regulated in CD103+CD11b+ (R2) DCs but not in CD103+CD11b–DCs (R1) isolated from the 11c^{AhR-/-} mice (Fig. 5). Conversely, the expression of a member of the dickkopf Wnt signalling pathway inhibitor (Dkk), Dkk3 was significantly up regulated in AhR-deficient intestinal macrophages (Fig. 5). Although significant differences in the expression of Wnt components were observed, we would caution that this is not a direct representation of the levels of secreted Wnt proteins.

Immunophenotyping of 11c^{AhR-/-} mice did not reveal marked mucosal T cell immunity. Since Wnt/ β -catenin signalling in DCs is crucial for intestinal tolerance¹⁷, our results directed us to study the activation status of these cells as well as known markers associated with tolerogenic DCs and/or macrophages. Based on our FACS analysis and expression datasets, we saw an up regulation of activation markers (CD86 and MHCII) in AhR-deficient intestinal macrophages and also a decrease in IL-10 expression in these cells (Supplementary Figure S5). For intestinal DCs, activation of these cells was less pronounced or no difference was observed when AhR was absent (Supplementary Figure S5). However, TGF β 1 and aldehyde dehydrogenase 1 family member A2 (ALDH1a2) were reduced in the R2 DCs (Supplementary Figure S5). The known high efficacy of intestinal CD103+ DCs in driving the development of Foxp3+ regulatory T cells (T_{reg}) has been attributed to its ability to

Figure 3. Differentiation of progenitors into secretory cell types are reduced in organoids co-cultured with AhR-deficient DCs. (a–d) CD11c+MHCII^{hi} DCs from the MLNs of 11c^{AhR+/+} or 11c^{AhR-/-} mice were pooled ($n = 3$) and co-cultured with isolated small intestinal crypts from AhR^{fl/fl} mice. Co-cultures were kept for five days. (a) Relative expression of epithelial lineage markers, transcription factors and proliferative markers were examined via quantitative RT-PCR. Each symbol represents a single biological replicate and data presented as mean $\pm$ SEM. Dataset shown was one out of two independent experiments performed with similar results. (b) Bright-field images of co-cultures are shown at different optical zooms. (c) Schematic showing how sizes of organoids were measured. (d) Size of organoids co-cultured presented as $\pm$ SEM from the two groups. Student's t-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

induce TGF- β and retinoic acid (RA) signalling (via the action of ALDH1a2) respectively, in responding naïve T cells^{34,35}. Thus, our results here suggested a possible change in the LP T cell populations. Following, we did not

Figure 4. Classical cell-surface markers expressions in intestinal APCs were down regulated in the absence of AhR. (a) Live/MHCII^{hi} cells were gated and the three major subsets of APCs in the SI LP are presented as shown based on their expression of markers: CD11c, CD11b, CD103 and F4/80. (b–c) Quantification of the three major subsets of APCs as defined in (b), shown as percentages among total live/MHCII^{hi} cells. Results are mean $\pm$ SEM pooled from 5 independent experiments ($n \geq 10$). (d) Quantification of MLN CD103+ DCs as mean percentages $\pm$ SEM is shown ($n = 6$). (e) Mean fluorescent intensity (MFI) representing expression levels of various cell surface markers of gated populations of all three groups are shown. Data are mean $\pm$ SEM pooled from 4–5 independent experiments ($n \geq 9$). Mann-Whitney test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant.

find any significant differences in T_{reg}, IL-17 and/or IFN γ producing CD4+ T cells in the small intestinal LP (Fig. 6a,b). We also did not observe any difference in the levels of fecal IgA, when comparing both groups of animals (Supplementary Figure S5). Nonetheless, in the MLNs, a two-fold increase in IFN γ producing CD4+ T cells was detected in the 11c^{AhR}-/- mice (Fig. 6a,b), indicative of a change in inflammatory status that was restricted to the lymphoid organ but not penetrating into the intestinal mucosal at steady state. Taken together, our results show that AhR signalling in intestinal APCs may be redundant for the maintenance of T_{reg} while the loss of AhR would be consistent with increased immune activation intrinsically at steady state.

Discussion

In this study, we uncovered that the loss of an environmental sensor in intestinal sentinel cells under steady state conditions is associated with abnormal intestinal epithelial development and aberrant Wnt signalling in these APCs. This was coupled to heightened sensitivity to DSS, which is known to be toxic to intestinal

Figure 5. Intestinal LP APCs from 11c^{AhR}^{-/-} mice exhibit aberrant expression of Wnt signalling constituents. Quantitative RT-PCR performed on cDNA generated from FACS sorted populations corresponding to R1, R2 and R3 APC subsets. Each symbol represents data from one single mouse. Data presented as $\pm$ SEM were pooled from 3–5 independent experiments. Mann-Whitney test: *P < 0.05; **P < 0.01; ns, not significant.

epithelial cells, highlighting an intricate relationship between intestinal APCs and IECs during health and disease.

Cell types of different ontogeny (epithelial, stromal and hematopoietic lineages), found at barrier interfaces between the host and the external environment, are each armed with in part overlapping receptor mediated mechanisms to respond to the fast changing microenvironment^{36–39}. These overlapping systems make mechanistic studies a true challenge as each cell-type often contribute, in its own way, in response to a given exogenous stimuli resulting in a combinatorial outcome. When we abrogated AhR in intestinal CD11c positive cells, the expression pattern of Wnt target genes increased along with an altered morphogenesis of the small intestinal epithelium of the 11c^{AhR}^{-/-} mice as observed. Our data suggests that DCs and macrophages may play a role in modulating Wnt signalling of cells that are anatomically near or in close proximity such as the IECs. In support, a previous study has shown that intestinal DCs and macrophages are capable of participating in Wnt signalling, including the expression of Wnt ligands¹⁷, highlighting the possibility of a cross-talk via secreted Wnts between the immune cells and the epithelium at steady state. Following, a study by Koch and colleagues has shown that

Figure 6. AhR-deficiency in intestinal APC subsets did not result in overt mucosal immunity. (a,b) Isolated lymphocytes from the small intestinal LP or the MLN re-stimulated for 4 hours *ex vivo*. (a) Representative FACS plots showing intracellular staining for Foxp3, IFN γ or IL-17A in CD4+ T cells. (b) Graphs illustrate mean percentages within the CD4+ population $\pm$ SEM. Each symbol represents one mouse. Data were pooled from 3–4 independent experiments. Mann-Whitney test: *P < 0.05; **P > 0.01; ns, not significant.

Dkk1 (another member of the dickkopf family of Wnt antagonists) was up regulated to the highest levels in DCs, relative to other hematopoietic lineages in the colon of DSS challenged mice⁴⁰. Interestingly, Dkk1 KO mice were found to recover faster but develop ectopic crypt architectures during wound repair. Thus, the increased expression of Dkk3 by the R3 subset of APCs in our $11c^{AhR-/-}$ mice at steady state conditions may be of interest for future studies. Taken together, it seems that intestinal DCs and macrophages could dampen or perturb homeostatic Wnt signalling in neighbouring cells via intrinsic AhR activity.

Our *in vivo* data from the $11c^{AhR-/-}$ mice has shown a slight but statistically significant reduction in Paneth cell numbers per crypt and an increase in Olfm4+ intestinal stem cells. In some mouse models where Paneth cells are dysfunctional or absent, significant increase in Wnt target genes expression as well as increased Olfm4+ stem cells were observed^{41,42}. Our results, presented here, mirrors what has been reported due to the reduction of Paneth cells in our $11c^{AhR-/-}$ mice. Furthermore, as differentiation of secretory subtypes is dependent on Wnt activation in general, the opposing effect on Paneth cells versus goblet cells in the small intestines of $11c^{AhR-/-}$ mice is noteworthy. Our co-culture experiments suggest that AhR in CD11c expressing cells could participate in modulating the specialisation of IECs via Math1, adding an additional layer of regulation. In the experiment where we increased the DC to crypt ratio, organoids co-cultured with AhR-deficient DCs had reduced expression of Sox9 but increased levels of Muc2 versus the control group. This result matches closely to the *in vivo* situation of reduced Paneth cells but increased number of goblet cells in the $11c^{AhR-/-}$ mice. Hence, differential Wnt activity levels and/or different Wnt ligands may account for the specialisation of different epithelial cell types observed. In support, the inhibition of β -catenin/Tcf4 activity has been demonstrated to promote goblet cell differentiation in colonic tumours⁴³, highlighting the heterogeneity of Wnt activation on secretory cell type specification. Following, the reduced size of organoids may be a reflection of decreased proliferation as evidenced in the relative levels of PCNA expression when co-cultures were setup at a ratio of 5 DCs to 1 isolated crypt. However, the possibility of indirect effects of AhR ablation in APCs on IEC differentiation cannot be formally excluded.

The intestinal stem cell niche comprises of not only the epithelial cells but also cells of stromal and hematopoietic origins⁴⁴. Stromal cells such as myofibroblasts and fibroblasts are found encircling the crypt regions and in the lamina propria, play a pivotal role in mucosal immunity⁴⁵ in addition to providing Wnt ligands within the stem cell niche for example^{45,46}. The observed differences here, may be an indirect effect of AhR signalling in DCs and macrophages, where changes in cytokines and/or growth factors such as TGF β 1^{47–49} will have yet unknown consequence on surrounding sub-epithelial myofibroblasts thereby impacting the intestinal stem cell pool. Importantly, the exact mechanisms of action of specific Wnt ligands and Wnt antagonists on stem cell biology and/or differentiation are still debatable. Recent work has demonstrated that non-epithelial and non-stromal derived Wnts are sufficient in maintaining intestinal villus-crypt structures and homeostasis *in vivo*⁵⁰. In other words, supplementary Wnt sources from the microenvironment such as those from LP DCs, macrophages and lymphocytes may further complicate the interpretation of Wnt-signalling and intestinal biology among others.

Both Paneth cells and goblet cells participate in controlling the resident microbes via secretion of anti-microbial peptides and mucins respectively. A change in the composition of these cells suggests a skewing of microbial populations in the 11c^{AhR^{-/-}} mice, an observation demonstrated in an earlier study without any detectable changes to barrier integrity, albeit in the whole AhR KO animals²². Interestingly, goblet cells have been described to be capable of transferring antigens to CD103+ DCs for subsequent antigen presentation¹¹, critical for adaptive immune responses in the gut against potential microbial invasion. Consequently, favouring goblet cell differentiation over Paneth cells, mediated by AhR-deficient APCs, raises the possibility that AhR could participate in immune regulation by increasing or decreasing antigen presentation depending on the availability of AhR ligands. Intestinal CD103+ CD11b+ (R2) DCs are known to be the major population among the CD103+ DCs in the small intestines⁵¹ and responds to luminal microbes by migrating into the epithelium for antigen sampling⁹. Recently, R2 DCs and CD103–CX3CR1+ (R3) macrophages have been reported to act cooperatively via gap junctions, important for tolerance induction via R2 DCs in the MLNs⁵². In parallel, R3 cells were the first to be identified to extend dendrites into the lumen to sample antigens¹⁰, thereby mediating the antigen transfer from macrophages to the DCs⁵². Corroboratively, our data that suggests aberrant Wnt signalling in both R2- and R3- but not in R1-gated AhR-deficient APCs highlights the unique environmental sensing functions of different SI LP APC subsets. A loss of function of AhR in sentinel cells could therefore predispose individuals to environmentally linked diseases where the intestinal epithelium plays a critical role. Nonetheless, future work aimed at understanding the effects of AhR activation on the secretion of Wnt signalling agonists and/or antagonists in various intestinal APC subsets is highly warranted.

In recent years, the AhR has been closely studied for its role in immunological responses, where intrinsic expression of AhR in various immune cells have been linked to allergy⁵³, autoimmunity⁵⁴, immune-invasion of tumorigenic cells³², mucosal immunity⁴ and immune disease tolerance⁵⁵. However, as AhR ligands are structurally very diverse and together with evidences supporting ligand specific gene regulation as being cell type and context dependent⁵⁶, the field has been plagued by the promiscuity of the AhR. Thus, we used a loss of function approach specifically in CD11c-expressing cells in hopes to alleviate confounding effects due to the use of different agonists to study the role of AhR in these cells. In this study, we observed deregulated cell-surface receptors expression including elevated co-stimulatory molecules via FACS analysis of different intestinal LP APC subsets when AhR was absent. Previous studies using different AhR ligands or DCs derived from various tissue origins have reported varying results^{49,57,58}. Yet the results are consistent as AhR activity was shown to regulate the differentiation and/or maturation and hence function of these APCs. Following, ligand induction of AhR, has been shown to induce tolerogenic CD103+ DCs, preferentially driving T_{reg} responses in a RA dependant manner to which ameliorate disease progression in a mouse model for multiple sclerosis⁵⁴. The immune-regulatory effect of AhR activation in DCs was further demonstrated when immuno-suppressive IL-10 cytokine secretion upon LPS stimulation in AhR-deficient bone marrow derived DCs was reduced compared to wild-type controls⁴⁷. Also, it was reported that in most cases, the immune-dampening effects of ligand induced AhR signalling in inflammatory conditions was attenuated when AhR is specifically ablated in the DCs^{25,26}. Interestingly, specific ablation of AhR in DCs and some subsets of macrophages did not result in overt inflammation in a skin inflammation model, suggesting tissue restricted effects of AhR²⁷. In parallel, we found that DCs in the small intestinal LP of the 11c^{AhR^{-/-}} mice expressed lower levels of TGF β 1 and ALDH1a2 while macrophages expressed less IL-10 respectively in the absence of AhR at steady state. Surprisingly, we found that T_{reg} populations or fecal IgA levels from 11c^{AhR^{-/-}} mice when compared to their littermate controls were not significantly different. We speculate that compensatory mechanisms might be at play to explain our findings for example, intestinal epithelial cells could contribute via the provision of proliferation-inducing ligand (APRIL) and B cell-activating factor (BAFF) upon Toll-like receptor activation to promote IgA secretion from plasma cells⁴⁴ while stromal cells may also contribute to the generation of T_{reg} cells via various mechanisms⁴⁵. In agreement, intestinal T_{reg} populations in AhR KO mice were at similar levels as wild type controls²², highlighting the role of other cell types in maintaining an immuno-tolerant environment at steady state. Moving on, while we did not find any differences in inflammatory CD4+ T cells in the LP of 11c^{AhR^{-/-}} mice, we found a two-fold increase in CD4+ IFN γ + T cells in the MLNs. Our mice did not get sick or display signs of weight loss compared to controls, suggesting that the tilt towards Th1 immunity is environmentally-dependent, as illustrated by the development of spontaneous colitis in AhR KO mice bred in certain animal facility⁵⁹ but not others^{60,61}. Collectively, it appears that the AhR plays a crucial role in APC function by shaping their preference towards immunity or tolerance in response to environmental cues.

The administration of AhR ligands have been shown to ameliorate colitis severity in various experimental models^{62–64}. A protective role of AhR signalling in controlling chronic intestinal inflammation was further demonstrated by complementary studies highlighting the increased susceptibility of AhR KO mice to chemically induced colitis^{22,65}. Interestingly, the increase in acute phase response genes during DSS colitis as well as the increase in goblet cell numbers in the 11c^{AhR^{-/-}} mice detected resembled phenotypes reported in patients with Crohn's Disease (CD) but not Ulcerative Colitis (UC)^{66,67}. Also, significant reduction of AhR protein in the

inflamed areas of the bowel in patients suffering from CD but not UC has been observed⁶⁸. Taken together, the $11c^{AhR-/-}$ animals may be an interesting model to examine the pathogenesis of distinct categories of inflammatory bowel diseases.

The notion of retrospective interaction from the DCs to the intestinal epithelial cells has only recently garnered more attention, shown in a model of intestinal inflammation⁶⁹. The current body of research has focused more on signals originating from the intestinal epithelial cells⁴⁴ or mesenchyme cells⁴⁵, acting on the immune system but seldom the reverse. This could be due to the classical view that immune subsets are mostly involved in inflammatory pathways via the secretion of cytokines and chemokines and not participate actively in the context of homeostatic regulation. Our findings provides an interesting aspect on how cell types of distinct developmental origins could sense and provide 'common' signals (possibly via Wnt signalling) to find the right balance between stem cell maintenance, proliferation and/or differentiation in IECs, in response to the changing external environment.

In summary, our data stressed the critical involvement of AhR signalling in the homeostasis of intestinal APC subsets, consequently modulating intestinal epithelium morphogenesis and mucosal immunity. The abundance of AhR ligands in the intestinal tract and the fact that the AhR has been evolutionarily conserved from nematodes to humans imply an essential role for AhR to facilitate APC adaptation to maintain intestinal homeostasis in mammals.

Materials and Methods

Mice. *Itgax-Cre* and *Lck-Cre* mice purchased from The Jackson Laboratory were crossed with $AhR^{fl/fl}$ mice on a B6 background to generate dendritic cell specific ($11c^{AhR-/-}$) or T cell specific ($Lck^{AhR-/-}$) AhR KO mice. Mice aged 8–14 weeks were used for experiments. All mice were bred and housed at the SingHealth Experimental Medicine Centre, Singapore. This study was conducted in accordance with institutional guidelines at the SingHealth Experimental Medicine Centre, Singapore. All animal protocols performed were approved by the SingHealth institutional animal care and use committee.

Dextran sodium sulphate induced colitis. Mice aged 8–10 weeks were given 2% DSS (TdB consultancy AB) in their drinking water for 10 days, which were replaced with fresh preparations every third day. The body weights of mice were monitored daily from day 0 where DSS was given on Day 1. Colon lengths were measured in centimetre scale, livers were harvested, snap-frozen and stored at -80°C at end point.

Intestinal epithelium scrapings and real-time PCR. Intestines were excised, opened longitudinally and fecal contents washed off using cold PBS. The small intestine was divided into 5 equal lengths and the most distal section was denoted as the ileum. Scrapings were performed using sterile blades on the surface of the intestines lightly, leaving the muscular layers intact. Tissues collected were snap-frozen in liquid nitrogen to minimize RNA degradation. Thereafter, total RNA was isolated from frozen tissues using RNeasy Mini Kit (Qiagen). 2 μg of total RNA was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Biorad). Thermal cycling condition was performed as follow: 25°C for 5 mins, 42°C for 30 minutes and reaction terminated at 85°C for 5 minutes. cDNA was pre-diluted with dH_2O to a concentration of 10 ng/ μl where 1 μl was used per RT reaction. Detection method used was based on Fast SYBR Green Master Mix reagent (Applied Biosystems) chemistry and the ABI 7500 fast real-time PCR system (Applied Biosystems). Relative gene expression analyses were done using the $\Delta\Delta\text{Ct}$ method with normalization to *Hprt*. The sequences of primers used are presented in Table S1.

In situ hybridization. The *in situ* probes targeting *Olfm4* and *cryptdin4* used in this study were previously described¹⁴. To ensure probe specificity, both sense and antisense probes were generated by *in vitro* transcription using DIG RNA labeling mix (Roche) according to previously published detailed methods⁷⁰.

Goblet cell counting. Periodic acid-Schiff staining on paraffin-embedded intestinal sections was done and slides were subsequently scanned and visualised using the Aperio ImageScope software. Stained goblet cells were counted per villus and the respective villus length was measured. At least 15 villi were examined per mouse in blind fashion.

Isolation of intestinal lamina propria leukocytes. Peyer's patches were first removed from the small intestines and thereafter, the intestines were opened longitudinally, cut finely and washed vigorously with ice-cold HBSS. The epithelial layer was then disrupted via incubation for 20 minutes in HBSS with 5% FBS, 2.0 mM EDTA and 1 mM DTT at 37°C with agitation, followed by 20 minutes incubation in HBSS with 5% FBS and 2.0 mM EDTA with agitation. Following each incubation step, tissues were vortexed briefly and the supernatant was discarded. Following, tissues were washed and then minced and incubated at 37°C for 1.5 hours in complete IMDM (Invitrogen) with 10% FBS, P/S and collagenase D at 1.5 mg/ml (Roche) with shaking. Digested tissues were homogenised and supernatants were first passed through a 100- and then 40- μm cell strainer and pelleted by centrifugation. Leukocytes were then enriched using a discontinuous Percoll (GE healthcare) gradient. After centrifugation at 2,800 rpm for 20 minutes at room temperature with zero deceleration, cells were collected at the 40/70 interface. Collected cells were washed twice and re-suspended in cold PBS with 2% FBS.

Flow cytometry, staining and PCR. Isolated intestinal LP cells were routinely treated with anti-Fc γ receptor antibody for 5 mins on ice before staining with antibodies or corresponding isotype controls from eBioscience for 30 mins on ice unless otherwise specified: FITC-anti-CD11b (M1/70; BioLegend), PE-anti-CD11c (N418), PerCP-Cy5.5-anti-MHC II (I-A/I-E, M5/114.15.2; BioLegend), APC-anti-CD103 (2E7), APC-anti-CD80 (16-10A1), APC-anti-CD45 (30-F11), PE-Cy7-anti-F4/80 (BM8), APC-Cy7-anti-CD86 (GL-1; BioLegend), V500-anti-MHC II (I-A/I-E, M5/114.15.2; BD Biosciences). Live cells were determined using the LIVE/DEAD

Fixable Dead Cell Violet Stain Kit (Invitrogen) or DAPI staining. All flow cytometry assays were performed on FACS Canto II or Aria II for sorting (BD Biosciences) and analysed with FlowJo software (Tree Star). Genomic DNA/RNA from FACS sorted DC populations were isolated using AllPrep DNA/RNA Mini Kit (Qiagen) and excision of floxed AhR exon 2 were confirmed by PCR following previous publication²⁴.

Ex vivo cultures and intracellular staining of CD4+ cells. Intracellular Foxp3 staining was performed using the Foxp3 fixation/permeabilization staining kit (eBioscience). For *ex vivo* cultures, cells from the MLN or the LP were stimulated with cell stimulation cocktail (eBioscience) containing PMA, ionomycin, brefeldin A and monensin for 4 hours at 37 °C with 5% CO₂. Following, surface staining with FITC-anti-CD4 (RM4-5) and then intracellular staining with PE-anti-IL-17A (ebio17B7) and APC-anti-IFN γ (XMG1.2) was done using IC fixation buffer and permeabilization buffer (eBioscience).

Isolation of small intestinal crypts and co-culturing with mesenteric lymph node dendritic cells. Intestinal crypts were harvested according to previously published protocol with slight modifications⁴⁶. Briefly, whole small intestines were opened longitudinally, washed and cut into fine pieces. Then, tissues were left in cold PBS with EDTA (2 mM), followed by washing with cold PBS with agitation for repeated rounds to remove the villus while enriching for crypts in the supernatant. Collected supernatants containing mostly crypts were spun down at 200 g for 2 mins at 4 °C with soft brake on to facilitate removal of single cells during washes. While crypts were being prepared, mesenteric lymph nodes were collected, dissected and digested for 30 mins with Collagenase D (Roche) at 1.0 mg/mL. Dendritic cells were then enriched using the AutoMACS separator (Miltenyi Biotec) and anti-mouse CD11c beads. Cells were then stained and CD11c+ MHCII^{hi} cells were FACS sorted using FACS Aria II. Isolated crypts and DCs were mixed together at 1:1 or 5:1 DC to isolated crypts in 50 μ L of matrigel (Corning Incorporated) droplet seeded onto each well of a 48-well plate. Co-cultures were maintained for five days where seeding day was considered day one in Advanced DMEM/F12 (Life Technologies) media with HEPES buffer plus supplements (N2 and B27), glutaMAX (Life Technologies), Y-27632, N-acetyl-L-cysteine (Sigma Aldrich), Penicillin/Streptomycin and recombinant proteins EGF, Noggin (Peprotech) and R-spondin-1 (R&D Systems). Subsequently, co-cultured organoids were treated with Trizol reagent (Life Technologies) for isolation of total RNA and stored at –80 °C for use later.

Immunofluorescence staining. Co-cultures were fixed with 2% PFA for 10 minutes and washed with PBS before staining with anti-mouse-CD11c-PE and DAPI (1 μ g/mL) in FACS staining buffer (2% FBS in PBS) for an hour at room temperature. Care had to be taken in between washes, as Matrigel (Corning Incorporated) tend to depolymerise upon fixation. Images were taken from fixed co-cultures at Days *in vitro* (DIV) 1, 3 and 5 using the Nikon Eclipse Ti inverted fluorescence microscope at 40X objective.

FACS sorting for gene expression analysis. Intestinal LP leukocyte preparations were first cleared further of dead cells using the dead cell removal kit (Miltenyi Biotec). Following, CD11c + LP cells were magnetically enriched using anti-CD11c beads (Miltenyi Biotec) and cells were sorted using the FACS Aria II (BD Biosciences) to a purity of >98%. Due to limited cell numbers per mouse, 500 single cells/events per APC subset from a single mouse were sorted into individual 0.2 ml tubes for direct cell-lysis and subsequent reverse-transcription to generate cDNA for downstream RT-PCR assays as described previously⁷¹. In brief, cell lysis was done on ice, in 0.2 mL tubes containing 10U RNaseOUT enzyme (Invitrogen), 0.15% (v/v) Tween-20 (Biorad) and 0.2 mM DTT at a final volume of 12 μ L with addition of nuclease-free water. For direct reverse transcription, 5X VILO Reaction Mix and 10X SuperScript Enzyme Mix (Invitrogen) were added to the cell-lysis tube to a final reaction volume of 20 μ L. Thermal cycling conditions performed were as follow: 25 °C for 10 minutes, followed by 42 °C for 90 minutes and reaction terminated at 85 °C for 5 minutes. cDNA derived were subsequently used for microfluidics-based RT-PCR assay platform (Fluidigm). Specific target amplification (STA) was done prior to RT-PCR reactions. Resulting cDNA, 2X TaqMan PreAmp Master Mix (Applied Biosystems), 500 nM (10X) of pooled primers mixtures were topped up to a final volume of 5 μ L with nuclease-free water followed by thermal cycling conditions as follow: 95 °C for 10 minutes, 14 cycles of 95 °C for 15 seconds, 60 °C for 4 minutes and finally hold at 4 °C. Unincorporated primers from the STA reaction were digested via exonuclease I (New England Biolabs) treatment and the resulting samples were diluted 5-fold for use in RT-PCR assays using the 48.48 Dynamic Array IFC on BioMark HD system (Fluidigm). The $\Delta\Delta$ Ct values were computed using the Real Time PCR analysis software (Fluidigm) with *Polr2a* as endogenous control. In the event where, for example, the R1 subset of DCs of a particular mouse did not reach the required 500 cells, the data point was excluded from analysis.

Fecal IgA detection by ELISA. 1–2 fecal pellets were collected in 2 mL tubes per mouse and weighed. Thereafter, 1X PBS containing complete protease inhibitor cocktail (Roche) were added to feces to achieve a final concentration of 100 mg/mL. Complete suspension of fecal pellets were ensured by grinding using pestles plus vortexing. Suspensions were allowed to stand on ice for an hour. Next, samples were centrifuged at 15,000 rpm for 15 minutes and supernatants were collected. Prior to loading, supernatants were pre-diluted 10 times. IgA concentrations were then determined using Mouse IgA ELISA Ready-SET-Go! Kit (eBioscience).

Statistics. Student t tests (two-tailed), Mann-Whitney tests (two-tailed) and one-way ANOVA with Dunnett follow up tests were conducted using the Prism 6 (GraphPad) software.

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Author Contributions

S.H.C., P.K. and S.P. designed research; S.H.C., P.K., C.D.B. and T.W.L. performed research; K.K., Y.F.K. and T.W.M. contributed new reagents/analytic tools; S.H.C. and P.K. analyzed data; S.H.C. wrote and S.P. edited the paper.

Additional Information

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SUPPLEMENTARY INFORMATION

Title: Ablating the aryl hydrocarbon receptor (AhR) in CD11c+ cells perturbs intestinal epithelium development and intestinal immunity.

Authors' list: Song Hui Chng, Parag Kundu, Carmen Dominguez-Brauer, Teo Wei Ling, Kaname Kawajiri, Yoshiaki Fujii-Kuriyama, Tak Wah Mak, Sven Pettersson

Supplementary Figure S1. Specificity and efficacy of Cre-mediated AhR deletion in 11c^{AhR-/-} mice. Relative *Ahr* expression by quantitative RT-PCR assays on MLN DCs (n=6), small intestinal LP APCs (n=6-10) and CD11c+MHCII⁻ cells (n=4) within the lymphocyte gate harvested from 11c^{AhR+/+} (Cre-) or 11c^{AhR-/-} (Cre+) littermates as shown. Values are mean ± SEM, Mann-Whitney test: ns, not significant. FACS sorted populations from respective groups as indicated where presence or absence of floxed *Ahr* exon 2 were probed by PCR and products separated by gel electrophoresis (n=2). FACS plot showing gating strategy for lymphocytes and subsequent quantification of CD11c+MHCII⁻ cells, where values show mean ± SEM (n ≥12). Student's t-test: ns, not significant. Mac, Macrophages.

Supplementary Figure S2. 2% DSS induced colitis in mice. Body weight of males measured in grams at 10 weeks of age. Values are mean $\pm$ SEM for respective groups (n =6). Graphs depicting daily percentage of initial weight of 11c^{AhR-/-} and Lck^{AhR-/-} mice against the control group 11c^{AhR+/+} with all statistical differences as shown where applicable (n $\geq$ 8 per group), Student's t-test: **, P < 0.01; ***, P < 0.001.

Supplementary Figure S3. Extended tracts of small intestinal sections comparing $11c^{AhR+/+}$ mice with littermate controls. Representative Ileal sections of respective groups as shown for *in situ* hybridizations performed to detect Paneth cells (top panel), Stem Cells (middle panel) and carbohydrate-rich cells such as Goblet cells in the villus (bottom panel) via PAS staining.

Supplementary Figure S4. Co-culture experiments reveal DC- and AhR- dependent effects on *ex vivo* organoid growth and differentiation. Immunofluorescence images of dendritic cells (red and arrows) embedded in Matrigel with co-cultured organoids on days *in vitro* (DIV) 1, 3 and 5, counterstained with DAPI to show nuclei. RT-PCR assays were performed on DIV5 samples and each data point represents a single biological replicate and presented as mean $\pm$ SEM. One-way ANOVA with Dunnett multiple comparisons tests: *, $P < 0.05$; **, $P < 0.01$ where 'No DC' group serves as the control for mean comparisons were performed as shown. Mann-Whitney tests for mean comparisons between DC^{AhR+/+} and DC^{AhR-/-} groups were not statistically significant except for PCNA ($P < 0.05$).

Supplementary Figure S5. AhR-deficient APCs display a more activated, immunogenic phenotype. Maturation markers expression levels shown as MFI $\pm$ SEM pooled from 3-5 independent experiments from respective gated APC subsets as shown (n =5-13), Mann-Whitney test; **, P < 0.01; ***, P < 0.001. Quantitative RT-PCR assays on *Tgfb1*, *Aldh1a2* and *Il10* expression in FACS sorted APC subsets accordingly. Bar graphs represent mean $\pm$ SEM from 2 independent experiments (n =4-5), Mann-Whitney test: *, P < 0.05; **, P < 0.01. Mean fecal-IgA concentration $\pm$ SEM as detected by ELISA is shown. Data presented is representative of two independent experiments (n $\geq$ 10), Student's t-test: ns, not significant

Fluidigm 48.48 array			
Category (n)	Gene names:	Forward Primer 5'-3'	Reverse Primer 5'-3'
Wnt receptors and mediators (13)	Fzd3	TGTCCGTACCAGGTTACTCAGATGA	GAATCCCAACTATGAGAGCCATGA
	Fzd6	TGGACACTTTTGGCATCCGA	TATAGCCTTGGTCCCCGGAA
	Fzd9	TAAGGACTTCGCGCTGGTTT	AAGATAATCGGGCGCTCTGG
	Fzd10	GCATGGCCAGCTCTTTATGG	GTTGGCTTCAATGGCCTCAT
	Wnt3	CAAGCACAACAATGAAGCAGGC	TCGGGACTCACGGTGTTTCTC
	Wnt3a	TCGGAGATGGTGGTAGAGAAA	CGCAGAAGTTGGGTGAGG
	Wnt2b	CACCCGGACTGATCTTGTCT	GCCACAACACATGATTTACA
	Wnt5b	GACGCCAACTCCTGGTGGT	GCATAACTCTGCCAAAGACAGATG
	Wnt7a	CGACTGTGGCTGCGACAAG	CTTCATGTTCTCCTCCAGGATCTTC
	Wnt8b	TTGGGACCGTTGGAATTGCC	AGTCATCACAGCCACAGTTGTC
	Wnt9a	GCAGCAAGTTTGTCAAGGAGTTCC	GCAGGAGCCAGACACACCATG
	Wnt11	CGTGTGCTATGGCATCAAGT	GCTCGATGGAGGAGCAGTTC
	Dkk3	CGAGAGGTGGAGGAGCTGATG	GTCTCCGTGCTGGTCTCATTG
Wnt Targets (6)	Axin2	GAGCAGCTCAGCAAAAAGGG	ACTGTCTCGTCGTCCCAGAT
	BMP4	CTTGAGTACCCGGAGCGTC	AAAGCAGAGCTCTCACTGGTC
	Sox9	AGGAAGCTGGCAGACCAGTA	TCCACGAAGGGTCTCTTCTC
	C-myc	CAGCCCTGAGCCCCTAGT	TCTCCACAGACACCACATCAA
	Wisp1	TGATGACGCAAGGAGACCAC	CCGGGCATTGACGTTAGAGA
	Wisp2	GTTTTGTGCCGCTGTGATG	CTGAGGAGGGCTGGATTG
Cytokines (8)	TNF α	CAAATGGCCTCCCTCTCAT	CTCCTCCACTTGGTGGTTTG
	IL-1 β	GCTGAAAGCTCTCCACCTCA	GGCCACAGGTATTTTGTCTG
	IL-6	CAAAGCCAGAGTCCTTCAGA	GAGCATTGGAAATTGGGGTA
	IL-12A	GTGTCAATCACGCTACCTCC	TTTCTCTGGCCGTCTTCACC
	IL-23A	GCACCTGCTTGACTCTGACA	ATCCTCTGGCTGGAGGAGTT
	TGF- β 1	GTGTGGAGCAACATGTGGAA	CGTCAAAAGACAGCCACTCA
	IL-10	ATCGATTTCTCCCCTGTGAA	TCATTCATGGCCTTGTAGACAC
	IFN γ	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
Enzymes and TFs (4)	ALDH1a2	ACCGTGTTCTCCAACGTCCTGAT	TGCATTGCGGAGGATACCATGAGA
	Blimp1	GACGGGGGTACTTCTGTTCA	GGCATTCTTGGGAAGTGTGT
	IDO1	CCATGGCGTATGTGTGGAAC	AGAGCTCGCAGTAGGGAACA
	IRF4	AATCCCCATTGAGCCAAGCA	TCGTCGTGGTCAGCTCTTTC
Notch Signalling (7)	Notch1	CGGTGAACAATGTGGATGCT	ACTTTGGCAGTCTCATAGCT
	Notch2	GTGGAGGCGACTCTTCTGCT	GCTGGGAGTCACGTTATACT
	Jagged1	GCCGAGGTCCTACACTTTGCT	GTGGGCAATCCCTGTGTTTT
	Jagged2	GAGGTCAAGGTGGAAACAGT	TGTCCACCATACGCAGATAA

	Hes1	TCAGCGAGTGCATGAACGAG	CATGGCGTTGATCTGGGTCA
	Hey1	TGAGCTGAGAAGGCTGGTAC	ACCCCAAACCTCCGATAGTCC
	Dll4	GCACCAACTCCTTCGTCGTC	GTTTCCTGGCGAAGTCTCTG
AhR pathway (4)	AhR	AGCCGGTGCAGAAAACAGTAA	AGGCGGTCTAACTCTGTGTTC
	Cyp1a1	CAGGATGTGTCTGGTTACTTTGAC	CTGGGCTACACAAGACTCTGTCTC
	Cyp1a2	CCATGTGCTTTGGGAAGAACTT	GTCCTTGCTGTTATTACGATGTT
	Cyp1b1	CCGAAAAGAAAGCGTCTGG	GAACATCCGGGTATCTGGTAAA
Housekeeping genes (6)	Ppia	CACCGTGTTCTTCGACATCA	CAGTGCTCAGAGCTCGAAAGT
	Polr2a	TGGTCCTTCGAATCCGCATC	GGACTCAATGCATCGCAGGA
	Hprt	AGCCAAATACAAAGCCTAAGATGA	GGACGCAGCAACTGACAT
	Tbp	GCACAGGAGCCAAGAGTG	AGGGAACCTTCACATCACAGC
	Hmbs	CCACGGGAGACAAGATTC	ACAGCATCACAAGGGTTT
	β-actin	CTGTATTCCCCTCCATCGTG	CCTCGTCACCCACATAGGAG
Co-culture experiments			
Epithelial lineage markers	Lzy1	GAGACCGAAGCACCGACTATG	CGGTTTTGACATTGTGTTTCGC
	ChgA	CCCGAAGTGACTTTGAGGAA	GATGGCTGACAGGCTCTCTA
	Muc2	ACAAAAACCCCAGCAACAAG	GAGCAAGGGACTCTGGTCTG
	IAP	GGTATCATCCCAGCTGAAGAG	CATCCCGTCTCCCAGGAA
	Lgr5	CCCAATGCGTTTTCTACGTT	AGGCTCGGTTCCCTGTTAAT
Transcription factor	Math1	TCCCGTCCTTCAACAACGAC	CTCTCCGACATTGGGAGTCTG
Proliferation markers	PCNA	ACCTCACCAGCATGTCCAA	TCTTGATTTGGTGCTTCGAATA
	Cyclin-D1	AGTGCGTGCAGAAGGAGATT	AGGAAGCGGTCCAGGTAGTT

Supplementary Table 1

Report

Neuron

Microbiota Controls the Homeostasis of Glial Cells in the Gut Lamina Propria

Highlights

- The glial cell network of the gut mucosa develops after birth
- Mucosal glial cells are a continuously renewed homeostatic cell population
- Microbiota regulates the development and homeostasis of mucosal glial cells

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In Brief

Mucosal glial cells are important for maintaining the intestinal epithelial barrier and regulating immune responses in the gut. Kabouridis et al. demonstrate that signals from microbiota regulate the postnatal development and the continuous renewal of this protective cell network.

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CellPress

Microbiota Controls the Homeostasis of Glial Cells in the Gut Lamina Propria

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SUMMARY

The intrinsic neural networks of the gastrointestinal tract are derived from dedicated neural crest progenitors that colonize the gut during embryogenesis and give rise to enteric neurons and glia. Here, we study how an essential subpopulation of enteric glial cells (EGCs) residing within the intestinal mucosa is integrated into the dynamic microenvironment of the alimentary tract. We find that under normal conditions colonization of the lamina propria by glial cells commences during early postnatal stages but reaches steady-state levels after weaning. By employing genetic lineage tracing, we provide evidence that in adult mice the network of mucosal EGCs is continuously renewed by incoming glial cells originating in the plexi of the gut wall. Finally, we demonstrate that both the initial colonization and homeostasis of glial cells in the intestinal mucosa are regulated by the indigenous gut microbiota.

INTRODUCTION

Glial cells encompass diverse neuroectodermal cell populations that are essential for the organization and function of the nervous system (Verkhratsky and Butt, 2007). In addition to their roles in providing support and nourishment for neurons, glial cells regulate synaptic transmission (Clarke and Barres, 2013), maintain the blood-brain barrier (Alvarez et al., 2013), and mediate communication between the nervous and immune system (Jensen et al., 2013). Consequently, glial cell deficits are associated with developmental, degenerative, and inflammatory disorders of the nervous system (Skaper et al., 2014). The enteric nervous system (ENS) encompasses the intrinsic neural circuits of the gastrointestinal tract (GI), which are organized into a vast network of interconnected ganglia distributed into two concentric layers within the gut wall, the outer myenteric (MP) and the inner submucosal (SMP) plexus (Furness, 2006). The ENS regulates most aspects of GI physiology, such as peristalsis, blood supply to the gut wall, and secretion (Furness, 2006), and consti-

tutes a relay station in the bi-directional neuro-endocrine pathways that connect the digestive system and the brain (gut-brain axis) (Collins et al., 2012). In rodents, enteric neurons are born during embryogenesis and early postnatal life and are restricted to the ganglia (Laranjeira et al., 2011; Liu et al., 2009; Pham et al., 1991). Enteric glial cells (EGCs) outnumber enteric neurons by 4:1 and are located within ganglia and extraganglionic sites, including the smooth muscle layers and the intestinal mucosa (Boesmans et al., 2015; Gershon and Rothman, 1991; Gulbransen and Sharkey, 2012; Rühl, 2005). In contrast to enteric neurogenesis, low levels of gliogenesis have been observed in enteric ganglia of unchallenged adult rodents, although the destination of the newly generated glial cells and their function remains unclear (Joseph et al., 2011). Based on morphological features and location, EGCs are subdivided into distinct subtypes that share molecular and functional characteristics (Boesmans et al., 2015; Gulbransen and Sharkey, 2012). Despite the realization that the different subpopulations of EGCs make critical and unique contributions to intestinal homeostasis, the dynamic relationship between spatially segregated EGCs, the physiological signals that regulate their steady-state equilibrium, and their response to trauma or disease remain unknown.

One of the subpopulations of EGCs that has generated considerable interest recently is located within the intestinal mucosa (Gulbransen and Sharkey, 2012; Rühl, 2005). In addition to their neuroprotective function, these mucosal EGCs (mEGCs) are thought to play crucial roles in maintaining the intestinal epithelial barrier and regulating immune responses in the mucosa (Bush et al., 1998; Neunlist et al., 2013; Rühl et al., 2004; Savidge et al., 2007). The residence of mEGCs within the most dynamic layer of the gut wall and their interactions with highly regenerative and remodeling tissues, such as the intestinal epithelium and the mucosal immune system, raise interesting questions regarding their development and homeostasis. These questions acquire renewed urgency given the emerging effects of microbiota on the organization and function of multiple GI tissues.

Here we have examined the developmental profile of mEGCs and their maintenance in adult mice. Our analysis shows that, in contrast to neural projections, mEGCs colonize the intestinal mucosa after birth. By performing inducible lineage tracing experiments we demonstrate that the network of mEGCs is maintained throughout life by the continuous supply of new glial cells

Figure 1. The Network of mEGCs Develops after Birth

(A) S100 β immunostaining of a vibratome cross-section from the ileum of an adult wild-type mouse. In addition to the myenteric (mp) and submucosal plexus (smp), EGCs (arrows) are also found within the lamina propria around the crypts and within villi. (B) Highly branched GFP $^{+}$ glial cells (arrows) within the mucosa in the ileum of *Sox10::Cre;MADM^{GR/EG}* mice. (C–E) S100 β immunostaining of cryosections of the mucosa of P0 (C), P10 (D), and adult (E) mice. Arrows in (D) and (E) point to mEGCs. (F) Quantification of glia $^{+}$ VC units (GFP and S100 β antibodies on sections from *Sox10::Cre;R26REYFP* mice) demonstrating that the network of mEGCs develops postnatally. Data are represented as mean of all glia $^{+}$ VC units $\pm$ SEM. One-way ANOVA, p value < 0.0001 ; Tukey post hoc test showed that comparison of E16.5 to P0, P10 to P18–P27, and P27–P38 to Adult was not significant (NS). However, comparison of E16.5 to P10, P18–P27, P27–P38, and Adult; P0 to P10, P18–P27, P27–P38, and Adult; P10 to P27–P38 and Adult; and P18–P27 to Adult was significant (*** $p < 0.0001$). Comparison of P18–P27 to P27–P38 was also significant (*** $p = 0.0003$). The F (DFn, DFd) value is 227.6 (5, 12). Scale bars: 100 μ m (A and B); 50 μ m (C–E).

extending from the MP and SMP to the lamina propria between crypts and within villi (Figure 1A). To characterize in detail the morphology of mEGCs, we combined the *Sox10::Cre* driver (Matsuoka et al., 2005) with the *MADM-6^{GR}* and *MADM-6^{RG}* alleles (Zong et al., 2005) in order to express green fluorescent protein (GFP) in subsets of peripheral glial cells (Boesmans et al., 2015). mEGCs were highly branched (Figure 1B; Movie S1) and contacted several mucosal tissues, including the epithelium, blood vessels, and neurites (Figure S1; Movies S2 and S3) (Bohórquez et al., 2014; Liu et al., 2013). The apparent interaction of mEGCs with highly regenerative and remodeling tissues of the mucosa prompted us to

originate in the peripheral plexi. Finally, by analyzing germ-free (GF), conventionalized, and antibiotic-treated mice we provide evidence that the postnatal settlement of mEGCs in the intestinal mucosa and the ongoing supply of glial cells to the lamina propria in adult mice are regulated by the gut microbiota. Our work provides insight into the role of environmental factors in the development of glial cells and their homeostasis in adult animals.

RESULTS

The Network of mEGCs Develops after Birth

Immunostaining of sections from adult mouse intestine for the glia-specific marker S100 β displayed a dense network of EGCs

examine their own dynamic properties. For this, we first analyzed the developmental profile of mEGCs by immunostaining intestinal sections from *Sox10::Cre;R26REYFP* reporter mice at different embryonic and postnatal stages with combinations of antibodies for S100 β , YFP, and the neuronal marker PGP9.5. Neuronal fibers emanating from enteric ganglia were observed in $\sim 50\%$ of villi at embryonic stage (E) 16.5 and in the majority of villi at postnatal day (P) 0 (Figure S2). In contrast, the lamina propria along the villus-crypt (VC) units (Figure S2) was essentially devoid of glial cells at both E16.5 and P0 ($n = 3$; Figures 1C and 1F). The small fraction of glia $^{+}$ VC units identified at these stages contained a single glial cell (Figure 1F). At P10 ($n = 3$) the percentage of glia $^{+}$ VC units increased significantly and units with more

Figure 2. Inducible Lineage Tracing of mEGCs

Distribution of confetti⁺ cells in the ileum of tamoxifen-treated *Sox10::CreER^{T2};R26RConfetti* mice at T0 (A), T15 (B), and T90 (C). Boxed areas in (A)–(C) correspond to panels (A')–(C'), respectively. (D and D') Confetti⁺ cells in a flat-mount preparation of myenteric ganglia from *Sox10::CreER^{T2};R26RConfetti* mice at T0. Arrows in (D) and (D') indicate Sox10⁺ Confetti⁺ glial cells. Asterisks in (D) indicate the position of confetti-negative enteric neurons. (E) Quantification of VC units with confetti⁺ glial cells at T0, T15, and T90. Data represented as mean $\pm$ SEM. Significant differences between the ages have been obtained with the two-way ANOVA, $p < 0.0001$, Tukey post hoc test, **** $p < 0.0001$ (T0–T15; T0–T90), $p = 0.7173$ (T15–T90). Although VC units with monochromatic glia showed no significant change in number ($p > 0.1$), VC units with polychromatic glia showed significant differences: **** $p < 0.0001$ (T0–T15; T0–T90), $p = 0.6222$ (T15–T90). The F(DFn, DfD) and p values for Factor 1: T0 Vs T15 Vs T90 is 29.94 (2, 18) with $p = 0.08$; Factor 2: polychromatic Vs monochromatic is 3.36 (1, 18) with **** $p < 0.0001$ and interaction of Factor 1 with Factor 2 is 8.104 (2, 18) with ** $p = 0.0031$. cm, circular muscle layer; lm, longitudinal muscle layer; mp, myenteric plexus. Scale bars: 100 μ m (A–C); 50 μ m (A'–C'); 20 μ m (D and D').

than one glial cell could also be identified (Figures 1D and 1F). These parameters increased further in adult ($P \geq 60$, $n = 3$) mice (Figures 1E and 1F). To examine the potential role of weaning on the maturation of the mEGC network, we quantified mEGCs in the ileum of mice whose age ranged from P18 to P38. At the time of gut harvesting, three animals (P18, P21, and P27) were still at the home cage with their mother (pre-weaning group), while the remaining (P27, P32, and P38) had been weaned 7 days earlier (post-weaning group). The average values of the pre-weaning and post-weaning groups were significantly different but very similar to those from P10 and adult animals, respectively (Figure 1F). Moreover, comparison of the values of the two animals that belong to different groups but have same age (P27) suggests that weaning contributes to the expansion of mEGCs observed after P10. Together, our experiments suggest that the mEGC network develops in response to signals associated with adaptation of the GI tract to the postnatal environment of the lumen and nutrition.

The Network of mEGCs Is Continuously Supplied with Glial Cells Originating in the Ganglionic Plexi

Given the regenerative and remodeling capacity of the intestinal mucosa, we considered the possibility that mEGCs detected within the lamina propria at any given moment represent a snapshot in the life cycle of a highly dynamic and incessantly renewing neuroectodermal cell population. Moreover, since ganglionic EGCs are capable of responding to injury and other insults (Joseph et al., 2011; Laranjeira et al., 2011), we posited that the ganglionic plexi of the outer gut wall constitute the main source of incoming mEGCs in adult animals. To test this hypothesis directly, we performed inducible lineage tracing of EGCs in adult

mice by combining the *Sox10::CreER^{T2}* transgene, which drives expression of tamoxifen-inducible Cre recombinase in peripheral glial cells (Laranjeira et al., 2011), with the Cre-dependent multicolor *R26R-Confetti* reporter (Snippert et al., 2010). Transgenic mice 8–12 weeks old ($n = 4$) were administered tamoxifen and analyzed 4 days later (T0) to establish the baseline labeling pattern, and 15 or 90 days post-induction (T15 and T90, respectively; $n = 4$ for each time point) to follow the fate of labeled cells. As expected, at T0 confetti⁺ glial cells were identified within the MP and (to a lesser extent) the SMP (Figures 2A, 2A', 2D, and 2D'). However, at this stage only 18% of VC units identified on sections contained labeled glial cells and the majority of them were monochromatic displaying a single confetti⁺ glial cell (Figures 2A and 2E). At T15 and T90, the number of confetti⁺ cells in the plexus was similar (or sometimes even increased), suggesting that the population of glial cells labeled at T0 remains stable at least during the post-induction period analyzed (Figures 2B, 2B', 2C, and 2C'). Interestingly, at these stages numerous confetti⁺ EGCs were found in the majority of VC units (~72% at T15 and ~65% at T90) and were distributed along the entire crypt-villus axis (Figures 2B, 2B', 2C, 2C', and 2E). In contrast to T0, the majority of confetti⁺ VC units at T15 and T90 were polychromatic containing several glial cells labeled with different fluorescent reporters (Figures 2E and S3). The dramatic increase in the number of confetti⁺ cells within VC units and villi and their monochromatic to polychromatic switch at T15 and T90 indicated that during the post-induction period they are colonized by multiple lineally unrelated mEGCs originating from a labeled pool of glial cells in the ganglionic plexi.

To provide further support for the centripetal flow of glial cells along the serosa-lumen axis in adult mice under physiological

Figure 3. Formation of the mEGC Network Depends on Microbiota

Cross-sections from the ileum of CONV (A), GF (B), and CONV-D (C) mice immunostained for S100β. Arrows indicate glial cells. (D) Quantification of S100β⁺ cells in the three conditions. One-way ANOVA, p value = 0.0093, Tukey post hoc test, $^*p = 0.0128$ (CONV versus GF), $^*p = 0.0260$ (GF versus CONV-D). Glial populations were not significantly different between CONV and CONV-D mice. The $F(DFn, DFd)$ value is 7.086 (2, 12). (E) Percentage of VC area positive for S100β is represented in all three conditions. One-way ANOVA, p value < 0.05, Tukey post hoc test, $^{**}p = 0.002$ (CONV versus GF), $^*p = 0.018$ (GF versus CONV-D). The $F(DFn, DFd)$ value is 10.70 (2, 12). Note that the glial cell number and distribution and the area of VC units positive for S100β was similar between CONV and CONV-D animals, but significantly different from GF mice. Scale bars: 100 μm (A–C). CONV = conventional; GF = germ-free; CONV-D = conventionalized.

conditions, we combined the *hGFAP::CreER^{T2}* transgene, which marks a relatively small subset of EGCs (Laranjeira et al., 2011), with the *R26RConfetti* and *R26REYFP* reporters. Three to four days after tamoxifen administration (T0) to 8- to 12-week-old mice from both driver-reporter combinations, sparsely labeled glial cells were detected predominantly in the MP (Figure S3). At this stage, no labeled glial cells were identified in the lamina propria (0/169 YFP⁺ cells detected on sections from the ileum of *hGFAP::CreER^{T2};R26REYFP* mice; $n = 4$). In contrast, 8–12 weeks post-induction ~9.5% of YFP⁺ cells were located within the mucosa (Figure S3; 24/253 YFP⁺ cells identified; $n = 4$) with the remaining residing within the ganglia. Taken together, our in vivo lineage tracing experiments demonstrate that in adult mice the pool of mEGCs is continuously supplied by new cells originating from Sox10⁺ and GFAP⁺ glia located within the peripheral ganglia.

Microbiota Is Required for the Postnatal Development of mEGCs

Recent work has demonstrated that the gut microbiota influences the function of neuronal circuits (Diaz Heijtz et al., 2011), but much less is known about the communication between the ENS and intestinal microflora. The GI tract is colonized immediately after birth with micro-organisms that increase in number and complexity after weaning (Nicholson et al., 2012). Since the postnatal development of mEGCs coincides with the establishment and maturation of microbiota, we examined the potential link between the two processes by comparing the mEGC network in the ileum of 8-week-old conventionally raised (CONV; $n = 6$) and germ-free (GF; $n = 5$) mice (Figure S4). Interestingly, although no obvious difference in S100β immunostaining was observed in the MP and SMP between the two groups, the average number and density of mEGCs in the mucosa of GF mice was significantly reduced relative to CONV animals (Figures 3A, 3B, 3D, and 3E). The reduction of mEGCs in GF mice

was not uniform along the crypt-villus axis since mEGCs were essentially eliminated from the villi but those at the level of the crypts were less affected (Figures 3A and 3B). To examine whether the requirement of the gut microbiota for normal EGC development is restricted to a critical early postnatal period, 4-week-old GF mice were conventionalized (CONV-D) by gavage feeding of microbiota and subsequent co-housing with CONV animals (Figure S4) and intestinal sections were immunostained for S100β 4 weeks later. In comparison to GF mice, the average number of mEGCs in the mucosa of CONV-D animals ($n = 4$) was increased and the network of glial cells within the lamina propria of villi was restored (Figures 3B–3E). We conclude that normal development of the mEGC network depends on gut microbiota but the ability of EGCs to invade the intestinal mucosa is not restricted to the early postnatal life.

Microbiota Regulates the Continuous Centripetal Flow of mEGCs along the Serosa-Lumen Axis

To examine whether gut microbiota is required for maintaining the normal complement of mEGCs throughout life, 8- to 12-week-old mice were treated with antibiotics for 3 weeks and the ileum was immunostained for S100β. Antibiotic treatment reduced the number of micro-organisms within the gut lumen and led to cecum enlargement (Reikvam et al., 2011) (Table S1; Figure S4). Importantly, it also reduced the number of S100β⁺ glial cells within the mucosa (Figures 4A and 4B) with mEGCs within villi affected more relative to their counterparts around the crypts (Figures 4A and 4B). Analysis of gut sections from control and antibiotic-treated *Sox10::Cre;R26RConfetti* mice, in which all mEGCs were labeled by confetti-encoded fluorescent reporters, confirmed that antibiotic treatment reduced the number of mEGCs rather than the expression levels of the S100β marker (Figure S4). Finally, to examine whether antibiotics disrupt the homeostatic flow of EGCs along the plexus-crypt-villus axis, we combined antibiotic treatment and EGC lineage

Figure 4. Microbiota Controls the Continuous Supply of Glial Cells to the Intestinal Mucosa

(A and B) Sections of the ileum of control (A) and antibiotic-treated (B) wild-type adult mice. Arrows in (A) indicate glial cells. (C–F) Distribution of confetti⁺ glial cells in the ileum of tamoxifen-treated 8- to 12-week-old Sox10::CreER^{T2};R26RConfetti mice at T0 (C and D) and T15 (E and F) in the absence (C and E) or presence (D and F) of antibiotics. Arrows indicate confetti⁺ glial cells in the lamina propria. (G) The average number of confetti⁺ cells in VC units at T15 is reduced in antibiotic-treated animals. Data is represented as mean ± SEM. The distribution of the number of labeled glial cells per VC unit has been plotted using the non-linear paradigm, representing a different curve for each dataset as the best fit with a ***p value = 0.0004. Using the two-way

tracing in 8- to 10-week-old Sox10::CreER^{T2};R26RConfetti mice. Administration of antibiotics resulted in dramatic reduction of the polychromatic mEGC population observed in the mucosa two weeks after tamoxifen administration (Figures 4C–4G). Taken together, these experiments argue that signals emanating from gut microbiota initiate and sustain the flow of mEGCs from the peripheral plexi to the lamina propria.

DISCUSSION

Despite extensive literature on the self-renewal and remodeling of non-neuroectodermal intestinal tissues (Belkaid and Hand, 2014; Clevers, 2013; Stappenbeck et al., 2002), only limited information is available on the homeostasis of the intrinsic neuroglial networks of the gut wall. Indeed, the ENS is often portrayed as an assembly of ganglionic units that form during development and are maintained, perhaps unchanged, throughout life. Here we challenge this view and demonstrate that the ostensibly rigid neuroglial networks in the gut of adult animals encompass highly dynamic populations of EGCs with previously unrecognized hierarchical relationships. The extensive network of mEGCs, which develops postnatally over a period entailing dramatic changes in GI microenvironment and physiology, continues to be renewed in adult animals by incoming glial cells originating in the ganglionic plexi of the gut wall. We also demonstrate that both the initial establishment and the homeostasis of mEGCs are controlled by the gut microbiota (Figure S4).

In our lineage tracing studies, the supply of new mEGCs to the intestinal mucosa was not associated with reduction of the labeled cell population in MP and SMP, suggesting that the subset of glial cells targeted by our reporter system is long lived. It would be interesting to determine whether the continuous centripetal flow of mEGCs is supported by dedicated progenitors or differentiated glial cells, which under certain conditions acquire self-renewing potential. Irrespective of the identity of mEGC progenitors in the ganglionic plexi, our current observations provide a rationale for the constitutive gliogenesis observed in the MP of adult rodents (Joseph et al., 2011; Laranjeira et al., 2011). Consistent with our current studies and in further support of the dynamic state of EGCs in the adult gut, pharmacogenetic ablation of proliferating glial cells in mice led to epithelial barrier breakdown and inflammatory degeneration of the mucosa (Bush et al., 1998; Savidge et al., 2007).

Although the majority of neurons found in MP and SMP of adult mice are already in place by the end of fetal life, enteric neurogenesis continues for several weeks after birth (Laranjeira et al., 2011) extending beyond the early postnatal period of microbiota establishment and maturation. Interestingly, GF rodents are characterized by reduction in specific subpopulation of enteric neurons and abnormal peristalsis, suggesting that microbiota influence the development and maturation of intestinal

ANOVA and Sidak's multiple comparisons test, significant differences were observed in the 0 and 2 cells per VC unit categories with **p values = 0.0014 and 0.0081, respectively. The F(DFn, DFd) and p values for Factor 1: With Vs Without antibiotic is 0 (1, 36) p > 0.99; Factor 2: number of glial cells in VC units is 21.47 (5, 36) ****p < 0.0001 and interaction of Factor 1 with Factor 2 is 6.353 (5, 36) with ***p = 0.0003. Scale bars: 100 μm (A–F).

neural networks (Abrams and Bishop, 1967; Collins et al., 2014; McVey Neufeld et al., 2013). Although the mechanisms by which luminal microflora impact the organization and function of enteric neural circuits remain largely unexplored, a recent study provided evidence for a reciprocal interaction between macrophages of the muscularis externa and enteric neurons that can be influenced by microbiota (Muller et al., 2014). The identification of EGCs as a major cellular target of intestinal microbiota in our study, in conjunction with the widely established roles of these cells in the survival and functional connectivity of enteric neurons, suggests that at least some of the motility defects observed in GF and antibiotic-treated rodents could be secondary to deficits in glial cell dynamics. For example, reduction of mEGCs that are associated with the projections of afferent neurons in the mucosa may result in altered input to the reflex circuitry that controls the pattern and frequency of peristaltic contractions of the gut wall.

The altered organization and properties of EGCs in GF rodents may also influence GI function independently of their effects on neural activity. Of particular interest in this context are the described roles of mEGCs in epithelial barrier function and immune responses in the intestinal mucosa (Bush et al., 1998; Neunlist et al., 2013; Rühl et al., 2004; Savidge et al., 2007). The implication of mEGCs in the function of multiple gut tissues, in conjunction with our current findings, suggests that the ongoing supply and turnover of glial cells to the lamina propria constitutes a novel microbiota-driven homeostatic mechanism essential for maintaining GI function under physiological conditions. It would be interesting to examine the dynamics of mEGCs in common inflammatory conditions of the gut (such as Crohn's disease) and whether altered homeostasis of these cells could be implicated in the pathogenesis of certain functional GI disorders (including irritable bowel syndrome) that are associated with abnormal motility (Longstreth et al., 2006) and changes in the microbial environment (De Palma et al., 2014; Mayer et al., 2014; Simrén et al., 2013). In that respect it is interesting that in our studies the network of mEGCs was restored within the intestinal mucosa upon conventionalization of GF mice at a stage beyond an early critical postnatal period. Although it is currently unclear whether this apparent plasticity of EGCs is manifested at all stages of adult life, it is conceivable that restoring glial function in patients with dysbiosis (abnormal composition of microbiome) could alleviate at least some of the dysmotility-associated symptoms.

As the ENS (and EGCs in particular) are known to express microbial pattern recognition receptors (such as TLR2 and TLR4) (Barajon et al., 2009; Brun et al., 2013), it will be of interest to determine whether microbiota control the dynamics of enteric glia directly or via an intermediary cell type. Irrespective of the mechanisms, our experiments identify EGCs as a major target of gut microbiota and suggest that microbes and their products regulate the development and maturation of other glial cell networks of the nervous system. Consistent with this view, previous studies have demonstrated transcriptome changes of differentiated astrocytes and reprogramming of Schwann cells exposed to lipopolysaccharides and leprosy bacilli, respectively (Hamby et al., 2012; Masaki et al., 2013). Changes in the organization and function of enteric glia, an essential component of a key relay

station (ENS) along the gut-brain axis, or potential deficits of CNS glia, could contribute to neuroendocrine and behavior abnormalities associated with changes in gut microbiota. Although our studies thus far have focused on the distal ileum, the identification of mEGCs in all intestinal segments, including the duodenum and the colon, suggests that homeostatic regulation of mucosal glia is a feature of the entire intestine. It will be interesting to determine whether microbiota regulates the homeostasis of mEGCs in all segments of the mammalian intestine and identify additional factors that contribute to the generation and supply of enteric glial cells to the intestinal mucosa. Understanding further the interplay between microflora and EGCs will provide an excellent model system to examine the effects of commensal and pathogenic micro-organisms on the host nervous system and help elucidate the pathogenesis and ultimately develop novel therapeutic strategies for GI disorders.

EXPERIMENTAL PROCEDURES

Experimental Procedures are described in [Supplemental Information](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, three movies, and experimental procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2014.12.037>.

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Neuron

Supplemental Information

**Microbiota Controls the Homeostasis
of Glial Cells in the Gut Lamina Propria**

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Snippert, Hans Clevers, Sven Pettersson, and Vassilis Pachnis**

Kabouridis et al. Fig. S1 related to Fig. 1

Kabouridis et al. Fig. S2 related to Fig. 1

Kabouridis et al. Fig. S3 related to Fig.2

Kabouridis et al. Fig. S4 related to Fig. 3 and Fig. 4

SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1. mEGCs contact multiple tissues in the intestinal mucosa.

(A, B) Glial processes (green, indicated by arrowheads) in the mucosa of *Sox10::Cre;MADM^{GR/RG}* mice are juxtaposed to crypt epithelia (identified by A33 immunostaining; cyan) (A) and the vascular network (identified by Rhodamine Dextran; red) (B). Scale bars: 0.5µm.

Figure S2, related to Figure 1. Intestinal mucosa is innervated prior to colonization by mEGCs.

(A, B) Most villi of *Sox10::Cre;R26REYFP* P0 mice have been colonised by neuronal processes (green). Arrows in (A) indicated PGP9.5⁺ neuronal processes. Scale bar: 100µm. (B) Quantification of villi with identifiable neuronal processes in the mucosa of *Sox10::Cre;R26REYFP* mice at different developmental stages. One way ANOVA, $P < 0.05$, Tukey post-hoc test, was performed. The F (dFn, dFd) and p value is 18.12 (3, 6) with $**p = 0.002$. (C) A villus-crypt (VC) unit corresponds to the lamina propria within a clearly identifiable villus and the associated crypt area above the submucosal plexus. Image is from a cryosection of the ileum of an adult *Sox10::Cre;R26REYFP* mouse. Arrows point to mEGCs identified DAPI⁺ nuclei and the associated GFP⁺ cytoplasm. Scale bar: 50µm.

Figure S3, related to Figure 2. Enteric glial cells continuously colonize the lamina propria of the gut.

(A, A', A'') A YFP⁺ cell (indicated by arrow) in a villus from the ileum of a tamoxifen-treated *Sox10::CreER^{T2};R26RConfetti* mouse at T15 co-expresses S100β. (B) A polychromatic villus from the ileum of a tamoxifen-treated *Sox10::CreER^{T2};R26RConfetti* mouse at T15. The presence of GFP⁺ nuclei (arrow) among the other fluorescent-labelled cells indicates that confetti signal corresponds to glial cell processes as well as glial cell bodies. This is independently confirmed by the superimposition of DAPI⁺ nuclei and confetti signal (C, C', C''). Scale bars: 50µm (A-B), 25µm (C-C''). (D, E) Cross sections from the gut of adult *hGFAP::CreER^{T2};R26RConfetti* mice injected with a single dose of tamoxifen and analysed at T0 (D) or at T90 (E). Arrows indicate isolated YFP⁺ cells in the myenteric plexus (D) and the myenteric plexus and a villus (E). Scale bars: 100µm (D-E).

Figure S4, related to Figure 3 and Figure 4. Microbiota is required to maintain the normal complement of mEGCs in the gut lamina propria.

(A) Direct comparison of the size of ceca (asterisks) dissected from CONV (conventional), GF (germ-free) and CONV-D (conventionalized) mice. (B-E) Treatment of adult *Sox10::Cre;R26RConfetti* mice with broad spectrum antibiotics result in enlargement of cecum (B, C) and loss of Confetti⁺ mEGCs (arrows) from the mucosa (D, E). Note the presence of Confetti⁺ ganglia in the myenteric and submucosal plexi of antibiotic treated mice (E). (F) The average number of confetti⁺ cells in VC units at T15 is reduced in antibiotic-treated animals. Data is represented as mean $\pm$ SEM. The distribution of the number of labelled glial cells per VC unit has been plotted using the non-linear paradigm, representing a different curve for each data set as the best fit with p value < 0.0001 . Using two-way ANOVA and Sidak's post-hoc test significant differences were observed in the 0 (****p<0.0001); 2 (*p=0.03); 3 (**p=0.0067) and 4 (*p=0.04) cells per VC unit categories. The F(DFn, DFd) and p values for Factor 1: With Vs Without antibiotic is 0 (1, 42) p>0.98; Factor 2: number of glial cells in VC units is 18.43 (6, 42) ****p<0.0001 and interaction of Factor 1 with Factor 2 is 23.46 (6, 42) with ****p<0.0001. Asterisks in panels A and B indicate the ceca. Scale bars in D-E: 100 μ m. (G) Model presenting the regulation of the homeostatic flow of glial cells from the peripheral plexus to the lamina propria of the gut mucosa. The microbiota of the gut lumen, either directly or indirectly, promote the transition of mature quiescent (Q) glial cells within the myenteric or submucosal plexi to an active (A) state which gives rise to mEGC (M) progeny that migrate into the mucosa. This model is consistent with the low levels of EGC proliferation and constitutive enteric gliogenesis that has been observed in the gut of adult rodents (Joseph et al., 2011; Laranjeira et al., 2011).

SUPPLEMENTAL TABLE

Table S1

Mice:	With antibiotics	Without antibiotics
Microorganisms:		
Aerobic bacteria		
0 days	$4.3 \times 10^6 (\pm 3.2 \times 10^6)$	$4.9 \times 10^6 (\pm 2.6 \times 10^6)$
10 days	<1	$1.0 \times 10^6 (\pm 0.34 \times 10^6)$
22 days	<1	$6.6 \times 10^5 (\pm 2.8 \times 10^5)$
Anaerobic bacteria		
0 days	$29.8 \times 10^6 (\pm 31 \times 10^6)$	$8.2 \times 10^6 (\pm 4.3 \times 10^6)$
10 days	<1	$4.2 \times 10^6 (\pm 5.0 \times 10^6)$
22 days	<1	$1.5 \times 10^6 (\pm 1.16 \times 10^6)$
Yeast		
0 days	$1.66 \times 10^5 (\pm 0.12 \times 10^5)$	$1.5 \times 10^5 (\pm 0.65 \times 10^5)$
10 days	<1	$2.0 \times 10^4 (\pm 0.65 \times 10^5)$
22 days	<1	$1.0 \times 10^4 (\pm 1.1 \times 10^4)$

Table S1, related to Figure 4. Bacteriologic analysis of faecal pellets in control and antibiotic-treated mice. Antibiotic treatment for 10 days reduced dramatically the number of aerobic and anaerobic bacteria and yeasts. Similar reduction was also observed at the end of the 3-week antibiotic course.

SUPPLEMENTAL MOVIES

Movie S1, related to Figure 1. mEGCs are highly branched. Shown is a surface-rendered reconstruction of confocal images of a single GFP⁺ glial cell (green) in a villus of *Sox10::Cre;MADM^{GR/RG}* mice.

Movie S2, related to Figure 1. mEGCs are closely associated with crypts. Shown is a surface-rendered reconstruction of confocal images of crypt epithelial cells immunostained for A33 (cyan) in close association with GFP⁺ glial cell network (green) from the ileum of a *Sox10::Cre;MADM^{GR/RG}* mouse. Parts of the vascular network are highlighted by Rhodamine dextran sulphate (red).

Movie S3, related to Figure 1. mEGCs interact closely with the vascular network of villi. Shown is a surface-rendered reconstruction of confocal images of a GFP⁺ glial cell (green) and Rhodamine Dextran labelled microvasculature (red) in a villus of a *Sox10::Cre;MADM^{GR/RG}* mouse.

MATERIALS AND METHODS

Animals

Generation of the *R26REYFP* (Srinivas et al., 2001), *R26RConfetti* (Snippert et al., 2010), *MADM-6^{GR}* and *MADM-6^{RG}* reporter (Tasic et al., 2012; Zong et al., 2005) and the *Sox10::CreER^{T2}* (Laranjeira et al., 2011), *hGFAP::CreER^{T2}* (Ganat et al., 2006) and *Sox10::Cre* (Matsuoka et al., 2005) transgenic mice have been described previously. The effect of microbiota on mEGCs was examined in wild-type C57Bl/6 mice which were raised either conventionally at specific pathogen-free conditions (CONV animals) or in germ-free isolators (GF animals). Sterility of isolators was analysed weekly by plating fecal homogenates onto different types of agar plates to detect aerobic and anaerobic bacteria and fungi. Mice maintained in GF conditions were given autoclaved food (LabDiet 5010/5021) and drinking water. Consistent with previous reports (Reinhardt et al., 2012), we consistently observed that the villi of GF mice were generally thinner relative to those from CONV animals. GF mice were conventionalised (CONV-D animals) by gavaging 100µl of homogenate generated by dissolving 2 fecal pellets from CONV animals in 1 ml of PBS. Following gavage CONV-D mice were co-housed with CONV animals. All mice were maintained in the animal facilities of NIMR (London, UK) and SingHealth Research (Singapore) in accordance with the regulatory standards of each institution and experiments were approved by the respective local ethical committees.

Tamoxifen administration for lineage tracing of EGCs

4-OH-tamoxifen was prepared at stock concentration of 10 mg/ml in corn oil with 10% ethanol. 8-12 week old *Sox10::CreER^{T2};R26RConfetti* mice were administered 4-OH-Tamoxifen (0.1 mg/gram body weight) intraperitoneally for two consecutive days and analysed 4 days (T0), two weeks (T15) or three months (T90) later without additional exposure to tamoxifen. *hGFAP::CreER^{T2};R26RYFP* mice were induced with a single injection of 4-OH-Tamoxifen (0.1 mg/gram body weight) and analysed at the indicated stages.

Antibiotic-induced depletion of gut microbiota

The effect of microbiota on the maintenance of mEGCs was analysed in 8-12 week-old wild-type (Parkes) and *Sox10::Cre;R26RConfetti* reporter mice that were supplied with a cocktail of broad-spectrum antibiotics in their drinking water for 3 weeks. The antibiotic cocktail consisted of ampicillin (1gr/L; Centaur Services Ltd.), metronidazole (1 gr/L; Centaur Services Ltd.), vancomycin (0.5 gr/L; Centaur Services Ltd.) and neomycin (0.5 gr/L; Sigma-Aldrich) (Reikvam et al., 2011). Drinking water was sweetened with 1% w/v of sucrose and

fresh antibiotic preparation was administered every 4 days. For the combination of EGC lineage tracing and antibiotic treatment, 8-12 week-old *Sox10::CreER^{T2};R26RConfetti* mice were induced with 4-OH-tamoxifen one week following addition of antibiotics to the drinking water and the distribution of labelled glial cells was analysed at T0 and T15. The effect of antibiotics on intestinal microflora was examined by quantitative analysis of aerobic and anaerobic bacteria and yeasts present in faecal pellets of *Sox10::Cre;R26RConfetti* mice. On day 0 of antibiotic treatment (prior to the addition of the antibiotic cocktail to the drinking water), on day 10 and on day 22 (conclusion of the treatment), mice defecated directly into pre-weighted sterile microcentrifuge tubes. Pellets were dissolved in PBS and 100µl of serial dilutions of faecal suspensions were plated onto duplicate horse blood agar plates for aerobic incubation (with 5% CO₂), anaerobic horse blood agar plates (incubation in anaerobic chamber) and Sabouraud Dextrose agar for yeast counts (air incubation). Following 3 days of incubation, plates were enumerated and the number of micro-organisms in the original sample was calculated and expressed as colony-forming units per milligram of faecal material (cfu/mg). Faecal bacterial load was determined for three mice in each of the antibiotic treatment and control groups (**Table S1**).

Marking the vascular network of MADM mice

To enable visualisation of the mucosal vascular system, *Sox10::Cre;MADM-6^{GR/RG}* were placed briefly under a heat lamp to dilate the tail vein, which was used to inject of 50µl of 2000kDa Rhodamine Dextran (10mg/ml in PBS; Life Technologies). 5 min after injection animals were culled by cervical dislocation.

Developmental profile of mEGCs

The developmental profile of mEGCs was analysed in the ileum of *Sox10::Cre;Rosa26EYFP* mice in which all enteric neurons and glia are labelled by GFP (Laranjeira et al., 2011). Since in rodents the cell somata of enteric neurons are located exclusively within the ganglia of the myenteric and submucosal plexus, GFP⁺ cells within the mucosa represent S100β⁺ glial cells. Due to the extensive branching of glial cells, we used the robust cytoplasmic GFP signal in association with the nuclear marker DAPI to quantify the experiments shown in **Figure 1F**. Glial cells were quantified in the area of the lamina propria that corresponds to villus-crypt (VC) units. Each VC unit represents a clearly identifiable villus and the associated crypt area extending up to (but excluding) the submucosal and myenteric plexus (**Figure S2**). Quantification is based on analysis of at least 200 VC units from each animal and at least three animals were used for each stage. For all stages included in the developmental analysis, gut was dissected and fixed in 4% paraformaldehyde (PFA) at 4°C overnight (O/N).

Following extensive washing with PBS 1cm segment of the ileum was placed in 30% sucrose (in PBS) and incubated O/N at 4°C. Gut samples were embedded in a mix of 15% sucrose and 7.5% gelatin in PBS. When solidified at 4°C, tissue blocks were immersed in isopentane on dry ice and stored at -80°C until processed further. 14µm sections were generated using a Leica Cryostat and placed on Superfrost Plus slides. For immunostaining, sections were permeabilised with 0.3% PBT (PBS/TritonX-100) for 10 minutes and incubated with blocking solution (1% Bovine Serum Albumin and 0.15% Glycine in 0.1% PBT) for at least 1 hour. Combinations of primary antibodies were diluted as required in blocking solution and incubated O/N at 4°C. Secondary antibodies were diluted in blocking solution and applied to sections at room temperature (RT) for 2 hours. Following three washes with 0.3% PBT, slides were mounted with mounting medium containing DAPI. Primary antibodies: S100β (rabbit polyclonal from DakoCytomation), PGP9.5 (rabbit polyclonal from Serotec) and GFP (rat monoclonal, clone GF090R, from Nacalai Tesque). All primary antibodies were used at a dilution of 1:1000. Secondary antibodies were AlexaFluor564-conjugated donkey anti-rabbit (Jackson ImmunoResearch) and AlexaFluor488-conjugated donkey anti-rat (Invitrogen) and used at a dilution of 1:500.

S100β immunostaining and fluorescent reporter detection in the gut of adult mice

Ileum from 8-12 week-old mice was immersed in 4% PFA in PBS for 4 hours at RT and fixed for a further of 16 hours in 2%PFA in PBS at 4°C. Following fixation, tissue was washed extensively and either processed immediately or stored at 4°C in PBS (with 0.05% sodium azide) for up to 1 week. 4-5mm long segments from the terminal ileum were embedded in 3% agarose in PBS and sectioned with a vibratome (Leica VT1000S) to obtain 50µm sections. For imaging of fluorescent reporters (MADM-GFP, Confetti) sections were mounted directly onto slides and analysed by laser confocal microscopy. Flat-mount preparations of myenteric ganglia were generated by peeling the longitudinal muscle layer of a freshly-dissected segment of the ileum along with the attached myenteric plexus (Laranjeira et al., 2011) followed by fixation for 30 min in 4% PFA in PBS. For immunostaining, sections or peels were blocked in 5% normal donkey serum in PBS/0.5% TritonX-100 for 2 hours at RT and incubated with primary antibodies diluted in blocking solution O/N. Following extensive washing sections or peels were incubated with secondary antibodies for at least 2 hours at RT. Primary antibodies: S100β (rabbit polyclonal from DakoCytomation, 1:1000), Sox10 (rabbit polyclonal from Santa Cruz, 1:200), and GFP (rat monoclonal, clone GF090R, from Nacalai Tesque, 1:1000). Secondary antibodies were AlexaFluor 564-

conjugated donkey anti-rabbit (Jackson ImmunoResearch, 1:500) and AlexaFluor 488-conjugated donkey anti-rat (Invitrogen, 1:500).

To assess the effect of GF conditions and antibiotics on mEGCs we examine >2000 VC units from each gut analysed. In virtually all cases, glial cells were eliminated from the villi but some S100 β cells were often observed in the lamina propria between crypts (**Figure 3B and Figure 4B**). Since the number and position of the residual glial cells varied along the crypt-villus axis, the effect of GF conditions and antibiotic treatment was quantified by counting the number of S100 β ⁺ glial cells present along the entire length of ~100 randomly identified VC units. To determine the area of VC units occupied by glial cells images of S100 β immunostainings were converted into binary images (8-bit grayscale) with ImageJ and the area of individual VC units was selected. The S100 β area scoring above the set threshold was expressed as fraction of the total area selected using the Measure command under the Analyse tool. An average value of the VC unit measurements was calculated for every mouse in each group.

Confocal microscopy and image analysis

Confocal images of adult gut cryostat (14 μ m) and vibratome (50 μ m) sections were acquired with Leica TCS SP5 with a DN6000 microscope assisted by the LAS AF software. The objective lenses used included a dry X20 (0.75 NA), Nose-dip X20 (1.0 NA) and an oil-immersion X40 (1.75 NA). Sequential scans were performed in for XFP excitations: nuclearGFP, the argon laser 488 nm line; for EYFP 514 nm line; for RFP a red diode laser emitting at 561 nm, and blue mCFP was excited using a laserline at 458 nm. In general GFP fluorescence was collected between 498–510nm, airy 1; EYFP fluorescence was collected between 521–560 nm, airy 1; RFP fluorescence was collected between 590–650 nm, airy 1; mCFP fluorescence was collected between 466–495 nm, airy 1.5. DIC was obtained while using 488 nm laser through transmission gate. All images were processed with either Adobe Photoshop CS4 (Adobe Systems) or ImageJ (Wayne Rasband, NIH). 3-D reconstructions and movies were generated using the 3D opacity mode in Volocity (Perkin Elmer, Waltham, MA).

Quantification and Statistical Analysis

Data have been obtained from at least three independent animals. N values have been mentioned in the text/figure legends. Data are mainly presented as mean $\pm$ standard error of the mean (SEM) unless otherwise mentioned. All error bars represent SEM. Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software) and Microsoft Excel

(Microsoft). Significant differences between data sets have been obtained with different tests as mentioned in the figure legends.

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Title: Establishing a co-culture system to study enteric glial cell functions.

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ABSTRACT

Recent findings have highlighted the emerging role of enteric glial cells (EGCs) in maintaining the integrity of the intestinal epithelial barrier, in addition to their well-established role in supporting the functions of enteric neurons. To dissect the significance of EGCs interaction with intestinal epithelial cells (IECs), establishing a co-culture system involving self-renewing intestinal organoids and primary EGCs is timely and relevant. Currently, the co-culture conditions for facilitating the interaction between EGCs and intestinal organoids *in vitro* is unknown. Here, using an inducible reporter line to label EGCs, we first selected a reliable culture condition to expand primary EGCs derived from mouse longitudinal muscle/ myenteric plexus for co-cultures with IECs. Following, culture media conditions designed for intestinal organoids previously was found to be sufficient in maintaining primary EGCs, albeit only when soluble factors from a commercially available basement membrane extract were added. Finally, we observed close association of EGCs with co-cultured intestinal organoids, reminiscent of the known *in vivo* contacts between these two cell types previously reported. Our results indicate an effective simulation of the *in vivo* microenvironment, providing a basis for future experiments where cell types found in the mucosa, in addition to intestinal epithelial cells could be tested for their interaction with EGCs.

(204 words)

INTRODUCTION

As the largest component of the peripheral nervous system (PNS), the enteric nervous system (ENS) is capable of co-ordinating diverse biological processes such as gastrointestinal motility independently of the central nervous system (CNS) inputs (Bayliss and Starling, 1901), earning itself the title of the 'second-brain' (Gershon, 1999). The ENS is structured mainly into two concentric plexuses, where the outer ring (myenteric plexus, MP) is sandwiched between the longitudinal muscle and circular muscle layers while the inner ring (submucosal plexus, SP) can be found within the submucosal layer. The ENS is comprised of a mixture of neurons and glial cells, where the latter outnumbers the enteric neurons by a factor of 4 or more (Gabella, 1981). Drawing parallels from the CNS, glial cells found within the ENS are also heterogeneous, as defined by distinct morphologies at different anatomical locations, possibly outlining multiple unique subtypes (Boesmans et al., 2015; Gulbransen and Sharkey, 2012). Recently, besides being responsible for providing aid and support for the health of enteric neurons, enteric glial cells (EGCs) has been shown to be important for modulating intestinal barrier functions and resistance against bacteria infection (Flamant et al., 2011; Neunlist et al., 2007; Savidge et al., 2007). The loss of EGCs via a genetic ablation technique was found to induce intestinal inflammation, further supporting the requirement of EGCs for intestinal barrier homeostasis and mucosal immunity (Bush et al., 1998; Savidge et al., 2007).

Several markers known to label astrocytes in the CNS or Schwann cells in the PNS such as the calcium-binding protein S100, the intermediate filament protein- glial fibrillary acidic protein (GFAP) and proteolipid protein 1

were also found to be expressed specifically by the glial cells within the ENS (Ferri et al., 1982; Jessen and Mirsky, 1980; Rao et al., 2015). Transcription factors such as Forkhead box d3 (Foxd3), SRY (Sex determining Region Y)-box10 (Sox10) and Sox2, commonly used to mark neural crest derivatives were also reported to be expressed by adult EGCs (Heanue and Pachnis, 2011; Hoff et al., 2008; Mundell et al., 2012). As mentioned above, only a few known markers exist and in most cases, different subsets of EGCs (based on their typical morphologies and cellular localisations) expressed one or more of these markers *in vivo* (Boesmans et al., 2015; Rao et al., 2015), making it difficult to define functionally distinct EGCs based on the current available markers.

In order to study the functions of EGCs, the research field has been utilising *in vitro* cultures and co-culture systems to address some of the open questions. EGCs in these systems were derived from a variety of sources; primary cells from mouse longitudinal muscle/myenteric plexus (LMMP) peels; rat myenteric plexus preparations or transformed cell lines (Abdo et al., 2010; Boesmans et al., 2015; Di Liddo et al., 2015; Grundmann et al., 2015; Savidge et al., 2007; Soret et al., 2013; Van Landeghem et al., 2009; von Boyen et al., 2004). In some cases, EGCs were co-cultured with a monolayer of intestinal epithelial cells (IEC) of human colorectal adenocarcinoma origins (Caco-2) to investigate the interactions between these two cell types (Savidge et al., 2007; Soret et al., 2013; Van Landeghem et al., 2009). Conceivably, most of these co-culture systems were based on the use of fetal bovine serum (FBS) in the culture medium, which could support both EGCs and Caco-2 cells. In overcoming the disadvantages of working with transformed cell lines,

a recent development where self-renewing organoids were derived from isolated intestinal crypts has greatly improved the prospect of culturing the intestinal epithelium at near physiological conditions (Sato et al., 2009). Similar to the intestinal organoid cultures, primary EGCs could also be cultured and maintained under serum-free conditions (Abdo et al., 2010; Boesmans et al., 2015). Importantly, given the plasticity of EGCs *in vivo*, it is very likely that the phenotype of primary EGCs would be different if cultured in the presence of FBS or in serum-free conditions. This conjecture has not been formally addressed and conditions suitable for the co-culture of primary EGCs with intestinal organoids at near physiological conditions is still lacking.

Here, we ensued to compare the phenotype of primary EGCs grown in the presence of FBS or in serum-free conditions directly. We noted both morphological and molecular differences in primary EGCs, distinctive of their respective culture conditions used. Next, we established a workflow of expanding primary EGCs for a short period, followed by confirming the identity of these cells by immuno-staining before co-culturing them with intestinal organoids in a three-dimensional matrix. We show that EGCs could form close associations with co-cultured organoids, reminiscent of the *in vivo* situation where EGCs can be seen surrounding individual crypt structures or in contact with a specific epithelial cell-type. Our results suggest that the co-culture system developed herein is suitable for the investigation of interactions between cell types in the lamina propria with a dynamic cellular component of the ENS, the EGCs.

RESULTS

Serum free media maintains EGCs in a progenitor-like state. To determine which culture conditions work best to expand and maintain primary EGCs for co-culturing later, we first examined the characteristics of EGCs grown in serum-free (G5) media or in the presence of FBS. Since Sox10 expression in the adult gut is restricted to EGCs (Bondurand et al., 2006; Kim et al., 2003; Laranjeira et al., 2011), we took advantage of an inducible line, the Sox10::iCreER^{T2} (Laranjeira et al., 2011) and crossed them to a reporter line available (R26R::CAG-tdTomato). Enzymatically dissociated LMMPs from the SER26-tdT adult animals yielded primary EGCs expressing tdT (red) upon tamoxifen injection, facilitating the live visualisation of these cells in culture. As shown in Figure 1 (A-D and E-H), Sox10-tdT⁺ cells grown in either conditions at days *in vitro* (DIV) 3 co-expressed known EGC markers such as FoxD3 (blue) and GFAP (green) as well as S100 (Figure 1, I-K and L-N, in green).

In order to assess the proliferative state of cells at a given time, we proceeded to add 5-ethynyl-2'-deoxyuridine (EdU) into the cultures to determine the proportion of Sox10-tdT⁺ cells that were in the S-phase of the cell cycle. We identified EdU⁺ cells among Sox10-tdT⁺ cells in both culture conditions however, the percentage of EdU⁺ Sox10-tdT⁺/total Sox10-tdT⁺ cells at DIV3 were significantly higher in G5 culture conditions compared to those cultured in the presence of FBS (Figure 2, A-D and E). To follow up with the nature of these Sox10-tdT⁺ EGCs in culture, we studied Sox2 and S100 β expression, which are markers for EGCs with ENS progenitor characteristics (Heanue and Pachnis, 2011) and differentiated EGCs (Young et al., 2003)

respectively, at different time points. We found that Sox10-tdT⁺ cells grown in the presence of FBS at DIV3 had significantly less of Sox2 immuno-reactivity (Figure 2F) but not S100 β (Figure 2G) when compared to cells grown in G5 media. Similarly, At DIV6, Sox10-tdT⁺ cells cultured in the presence of FBS, positively immuno-stained for Sox2 (Figure 3, A-C) was significantly lower, at $64.3 \pm 6.7\%$ compared to $99.7 \pm 6.2\%$ of Sox10-tdT⁺ cells cultured in G5 conditions (Figure 3, D-F and G). Following, S100 β immuno-reactivity showed 100% overlap with Sox10-tdT⁺ cells in FBS cultures at DIV6 (Figure 3, C and H). However, the percentage was significantly lower, at $71.4 \pm 6.9\%$ for cells cultured in the G5 media at the same time point (Figure 3, F and H). Of interest, GFAP immuno-reactivity within the total Sox10-tdT⁺ pool did not present any significant difference between the two different culture conditions examined at DIV6 (Figure 3I). These results indicate that Sox10-tdT⁺ cells retain progenitor characteristics and had higher proliferative rates when grown in G5 media conditions compared to culture conditions containing FBS.

Interestingly, we noticed a significantly higher proportion of DAPI⁺ nuclei that were not Sox10-tdT⁺ in FBS supplemented cultures at $30.5 \pm 4.7\%$ compared to $8.0 \pm 2.9\%$ of DAPI⁺ cells grown in G5 media (Figure 3, J, M and P). Based on previous publications (Bondurand et al., 2003; Morrison et al., 1999), these cells could be alpha-smooth muscle actin (α -SMA)⁺ myofibroblasts that differentiated during the culture period from neural crest derived progenitors or they could have been seeded at the start of the culture as they are highly abundant in the LMMP tissue preparations. As shown in Figure 3K, α -SMA immuno-staining revealed large numbers of myofibroblasts in FBS supplemented cultures but not so in G5 media conditions (Figure 3N).

Since we did not observe any obvious α -SMA and Sox10-tdT double positive cells in both culture conditions, these cells were likely to be seeded from before and maintained in cultures supplemented with FBS (Figure 3, L and O). Together, it appears that the G5 media condition was more permissive for the proliferation of EGCs but not smooth muscle cells in culture.

Differentially cultured EGCs show distinct morphological phenotypes. At least 4 EGC subtypes have been described *in vivo* previously, based largely on their distinct morphologies (Boesmans et al., 2015; Gulbransen and Sharkey, 2012). GFAP is the major protein component of intermediate filaments found in glial cells and believed to be important for determining the shape of astrocytes and other glial lineages such as EGCs (Eng, 1985; Jessen and Mirsky, 1980). Here, using antibodies against GFAP, we went on to compare the morphology of cells grown in either of the two conditions. As presented in Figure 4A, EGCs cultured in the presence of FBS appeared to display extensive GFAP⁺ fibres. In contrast, GFAP⁺ fibres in cells grown in G5 media were less extensive, indicating a discrete morphology of cells (Figure 4B). Furthermore, besides the distinct morphological properties observed when comparing both culture conditions, EGCs were seen to be consistently arranged in tight colonies in cultures containing FBS compared to a wider spread at DIV3 for EGCs cultured in G5 media conditions (Figure 4, E and F and Supplementary Figure 1, i and iii). Remarkably, these morphological distinctions were reversible, as seen with EGCs cultured first in the presence of FBS till DIV3 and then in G5 condition till DIV6 and *vice versa* (Supplementary Figure 1, ii and iv). Taken together, EGCs in culture were

responsive to different culture conditions and the associated morphological differences in various conditions were reversible.

Conditioned media from Matrigel supports proliferation of EGCs. Next, to perform co-cultures of EGCs to study their interaction with other cell types of interest such as IECs, sub-culturing of primary EGCs was necessary. Consequently, we harvested Sox10-tdT⁺ cells at DIV5 (grown in either FBS or G5 media) when they were 70-80% confluent and re-plated them (1:4) onto culture slides containing their respective culture supplements. Four days after the first passage, Sox10-tdT⁺ maintained in the presence of FBS were mostly large flat cells mixed with smaller sized cells with short processes (Figure 5A'). On the other hand, Sox10-tdT⁺ cells re-seeded into G5 media appeared mostly homogenous morphologically and became non-adherent, resembling the well-characterised enteric neurospheres (Figure 5B'). Remarkably, these spheres did not seem to increase in size over time (data not shown), which may reflect a quiescent state. Considering what we had observed so far where EGCs cultured in G5 media had higher proportion of proliferating cells and less α -SMA⁺ myofibroblasts, we decided to expand primary EGCs cultured in G5 conditions briefly and collected them at DIV3 for further co-culture experiments.

EGCs are known to interact with multiple cell types in the mucosa (Bohorquez et al., 2014; Kabouridis et al., 2015; Liu et al., 2013). For example, Sox10-tdT⁺ labelled cells can be seen associating with individual crypt structures and also in the villus (Supplementary Figure S2). We were keen on investigating the relationship of EGCs with the IECs in particular and proceeded to take steps in co-culturing these cells types. Culture condition

used to generate intestinal epithelial organoids *ex vivo* in a basement membrane matrix (Matrigel®) has been established recently, with high similarity to conditions used for culturing neuronal cells (Sato et al., 2009). We first looked at how the EGCs grown in G5 media till DIV3 respond to culture conditions designed for the culturing of intestinal epithelial organoids using organoid media. We found that cells cultured in organoid media remained mostly as single rounded cells, two days after the passage (DIV5) (Figure 6, Ai). Since intestinal organoids requires the use of a basement membrane matrix to be grown in culture, we reasoned that EGCs might require additional factors if cultured alone, such as factors from the Matrigel® for example. Thus we supplemented the organoid media with Matrigel®-conditioned media (supernatant collected 24 hours after allowing polymerised Matrigel® to sit in organoid media). At 2 days after passage (DIV5), Sox10-tdT⁺ cells grown in the presence of 40% Matrigel-conditioned media started spreading and formed processes (Figure 6, Aii). To confirm the identity of these cells, we fixed and stained them for GFAP (Figure 6B). Apart from scoring positive for GFAP immuno-reactivity, re-plated cells cultured with the addition of Matrigel® conditioned media were also positive for the incorporation of EdU, suggesting that these cells were EGCs and were cycling in these conditions (Figure 6B). Taken together, we have confirmed that culture conditions set for the growth of intestinal organoids were also suitable for the growth and maintenance of EGCs in culture.

Close proximity of EGCs with co-cultured intestinal organoids observed.

At 3-4 DIV, EGC cultures were harvested and re-suspended in Matrigel® containing freshly isolated crypts. The mixture of EGCs and isolated crypts

were then seeded in the form of droplets onto culture slides. After three days of co-culture, Sox10-tdT⁺ cells were observed to be in close proximity of growing organoids (Figure 7, A-C). Three-dimensional reconstruction of confocal images focused on the associations between Sox10-tdT⁺ cells and intestinal organoids revealed at least three types of associations. Processes from Sox10-tdT⁺ cells could be seen wrapping around the organoid and cell debris marked by DAPI staining (Figure 7A'); attached onto the surface of the organoid (Figure 7B'); or selectively in contact with a single cell on the organoid surface (Figure 7C'). Our results showed that EGCs could form close associations with intestinal organoids co-cultured within a basement membrane extract *in vitro*.

DISCUSSION

To study the multifaceted functions of EGCs, experiments involving the use of primary cultures or immortalised cell lines of EGC origins were conducted (Abdo et al., 2010; Boesmans et al., 2015; Boesmans et al., 2013; Di Liddo et al., 2015; Soret et al., 2013; Van Landeghem et al., 2009; von Boyen et al., 2004). Collectively, these studies have demonstrated that EGCs can be cultured and maintained in a variety of culture conditions. However, culture conditions meant for co-culturing EGCs with other cell types of interest such as IECs at near physiological conditions remained unknown.

We began by first comparing the characteristics of EGCs at DIV3 and DIV6, grown in either in the presence of FBS or in G5 media to determine the appropriate conditions for expanding EGCs initially for use later in co-culture experiments. Our results show that short-term cultures (3 DIV) of Sox10-TdT⁺ cells derived from the longitudinal muscle/myenteric plexus retain their expression of known glial markers when cultured in both media conditions tested. However, the phenotypic expressions of EGC markers were highly dynamic, as demonstrated by comparing cultures fixed on DIV3 with those fixed on DIV6, coherent with the plasticity of these cells previously reported (Boesmans et al., 2015; Di Liddo et al., 2015). In addition, the data presented here showed that despite having stable GFAP expression in both conditions, S100 β and Sox2 expression varied inversely at DIV6 suggests that G5 culture conditions promotes the induction and/or maintenance of progenitor cells while FBS containing cultures supports differentiation. The progressive loss of S100 β immuno-reactivity specifically in G5 media cultured cells, could imply a gradual process of dedifferentiation since the expression of s100 β in GFAP⁺

cells was found to indicate the loss of neural stem cell potential within the CNS for instance (Raponi et al., 2007). Further, a recent study comparing EGCs cultured in "standard" growth media (Schafer et al., 1997) versus one without the major growth factors such as (bFGF, EGF, NGF and GDNF) gave rise to cells that were phenotypically glial and permitted the formation of neurospheres (Di Liddo et al., 2015). Conversely, cells cultivated in the absence of these growth factors were more prone to become neurons; expressing classic pan-neuronal markers in culture with limited formation of neurospheres (Di Liddo et al., 2015). As such, it is plausible that the molecular signalling pathways specifying enteric neural crest derived progenitor cells (ENCCs) are closely tied to the differentiation program of EGCs and *vice versa*, which is an exciting area to be addressed since adult EGCs and ENCCs during development expresses Sox10 and also Sox2 in a subset of these cells but not in matured enteric neurons (Heanue and Pachnis, 2011). Elucidating culture conditions that potentiates the maintenance of a particular EGC subtype, if possible, coupled with molecular studies at single-cell resolution (Grun et al., 2015) *in vivo* may provide a viable platform to begin deciphering the putative functions of specific EGC subtypes.

Apart from the use of molecular makers, the morphologies of cells in general may provide clues to differential functions. The EGCs were traditionally viewed as chaperone cells that support and maintain the health of enteric neurons by 'gluing' them together in the enteric ganglia (the word 'glial' is derived from the word 'glue' in Greek). However, recent evidences argue that EGCs are also involved in various homeostatic regulations such as immune modulation, gastrointestinal motility patterns and mucosal secretion

(Gulbransen and Sharkey, 2012). Of interest, it appears that EGCs can be subcategorised into 4 different types based on their and cellular morphologies (Boesmans et al., 2015; Gulbransen and Sharkey, 2012). Here, we have seen distinct morphological phenotypes of cells via GFAP immuno-staining, demonstrating that primary EGCs adopt different morphologies depending on environmental cues such as variations in culture conditions. Moreover, these differences are reversible in culture when we switched the culture media from FBS supplemented to G5 media and *vice versa*. Of note, these changes were observed from cells cultured directly on a plastic surface and hence were not exactly how they should look in a three-dimensional environment. Nonetheless, further studies aimed at identifying unique gene expression profiles that matches distinct morphological phenotypes both *in vitro* and *in vivo* are warranted. The resulting metadata would allow the field to develop tools to setup cultures supporting the growth of a particular EGC subtype for instance, to understand the functions of these unique cells. Taken together, our *in vitro* data, which suggests that EGCs are plastic and possess the propensity to respond to external cues for adaptation may shed light on the supposed functions of these cells *in vivo*.

Recently, several studies have focused on the interaction of EGCs with intestinal epithelial cells at steady state conditions or when mice were challenged with infectious pathogens such as *Shigella flexneri* (Bohorquez et al., 2014; Flamant et al., 2011; Kabouridis et al., 2015; Van Landeghem et al., 2009). Embedded in these studies was the notion that molecular factors that support intestinal epithelial barrier (IEB) homeostasis could come from EGCs (Flamant et al., 2011; Gulbransen and Sharkey, 2012; Van Landeghem et al.,

2009). In support, confocal images from transverse sections of the ileum of the Ser26-tdT mice revealed a dense network of EGCs encircling individual crypt as well as within the villus, suggests that EGCs are well positioned for such a role. Following, it has recently been established that the migration of EGCs into the villus was dependent on the presence of the microbiota (Kabouridis et al., 2015) and together with the discovery of direct contacts between EGCs and structures termed 'neuropods' found on entero-endocrine/chromaffin cells (Bohorquez et al., 2014) advocate for the involvement of EGCs in responding to environmental cues, perhaps by providing a feedback to maintain the IEB. Relevant to the *in vivo* situation, the co-culture system that we have established here provides evidences of various types of contacts such as individual Sox10-TdT⁺ cells (EGCs) wrapping around growing organoids and EGCs that specifically 'target' a particular cell within the organoid. Of interest, signalling molecules known to regulate ENS development were found to be specifically expressed or secreted by intestinal stem cells and not by their recently divided sister cells (Munoz et al., 2012). Consequently, by examining if specific epithelial cell types were targeted by the EGCs in our co-cultures may shed light on the physiological importance of such interactions and allow us to perturb such interactions *in vitro* for more in-depth analysis.

In summary, we have established a stable protocol for the culture of primary EGCs for *in vitro* studies, coupled with a demonstration of close associations between co-cultured EGCs and growing intestinal organoids. We believe that such a system is a basis for future studies where other cell types for instance; immune cells and/or myofibroblasts could be added for a more

representative replication of the *in vivo* microenvironment, allowing for a broader scope of experiments to be performed.

MATERIALS AND METHODS

Animals. The *Sox10::CreER^{T2}* line was generated as previously described (Laranjeira et al., 2011) and crossed with *R26R-CAG-tdTomato* (The Jackson Laboratory) to generate SER26-tdT⁺ heterozygotes. Mice aged between 8-14 weeks were used for experiments. C57/BL6 mice wild type mice were used for the isolation of intestinal crypts. All mice were housed in the animal facility at the Francis Crick Institute, Mill Hill laboratory (London, UK) in accordance to regulatory standards. All animal experiments were approved under licences from the Home Office, UK.

Lineage tracing of EGCs via Tamoxifen administration. Working concentrations of tamoxifen were prepared from powdered stock (Sigma Aldrich), dissolved in 9 parts of corn oil (10mg/ml) and 1 part of ~99% ethanol. SER26-tdT⁺ mice were given intra-peritoneal injections of tamoxifen preparations at a dose of (0.1mg/gram of body weight) a day before they were humanely sacrificed for subsequent tissue harvesting.

Primary enteric glial cell cultures. Enteric glial cultures were modified from (Boesmans et al., 2015). Briefly, tissue preparations of LMMP were collected and washed in ice-cold HBSS with 5% FBS. Tissues were then minced and allowed to digest in collagenase (1mg/ml) for 20 mins shaking at 37°C followed by Liberase TL (Roche) at 1mg/ml for another 20mins shaking at 37°C. Digestion was halted with the addition of 10% FBS followed by triturating gently using glass Pasteur pipettes. Cell suspensions were washed twice, resuspended in DMEM/F12, 1%Penicillin/Streptomycin supplemented with 10% FBS and seeded overnight onto Permanox plastic slides (Thermo-Fisher Scientific). For serum-free EGC cultures, media was removed on the

second day and exchanged for DMEM/F12, 1%Penicillin/Streptomycin, 1% N2, 1% G5 and NGF 7s at 50ng/mL (all from Thermo Fisher Scientific). Cells were kept in a humidified incubator at 37°C with 5% CO₂ for 3, 5 or 6DIV.

Immuno-staining and EdU detection. Cultured cells were fixed with 4% PFA in PBS in the cold room for 10 minutes followed by permeabilisation with 0.3% Tx-100 in PBS (PBT). Next, 10% Normal Donkey Serum (Jacksons ImmunoResearch Laboratories) in 0.3% PBT was used for blocking for an hour before respective primary antibodies were added and incubated at 4°C overnight. Slides were washed 3 times with 0.3% PBT, and corresponding fluorescently labelled secondary antibodies were added for an hour in blocking buffer before another 3 round of washes with 0.3% PBT. Vectashield mounting medium (Vector Labs) was used for mounting. Primary antibodies used in this study included rabbit-anti-S100 β (1:500; DAKO), chicken-anti-GFAP (1:300; Abcam), goat-anti-Sox2 (1:200; Immune Systems, UK), rabbit-anti-FoxD3 (1:100; a gift from Dr. Patricia A Labosky, Vanderbilt University, US), mouse-anti- α SMA (1:400; Sigma-Aldrich). All secondary antibodies were donkey hosted with the respective conjugated fluorophores- 488, 568, 647 or Cy5 (1:500; Thermo Fisher Scientific). Stock concentration of EdU (10 mM) was diluted 1000X before being added into cultures an hour before cells were fixed. Click-iT Alexa Fluor 647 imaging kit (Thermo Fisher Scientific) was used for visualization of EdU incorporated in cells following manufacturer's recommendations.

Microscopy and image analysis. Confocal and fluorescent images were acquired with Leica TCS SP5 and Leica 4000B inverted microscope respectively. Standard profiles of laser excitations and emission filters for

secondary fluorophores were used, managed by the Leica Application Suite Advanced Fluorescence (LAS AF). All images were processed with either Adobe Photoshop CS5 (Adobe Systems) or Fiji (Schindelin et al., 2012). 3D reconstructions were generated using the 3D Project function in Fiji.

Tissue Processing. Intestinal tissues from SER26-tdT mice were cut longitudinally, washed and then immersed in 30% sucrose/PBS overnight. Samples were then embedded in sucrose-gelatin at 2:1/PBS before mounting onto aluminium foil receptacles using Tissue-tek O.C.T Compound (VWR) and left to freeze on dry ice. Sections of 12-20µm were cut using Microm HM 560 CryoStar cryostat, thaw-mounted onto SuperFrost Plus slides (both Thermo Scientific) and air-dried for 2 to 3 hours at RT followed by immuno-staining.

Isolation of small intestinal crypts and co-culturing with EGCs. Intestinal crypts were harvested according to a previously published protocol with slight modifications (Kabiri et al., 2014). Briefly, whole small intestines were opened longitudinally and washed before cutting into fine pieces. Then, tissues were treated in cold PBS with EDTA (2mM) for 5 mins, followed by washing with cold PBS with agitation for at least three rounds to remove the villus while enriching for crypts in the supernatant. Collected supernatants containing mostly crypts were spun down at 200g for 2 mins at 4°C with soft brake on to facilitate separation of single cells from the crypt pellet during washes. While crypts were being prepared, DIV3-4 EGCs grown in G5 conditions were harvested via 5 mins incubation with Trypsin/EDTA (0.25%), centrifuged and washed with medium containing 10% FBS. Isolated crypts and EGCs were then mixed together in 35 µL of matrigel (Corning Incorporated) and seeded

onto each well of 8 chamber Permanox Slides (Thermo Fisher Scientific). Co-cultures were maintained for 4 days in Advanced DMEM/F12 with HEPES buffer plus supplements N2, B27, glutaMAX (Thermo Fisher Scientific), Y-27632, N-acetyl-L-cysteine (Sigma-Aldrich), Penicillin/Streptomycin and recombinant proteins EGF, Noggin (Peprotech) and R-spondin-1 (R&D Systems). Co-cultures were then fixed with 2% PFA in PBS for 20 minutes at RT, washed, mounted and scanned by confocal microscopy.

Statistics. Mann-Whitney tests (two-tailed) were performed using the Prism 6 (GraphPad) software.

FIGURE LEGENDS

Figure 1. Sox10-tdT⁺ cells express known EGC markers when cultured in different conditions tested. (A-N) Representative confocal z-stacked images taken at the same magnification showing the expression of known EGC markers at DIV3 by Sox10-tdT⁺ (red) cells cultured in the presence of FBS (A-D, I-K) or in G5 media (E-H, L-N). (D and H) Co-expression of FoxD3 (blue) and GFAP (green) can be seen in Sox10-tdT labelled cells in both conditions. (K and N) S100 expression (green) co-localised with Sox10-tdT⁺ cells in both conditions as shown. Nuclei of cells were visualised by DAPI staining. Bar: 100µM.

Figure 2. G5 cultures had higher proportion of Sox10-tdT⁺ cells that incorporated EdU. (A-D) Proliferative Sox10-tdT⁺ cells at DIV3 in both conditions were detected via EdU incorporation (as shown in blue), where EdU was added 1 hour before fixation. (E-G) Graphs show mean $\pm$ SEM of counted (E) EdU⁺, (F) Sox2⁺ or (G) S100⁺ cells within Sox10-tdT⁺ cells expressed as percentage over the total number of Sox10-tdT⁺ cells. (n $\geq$ 4). Mann-Whitney test: **, P < 0.01; ****, P < 0.0001; ns, not significant.

Figure 3. G5 culture condition promotes progenitor-like characteristics in primary EGCs. (A-F, J-O) Representative images of cultured cells in both conditions till DIV6, showing (A, D) Sox2 (blue), (B, E) S100 (green) or (K, N) α -SMA (green) expression merged with (C, F, L, O) Sox10-tdT (red) labeled cells. (E-G) Graphs show mean $\pm$ SEM of counted (G) Sox2⁺, (H) S100⁺ or (I) GFAP⁺ cells within Sox10-tdT⁺ cells expressed as percentage over the total number of Sox10-tdT⁺ cells (n $\geq$ 4). (P) Quantification of Sox10-tdT⁺ cells over total number of counted nuclei per field of view as percentage mean $\pm$ SEM

(n=4). Mann-Whitney test: *, $P < 0.05$; **, $P < 0.01$; ns, not significant. Bar, 100 μ M.

Figure 4. EGCs display distinct morphological phenotypes in different culture conditions. (A-D) Representative z-stacked confocal images of (A, C) GFAP (green) expression detected in (B, D) Sox10-tdT⁺ (red) cells at DIV3. Bar, 50 μ m. (E-F) Typical colony arrangement of Sox10-tdT⁺ cells as shown from both culture conditions at DIV3. Images are representative of at least 3 independent experiments conducted, with similar observations ($n \geq 5$).

Figure 5. Sox10-tdT⁺ cells cultured in G5 media tend to form neurosphere-like bodies upon passage. (A, A', B, B') Representative images of Sox10-tdT labelled cells taken on (A, B) DIV5 or (A', B') DIV9 after passage on DIV5, in the respective culture conditions as shown.

Figure 6. Matrigel®-derived conditioned media promotes proliferation and maintains expression of EGC markers. (A) Primary EGCs cultured in G5 media were harvested at DIV4 and grown for another 2 more days in (i) organoid media only or (ii) in organoid media with 40% Matrigel® conditioned media. (B) Representative images of proliferating GFAP and Sox10-tdT co-labelled cells grown in the presence of 40% Matrigel® conditioned media. Bar, 100 μ m.

Figure 7. EGCs form close associations with co-cultured intestinal organoids. Primary EGCs harvested from DIV4 were co-cultured with intestinal crypts for 3 DIV. (A-C) Confocal z-stacked images show Sox10-tdT⁺ cells associating with or wrapping around individual organoid structures. Bars, 50 μ m. (A'-C') Still images captured of rotating views of the associations observed.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

SUPPLEMENTARY INFORMATION

Title: Establishing a co-culture system to study enteric glial cell functions.

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Supplementary Figure S1. EGCs are plastic and respond to external cues. Switching of culture conditions from FBS->G5 or from G5->FBS was done after 3DIV. Representative images of Sox10-tdT labelled cells taken on (i, iii) DIV3 and on (ii, iv) DIV6 as shown. (ii, iv) Top panel arrowheads highlight characteristic clustering of cells cultured in G5 (ii) while lower panel arrowheads refer to large, flat cells commonly found in FBS supplemented cultures (iv), highlighting the plasticity of these cells.

Supplementary Figure S2. EGCs are in close association with the intestinal epithelium. (Top panels) Ileum sections showing individual Sox10-tdT marked cells within the villus (asterisks) and presence of an EGC network at the level of the crypts. (Lower panels) Flat mount cross-section of the mucosal layer showing Sox10-tdT⁺ cells surrounding individual crypt structures as shown. Bars, 100μm.