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IMPACT OF GENETIC AND ENVIRONMENTAL FACTORS IN INTESTINAL INFLAMMATION

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Impact of genetic and environmental factors in intestinal inflammation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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A mi familia

ABSTRACT

The gastrointestinal tract, is continuously exposed to a wide variety of stimuli, including dietary-derived metabolites, environmental compounds and the microbiota. Since these antigens can be beneficial or detrimental for the organism, proper sensing of the luminal content is essential for orchestrating both pro-inflammatory and tolerogenic immune responses on demand and therefore, ensure the establishment of intestinal homeostasis. Disruption of these mechanisms might result in inflammation, a characteristic of intestinal disorders, such as inflammatory bowel disease (IBD). How environmental triggers modulate intestinal inflammation in genetically susceptible host leading to IBD is largely unclear. The gap in knowledge around this topic is likely due to the lack of versatile in vivo models allowing the testing of a wide variety of compounds in a cost-effective manner. The aim of my doctoral thesis was to understand how intestinal inflammation is impacted by genetic factors and environmental exposures.

In **study I and II** we analyzed how the sensing of dietary-derived metabolites modulate immune homeostasis. In **study I**, we found that retinoic acid receptor α (RARα) signaling in intestinal epithelial cells modulated lineage specification of secretory cells and the development of the intestinal immune system. Epithelial RARα signaling was essential for mounting protective responses against pathogen infection and maintaining intestinal homeostasis. In **study II**, we explored how combinatorial activation of ligand-activated transcription factors that sense dietary metabolites *in vivo* modulate cytokine signaling. We found that co-exposure with more than one ligand resulted in activation of the corresponding receptors, and in induction of specific cytokine profiles as a result of their interaction.

In **study III**, we investigated how GPR35, an IBD-risk gene, modulated intestinal immune homeostasis. We found that Gpr35 deficiency in macrophages resulted in exacerbated colitis, due to low expression of genes involved in corticosterone synthesis and tumor necrosis factor (*Tnf*). We also identified lysophosphatidic acid as a potential GPR35 ligand that induces *Tnf* expression in macrophages in a GPR35-dependent manner.

In **study IV**, we further examined how environmental factors modulate intestinal inflammation and found that perfluorooctane sulfonic acid (PFOS), an environmental pollutant, exacerbates intestinal inflammation. This was associated with impaired epithelial barrier function and systemic T cell responses.

Taken together, this doctoral thesis provides insight into how environmental and genetic factors modulate immune responses contributing to the maintenance of intestinal homeostasis.

LIST OF SCIENTIFIC PAPERS

I. Jijon HB, Suarez-Lopez L, **Diaz OE**, Das S, De Calisto J, Parada-kusz M, Yaffe MB, Pittet MJ, Mora JR, Belkaid Y, Xavier RJ, Villablanca EJ.

Intestinal epithelial cell-specific RARα depletion results in aberrant epithelial cell homeostasis and underdeveloped immune system

Mucosal Immunology, 2018 May;11(3):703-715. doi: 10.1038/mi.2017.91.

II. **Diaz OE**, Xue S, Luo X, Nava J, Appelblom A, Morales RA, Das S, Villablanca EJ.

Retinoic acid induced cytokines are selectively modulated by liver X receptor activation in zebrafish

Reproductive Toxicology, 2020 Apr;93:163-168. doi: 10.1016/j.reprotox.2020.02.009.

III. Kaya B, Doñas C, Wuggenig P, **Diaz OE**, Morales RA, Melhem H; Swiss IBD. Cohort Investigators, Hernández PP, Kaymak T, Das S, Hruz P, Franc Y, Geier F, Ayata CK, Villablanca EJ, Niess JH.

Lysophosphatidic Acid-Mediated GPR35 Signaling in $CX3CR1^{+}$ Macrophages Regulates Intestinal Homeostasis

Cell Reports, 2020 Aug 4;32(5):107979. doi: 10.1016/j.celrep.2020.107979.

IV. **Diaz OE**, Sorini C, Das S, Morales RA, Appelblom A, Luo X, Nava J, Frede A, Krais AM, Wincent E, Villablanca EJ.

Oral perfluorooctanesulfonic acid enhance neutrophil-mediated intestinal damage and expand systemic T cells during experimental colitis

Manuscript

PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Czarnewski P, Parigi SM, Sorini C, **Diaz OE**, Das S, Gagliani N, Villablanca EJ. Conserved transcriptomic profile between mouse and human colitis allows unsupervised patient stratification. *Nature Communications,* 2019 Jun 28;10(1):2892. doi: 10.1038/s41467-019-10769-x.

II. Lindheim L, Manti M, Fornes R, Bashir M, Czarnewski P, **Diaz OE**, Seifert M, Engstrand L, Villablanca EJ, Obermayer-Pietsch B, Stener-Victorin E. Reproductive and Behavior Dysfunction Induced by Maternal Androgen Exposure and Obesity Is Likely Not Gut Microbiome-Mediated. *Journal of the Endocrine Society*, 2018 Oct 15;2(12):1363-1380. doi: 10.1210/js.2018-00266.

III. **Diaz OE**, Morales RA, Das S, Villablanca EJ. Experimental Models of Intestinal Inflammation: Lessons from Mouse and Zebrafish. In: Hedin C., Rioux J., D'Amato M. (eds) *Molecular Genetics of Inflammatory Bowel Disease. Springer, Cham.* 2019 .doi: 10.1007/978-3-030-28703-0_3
Book chapter

CONTENTS

LIST OF ABBREVIATIONS

AGM	Aorta-gonad-mesonephros
AHR	Aryl hydrocarbon receptor
ASBT	Apical sodium-dependent bile salt transporter
CBC	Crypt base columnar
CCR9	Chemokine receptor 9
CD	Crohn's disease
CD	Cluster of differentiation
CHT	Caudal hematopoietic tissue
CXCL1	Chemokine ligand 1
dmPGE2	Dimethyl prostaglandin E2
dpf	Days post fertilization
DSS	Dextran sulfate sodium
EAE	Experimental autoimmune encephalomyelitis
EEC	Enteroendocrine cell
eQTL	Expression quantitative trait loci
GALT	Gut-associated lymphoid tissue
GPR	G-protein coupled receptor
GWAS	Genome-wide association studies
hpf	Hours post fertilization
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IL	Interleukin
ILC	Innate lymphoid cell
ISC	Intestinal stem cell
KLF4	Kruppel-like factor 4
LGR5	Leucine-rich-repeat-containing G protein-coupled receptor 5
LOAEL	Low observed adverse effect level
LTi	Lymphoid tissue inducer
LXR	Liver X receptor
MNP	Mononuclear phagocyte

NR	Nuclear receptor
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
PFAS	Per- and polyfluoroalkyl substances
PFBS	Perfluorobutane acid
PFHxS	Perfluorohexane sulfonic acid
PFOA	Perfluorooctane acid
PFOS	Perfluorooctane sulfonic acid
PP	Peyer's Patch
PUFA	Polyunsaturated fatty acids
RA	Retinoic acid
RAR	Retinoic acid receptor
ROR	RAR-related orphan receptor
ROS	Reactive oxygen species
SCFA	Short-chain fatty acid
SNP	Single nucleotide polymorphism
TA	Transit-amplifying
TCR	T cell receptor
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF	Tumor necrosis factor
UC	Ulcerative colitis
VEO	Very early onset

1 INTRODUCTION

The intestinal tract, as the largest barrier surface in our body, is continuously challenged by a wide variety of external and internal insults, including metabolites, microbial products and pollutants [8] etc. To distinguish between pathogenic versus innocuous antigens, complex interactions between intestinal epithelial cells (IECs), immune and non-immune cells, need to be tightly regulated. This ensures inflammatory immune responses against pathogens while also inducing tolerance towards innocuous antigens. Failure in maintaining this tight balance can lead to aberrant immune responses against commensal or food antigens, a hallmark of inflammatory disorders, such as inflammatory bowel disease (IBD) [9].

1.1 INTESTINAL EPITHELIAL CELLS

The intestinal epithelium, as a single layer of epithelial cells, also acts as a sensor of environmental signals and represents a barrier that protects the host from potentially damaging microorganisms and toxic compounds in the luminal content. Unique features of the intestinal epithelium, such as its architecture and regional compartmentalization, allow the performance of these functions. The intestine is divided mainly in the small intestine and colon. The small intestine (SI) is primarily responsible for the digestion and absorption of nutrients. The SI epithelial cells are organized in crypts and villi, which are tubular invaginations and finger-like protrusions, respectively [10]. Conversely, the epithelial lining of the colon is devoid of villi, which is responsible for water absorption and compacting the stools for rapid excretion [11].

The epithelial surface from both SI and colon is constantly renewed by pluripotent intestinal stem cells (ISCs), which reside in the bottom of the crypts and can give rise to all IEC lineages. ISCs have the ability of self-renewal and divide regularly to generate a transit-amplifying (TA) population of cells that are highly proliferative and differentiate into the different IEC types [12]. Fully differentiated cells migrate upwards along the villi and are lost at the villus tip. This cycle is completed in 3-5 days and is repeated continuously throughout the entire lifespan [10]. The epithelium of both the small and large intestine contains several specialized IEC types, which are briefly described below.

1.1.1 Secretory cells

Products secreted by epithelial cells increase the physical barrier between epithelial cells and the microbiota, which minimizes their interaction and the risk of bacterial penetration, while also regulating a wide variety of responses and modulating immune responses. The secretory lineage of IECs includes goblet cells, Paneth cells, enteroendocrine cells and tuft cells, whose roles are summarized below.

Goblet cells are specialized in the secretion of mucus, which provides the first line of defense against microbial pathogens and chemical damage [13]. In the SI, the mucus consists of an easily removable single layer, while in the colon it forms a double layer: a dense inner layer

impenetrable to the microbiota, and an outer layer where bacteria can reside. Besides creating a physical barrier, the mucus also contains many molecules to control the microbiota [14].

Paneth cells are located at the base of the crypts and have only been described in the small intestine. These cells are essential in limiting pathogen colonization as they produce the majority of antimicrobial peptides in the small intestine, including α-defensins, lysozyme, phospholipase A2 and ANG4 [15]. In addition, Paneth cells have a crucial role in the stem cell niche as they provide necessary signals for the growth and maintenance of the surrounding stem cells, such as Notch ligands [16]. Contrary to the other differentiated epithelial cell types, Paneth cells are the only ones that migrate down to the bottom of the crypt and are positioned in between stem cells, in a process dependent on EphB signaling [17]. Notably, Paneth cells have a life span of ~57 days, much longer than that of the other secretory cells [18].

The intestinal epithelium also contains several lineages of enteroendocrine cells (EECs), which differ in their location along the gastrointestinal tract and secrete specific hormones. EECs sense dietary elements and produce hormones that regulate a wide variety of processes [19]. Finally, tuft cells have long apical microvilli that project into the lumen, in line with their chemosensory function [20]. Succinate, a metabolic product of pathosymbionts, has been recently identified to be sensed by tuft cells and result in their activation [21]. Although representing a small fraction of intestinal epithelial cells during steady-state conditions, tuft cells expand rapidly in response to infection with helminths or protozoa, by producing IL-25 and promoting type 2 immunity. Upon pathogen sensing, IL-25 production by tuft cells stimulates group 2 innate lymphoid cells (ILC2s) in the lamina propria to secrete IL-13, which acts on epithelial cell progenitors and results in the hyperplasia of goblet and Tuft cells [22,23].

Fate determination of IECs towards the absorptive or secretory lineage is tightly controlled by the activity of the Notch pathway [24,25]. In addition, differentiation to specific IEC lineages is regulated by several transcription factors. Cell specification towards goblet cells is negatively regulated by Notch signaling [26], while other transcription factors, including Kruppel-like factor 4 (Klf4), promote their differentiation [27]. Moreover, the specification towards the enteroendocrine cell fate in the intestine is dependent on the transcription factor Neurogenin3 [28,29]. The differentiation of Paneth cells requires Sox9 [30]. Finally, tuft cell differentiation is dependent on the transcription factor Pou2f3 [31].

1.1.2 Absorptive cells

Absorptive enterocytes represent the majority of cells in the intestinal epithelial lining, particularly in the villi, produce hydrolytic enzymes that facilitate the digestion and are also responsible for nutrient absorption [8]. TA cells fated to the absorptive lineage divide several times before giving rise to enterocytes [32,33].

Sampling of the luminal content, especially of microbes, is essential for the maintenance of intestinal homeostasis. Microfold (M) cells are specialized IECs that are located in areas of

the epithelium that cover lymphoid follicles and transfer luminal antigens to the underlying immune cells [32,34]. The differentiation of epithelial precursors into M cells is dependent on RANKL (receptor-activator of NF-κB ligand), which is expressed by subepithelial stromal cells [35].

1.1.3 Intestinal stem cells

The intestinal crypts harbor the intestinal stem cells, which give rise to all the lineages of epithelial cells. Two pools of ISCs reside in the crypt, with different location and different function depending on the context.

Crypt base columnar (CBC) cells are undifferentiated cells which are continuously cycling and are located at the base of the crypt, intercalated with Paneth cells [36]. More than 30 years after their discovery, leucine-rich-repeat-containing G protein-coupled receptor 5 (*Lgr5*) was identified as the first CBC marker. This allowed lineage-tracing experiments, which showed that $Lgr5^+$ CBCs are pluripotent and can give rise to all IEC lineages over time, thus being *bona fide* intestinal stem cells [37]. Confirming this, sorted $Lgr5^+$ intestinal stem cells cultured *in vitro* under specific conditions can give rise to "organoids", structures with the crypt-villus architecture characteristic of the intestinal epithelium, where all IEC lineages are present [38].

While CBCs are located in the crypt base, cells with stem cell attributes located in position +4, immediately above the last Paneth cell, were identified by Potten [39]. These cells are more quiescent but can give rise to all IEC lineages and are considered as a reserve stem cell pool, since they can repopulate the $Lgr5^+$ CBCs pool if the latter are eliminated [40,41]. Although several markers for the population of +4 stem cells, such as *Bmi1*, *Tert, Hopx and Lrig1*, have been proposed, they are also expressed by $Lgr5^+$ CBCs [40-42]. Finally, analysis of the intestinal epithelium at the single-cell level identified a population of $clusterin^+$ (Clu^+) stem cells known as revival stem cells (revSCs) that expand following damage and are required for intestinal regeneration [43]. Thus, further studies are required to understand the relation between the different stem cell pools during homeostasis and regeneration.

1.2 INTESTINAL IMMUNITY

Below the epithelial layer lies the lamina propria, a loose connective tissue that contains the vast majority of immune cells in the intestine, including myeloid cells and lymphocytes, which are briefly described below **(Figure 1)**.

1.2.1 Myeloid cells

Mononuclear phagocytes (MNPs): Intestinal MNPs, including macrophages and dendritic cells, reside in the lamina propria along the intestinal tract and can also be found in gut-associated lymphoid tissues (GALT), such as Peyer's Patches (PPs) and mesenteric lymph nodes (MLNs) [44]. Under steady-state conditions two populations of MNPs exist in the intestinal lamina propria, functionally defined by their expression of CX3CR1 [44] or CD64 [45]. $CX3CR1^+$ $CD64^+$ macrophages are located close to the epithelium and are specialized in

antigen sampling. These cells are able to directly sample bacteria through dendrites that extend to through the epithelial layer without disturbing the intestinal barrier [46], in a CX3CR1-dependent manner [47]. On the other hand, dendritic cells (DCs) are characterized in the intestine by their expression of CD103. These cells are specialized in antigen-presentation and activation of T cells [48]. $CD103^+$ Dendritic cells can be further subdivided in two major subsets that differ in their expression of CD11b and whose ontogeny is dependent on different transcription factors [49]. Following antigen uptake, $CD103^+$ $CD11b^-$ migratory DCs are essential for the induction of T cell responses in the draining lymph nodes [50,51].

Neutrophils: In the event of a bacterial infiltration in host tissues, there are several mechanisms to control the bacterial spreading. Microorganisms are rapidly eliminated by cells with phagocytic activity, such as neutrophils and macrophages [52]. Besides their phagocytosis capability, neutrophils are well equipped to limit bacterial translocation through the production of reactive oxygen species (ROS), granules with antimicrobial activity and formation of neutrophil extracellular traps (NETs) [53,54]. Moreover, they are rapidly recruited to injured tissues through several signals produced by immune and non-immune cells and can further promote the recruitment of other cells by secreting or modifying chemokines and cytokines, potentiating the inflammatory conditions [54]. After infiltrating tissues, senescent neutrophils undergo apoptosis and are engulfed by macrophages, a process that aids in the resolution of the acute inflammation [55,56]. However, failure in the mechanisms that control their removal or apoptosis can lead to accumulation of their cytotoxic products, and result in chronic inflammation [57].

Finally, both neutrophils and macrophages can also promote mucosal healing by producing anti-inflammatory mediators that limit further infiltration and growth factors that stimulate the proliferation of epithelial cells, respectively [58,59]. Thus, understanding the role of myeloid cells at different stages of the inflammatory process can aid in the design of therapeutic approaches targeting them.

1.2.2 Lymphocytes

The gastrointestinal tract contains the largest population of B and T cells in the body and a vast network of secondary lymphoid organs, collectively termed as the GALT, where antigen presentation takes place [7,60]. $CD4^+$ T helper (T_H) cells differentiate into several subsets, characterized by their expression of cytokines and transcription factors. T_H1, T_H2 and T_H17 cells orchestrate responses against microorganisms and parasites. On the contrary, regulatory T cells (T_{reg}) produce anti-inflammatory cytokines (e.g., IL-10) that suppress unrestrained responses and are essential for the development of tolerance against dietary or microbial antigens [61]. $CD8^+$ T cells are also present in the intestinal mucosa but are mostly found among epithelial cells, and prevent the entry of pathogens while restoring the integrity of the epithelial barrier [62]. Moreover, γδ T cells can be found in the intestinal epithelium and lamina propria, where they mediate immune responses and limit bacterial penetration [63].

Innate lymphoid cells (ILCs) are enriched in mucosal and barrier surfaces, such as the gastrointestinal tract. They support tissue homeostasis by orchestrating responses against pathogens and promoting the formation of lymph nodes [64,65]. ILCs have been classified in three subtypes, termed group 1, 2 and 3, (ILC1s, ILC2s and ILC3s, respectively), based on their expression of signature cytokines and transcription factors [66]. Notably, ILC1s, ILC2s and ILC3s share remarkable functional and transcriptional similarity to the T_H1, T_H2 and T_H17 subsets, respectively, although their responses are not antigen-specific since they lack antigen specific receptors [67].

Activation of T lymphocytes takes place in the GALT, which maximize the probability of events of antigen presentation and subsequent activation. Upon activation, T cells are acquire gut-homing capabilities [68], by increasing their expression of the gut-homing markers integrin α4β7 and chemokine receptor 9 (CCR9), whose ligands MadCAM-1 and CCL25, respectively, are expressed in the lamina propria and by epithelial cells [69,70].

Regarding humoral responses, antigen-loaded dendritic cells migrate to the GALT and induce the differentiation of B cells into IgA-producing plasma cells [71]. IgA^+ plasma cells then migrate to the lamina propria and secrete IgA, which binds the commensal microbiota and limits its interaction with the epithelial barrier [72].

Altogether, several immune cell types with highly specialized functions, are located in the intestinal tract and are essential for the maintenance of immune homeostasis.

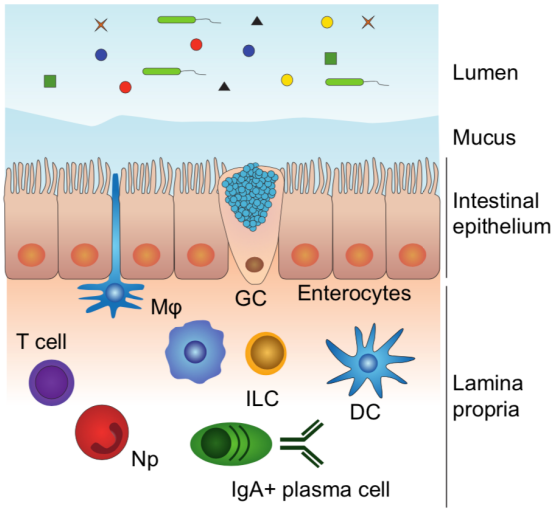

Figure 1. Overview of the intestinal immune system. A single layer of epithelial cells separates a wide variety of dietary-derived metabolites, microbial products and toxins from the host. Highly specialized epithelial cells, including goblet cells (GC), secrete products to limit the interaction between the microbiota and the host. Most intestinal immune cells are located in the lamina propria, directly underneath the epithelial cells. Both myeloid cells, including macrophages (Mφ), dendritic cells (DC) and neutrophils, and lymphocytes, such as T cells, innate lymphoid cells (ILC) and IgA^+ plasma cells, promote the maintenance of intestinal homeostasis. Modified from Parigi et al., (2015) [7]

1.2.3 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD), encompassing both ulcerative colitis (UC) and Crohn's disease (CD), is a relapsing and remitting disorder characterized by chronic inflammation of the gastrointestinal tract [73].

Ulcerative colitis (UC) involves inflammation in the colon, which starts in the rectum and can spread proximally, affecting it entirely or partially. In contrast, Crohn's disease (CD) affects any region of the gastrointestinal tract, although it involves mostly the ileum and colon, often

in a discontinuous fashion. While in ulcerative colitis the damage is mostly restricted to the mucosa, in Crohn's disease the inflammation can be transmural, and other complications such as strictures and fistulas can be present [73,74].

The peak of IBD onset is during adolescence and early adult life [75], although people of any age can be affected. IBD has been traditionally seen as a disease mostly prevalent in Western countries, with more than 1 million IBD patients estimated in the United States, and approximately 2.5-3 million estimated in Europe [76,77]. However, regions with a traditionally low prevalence, such as Africa, South America and Asia, have seen a marked increase in incidence in recent decades [78]. Hence, with more than 6.8 million patients estimated worldwide, and growing incidence in all continents, IBD has become a global disease [79,80].

The etiology of IBD has not been fully elucidated and it is believed to develop as an active immune response in genetically susceptible hosts as a consequence of exposure to microbial antigens and environmental triggers [81] **(Figure 2).** Family history represents a risk factor for developing IBD, although the concordance rate in studies in monozygotic twins is only 6-16% for ulcerative colitis and 35-58% for Crohn's disease [82,83]. Thus, since the contribution of genetics in IBD pathogenesis only explains a fraction of disease variability, this suggests that non-genetic components might have a predominant role in disease onset.

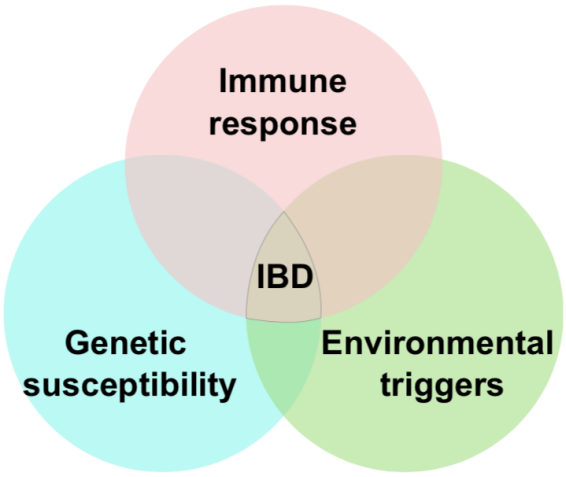

Figure 2. IBD is a multifactorial disease that develops as an active immune response to environmental triggers in the context of genetic susceptibility.

2 GENETICS IN IBD

2.1 FAMILIAL AND GENOME-WIDE ASSOCIATION STUDIES (GWAS) IN IBD

Family-based linkage studies were one of the earliest approaches to identify genetic risk factors for complex diseases. This approach had proven to be successful in the mapping of Mendelian disease genes, especially for rare diseases such as Huntington's disease and Alzheimer's disease [84,85]. Rare genetic variants that cause IBD-like intestinal inflammation, including those found in the *IL10RA*, *IL10RB* and *IL10* loci, where identified with this method [86,87]. Missense and nonsense variants in these loci are highly deleterious and have large effect sizes, usually leading to a very early onset (VEO) of the disease. These variants can result in a disbalance in immune homeostasis by affecting several functions, including pathogen clearance, epithelial barrier function and lack of immune inhibitory mechanisms [88].

In parallel, the advent of genome-wide association studies (GWAS) triggered a remarkable shift in our ability to examine the contribution of genetics to diseases, being extremely useful in the identification of common variants [89]. GWAS focus on the identification of variants that are more frequent in the population (5-50%), which usually have small effect sizes. However, GWAS are usually not able to distinguish the highly deleterious rare variants described above [88,90]. GWAS usually have large cohort sizes, which allows for the identification of several risk loci simultaneously, and to date, more than 200 risk loci have been associated to IBD [91-105]. Since UC and CD share similarities in terms of their pathological features it would be expected that they also share several risk loci. Indeed, most of the loci are associated with both diseases, although a few have been classified as UC -or CD-specific. This suggests that most of the biological mechanisms that are involved in UC are shared to some degree with CD and vice versa [103].

2.2 CHALLENGES IN STUDYING THE GENETIC CONTRIBUTION TO IBD

Risk loci identified by GWAS contain large clusters of single nucleotide polymorphisms (SNPs) that are in linkage disequilibrium. Therefore, it is difficult to distinguish the causal SNPs from the neutral variants by just following this approach, especially since the vast majority of GWAS hits are located in noncoding regions, with unknown effects. In order to identify the causal variants, fine mapping techniques use high-throughput imputations, dense genotyping, and methods for statistical refinement, to assign a probability to each variant in a locus to be the causal variant of that region being associated to the trait [106]. In this sense, a study integrating genetic and epigenetic fine mapping analyzing GWAS hits from 21 autoimmune diseases identified that approximately 90% of the causal variants are indeed found in noncoding regions [107]. Most of these variants were located in immune-cell enhancers and sometimes affecting transcription factors binding sites [107].

However, assigning a function to variants located in noncoding regions remains a challenge due to the incomplete knowledge of the regulatory activity of noncoding elements. Different techniques, including the analysis of chromatin openness and histone marks and the

prediction of transcription factor binding sites, could provide some information on their effect. Further, the study of expression quantitative trait loci (eQTL) can link variants with the gene whose expression is affected [108]. Combining all these approaches, a fine mapping study on selected IBD risk loci found 45 variants with a >50% probability to be the causal variant. Coupling the genetics fine mapping with data of expression quantitative trait loci (eQTL) and epigenetic marks, allowed them to propose a function for some of these variants [109]. Nevertheless, further research is required to improve our understanding of the regulatory activity of noncoding genomic regions.

Lastly, another significant hurdle is that many of the genes that are implicated through GWAS studies have an unknown function or interact in pathways that are not fully characterized or with unsuspected relevance in disease pathology [110,111]. To bridge this gap, the genetic manipulation of animal models will continue to provide important information about the functional consequence of individual genes in IBD pathology.

2.2.1 SNPs in the GPR35 gene

Among environmental sensors, the large family of G protein-coupled receptors (GPCRs) has been recently recognized to be activated by dietary and microbial metabolites. These receptors are expressed by different cell types throughout our body and have been shown to regulate immune responses and gut homeostasis [112,113]. Interestingly, GWAS have identified SNPs in *GPR35* associated with an increased risk of IBD [102,114]. Particularly, among the SNPs with the highest association to IBD in this locus, rs3749171 leads to a T108M substitution in the protein sequence, which could affect the efficiency of signaling through the receptor [102].

A challenge in the study of its role in intestinal homeostasis is the lack of *bona fide* GPR35 ligands. Although endogenous molecules including kynurenic acid, lysophosphatidic acid (LPA) and the chemokine CXCL17 have been reported to activate GPR35 [115-117], their potency differs between species and it is unclear if they can be produced physiologically at concentrations sufficient to activate the receptor [118].

Interestingly, this receptor is highly expressed by immune cells along the gastrointestinal tract, in both humans and mice [117]. Functionally, GPR35 is shown to promote the turnover of IECs in homeostatic conditions and enhance intestinal tumorigenesis in $Apc^{Min/+}$ tumor model [119]. On the other hand, impaired GPR35 signaling led to a worsened colitis in mice in the DSS model [120].

In the third manuscript included in this thesis we found that LPA-mediated GPR35 activation in macrophages contributes to the maintenance of intestinal homeostasis by inducing TNF production [3].

3 EXPOSOME IN IBD

The shift in the epidemiology of IBD, with a rapid rise in incidence in areas of the world with a traditional low prevalence, hints towards environmental exposures having a predominant role in IBD pathogenesis [79]. The largest increase in incidence in recent decades are seen in newly industrialized countries in Africa, Asia, and South America [121]. These regions have also seen improved access to health care and better infrastructure to record and study health outcomes, which improves the surveillance of IBD incidence globally [79]. Although this might influence the rise of IBD reported cases, younger immigrants from newly industrialized countries to the West have increased risk of developing IBD when exposed to the Western environment at an early age [122], thus suggesting that a change in environmental exposures might be a critical trigger.

Besides the shift from an agricultural to a manufacturing industry, societies in these countries have also experienced increased migration from rural to urban areas, which has been associated with an increased risk for the development of IBD [123]. This transition towards a more urbanized lifestyle has been accompanied with behavioral changes (e.g. increased smoking and more sedentary activities) and alterations in daily exposures (e.g. diet and pollutants). Although exposures to these environmental triggers have been associated with IBD pathogenesis, their mechanism of action remains vastly unclear. Thus, a better understanding of the effect of the "exposome", defined as the cumulative lifelong environmental exposures [124], in IBD, is crucial for reducing the risk of disease incidence or patients having a milder clinical course, and for developing therapeutic strategies [125].

Particularly, early-life exposure to some factors can have long lasting effects in IBD pathology. For instance, antibiotic exposure during childhood is associated with a higher risk of developing IBD, with the risk increasing with the number of courses of antibiotics used [126,127]. This highlights the impact of an alteration in the normal microbial ecology, termed dysbiosis, during the first year of life, a stage where the composition of the gut microbiota is established and converges to that found in the adult gastrointestinal tract [125,128]. Moreover, while the incidence of both pediatric and adult IBD in immigrants from South Asia in Canada was lower than that of non-immigrants, second-generation immigrants were at a similar risk of developing IBD to non-immigrant children, suggesting that early life or even *in utero* exposures to specific westernized lifestyle may have an influence [129].

3.1 ENVIRONMENTAL POLLUTANTS

Among environmental triggers, pollutants are considered a risk factor in IBD pathogenesis. As a consequence of human activity, pollutants have a wide distribution and can have an impact on entire populations, rather than at the individual level. Thus, understanding how they modulate immune responses is imperative.

Components of air pollution, associated with industrialization and urbanization, have been suggested to have detrimental health effects. Regarding the gastrointestinal tract, short-term exposure to ingested particulate matter increased intestinal permeability and enhanced the

expression of pro-inflammatory cytokines in mice, while long-term exposure also resulted in changes in microbiota composition [130].

However, epidemiological studies assessing the effect of air pollution in IBD have yielded conflicting results. Individuals younger than 25 years of age were more commonly diagnosed with CD and UC in areas with high levels of NO_2 and SO_2, respectively [131]. Conversely, another study reported an inverse association between IBD and particulate matter but a positive association with nearby traffic intensity [132]. Finally, a study on the number of adult IBD hospitalizations found an association between IBD-related hospitalizations and total air pollutant emissions as well as with its individual components [133]. Overall, future research is required to understand if there is a link between elements of air pollution and intestinal inflammation and to examine the underlying mechanism.

3.1.1 Per- and polyfluoroalkyl substances (PFAS)

In addition to molecules present in the air, we are continuously exposed to other classes of anthropogenic compounds. Among these, PFAS are a class of man-made compounds that have been vastly used in the production of a large variety of consumer goods, including firefighting foams, water repellent textiles, cookware and food packaging [134]. PFAS are defined as aliphatic substances where all the hydrogen atoms bound to at least one carbon atom have been replaced by fluorine atoms [135]. Due to the nature of the strong carbon-fluorine bonds, these compounds are highly persistent, have a global distribution and are found in the environment as a result of either their direct emission or degradation of precursor substances [135,136].

PFAS are resistant to metabolic degradation following their absorption [137,138] and their bioaccumulative potential increases with the length of the carbon chain [139]. PFAS are also known as ‘Forever Chemicals’ since their mean half-life in serum in humans has been estimated to be 4.8 and 3.5 years for perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), respectively, both with eight C atoms and the most widely characterized compounds from this family [139,140]. Considering this, in addition to the adverse health effects associated to their exposure (discussed below), PFOA and PFOS have been categorized as persistent organic pollutants (POP) in the Stockholm Convention [141]. Thus, their production and use has been restricted or eliminated in the Western world, although it is still produced in other regions, such as in China, due to the lack of cost-efficient alternatives [142].

As the production of PFOS and PFOA has been vastly reduced they have been replaced by other PFAS with smaller carbon chains, which are deemed less bioaccumulative [139]. However, a larger amount of the shorter chain PFAS might have to be used to achieve the same function performed by the longer chain compounds. Additionally, they can also be generated as byproducts of the longer chain pollutants and are widely distributed as a consequence. Thus, the general population might be exposed to higher levels of the smaller

chain compounds than those of the longer chain PFAS. This represents a concern, since their potentially hazardous effects have not been examined in detail.

Regarding the route of exposure, PFAS have been detected in drinking water sources and in wildlife animals [143,144], dietary intake being the primary route of exposure in humans **(Figure 3)** [2,145]. Following oral exposure, PFAS are well absorbed in the gastrointestinal tract, distribute mostly to the liver, blood, kidney and spleen and remain in these tissues [138,146,147].

Additionally, transporter proteins might help in the absorption of PFAS. PFOA and PFOS can be transported by organic anion transporters (OATs) and organic anion transporting polypeptides (OATPs), which are highly expressed in the kidney [148-150]. Moreover, perfluoroalkyl sulfonates, such as PFOS, have been shown to be transported by the apical sodium-dependent bile salt transporter (ASBT), which is expressed in the gastrointestinal tract, suggesting that they could be preferentially taken up in this manner [151]. However, this might not be the only route of uptake, as the authors suggest that passive diffusion of PFOS might also have an important role [151].

The interaction of PFAS with proteins in tissues and in the circulation might also help in their distribution and bioaccumulation, as more than 90% of PFOA in blood is bound to serum albumin in rats and humans [152] and PFOA also binds fatty acid binding proteins [153]. Although they persist for a shorter period in other species, its half-life in serum is still counted in months in rats, mice and monkeys [154]. These differences in species-specific pharmacokinetics must be taken into account when studying the long-term consequences of PFAS exposure.

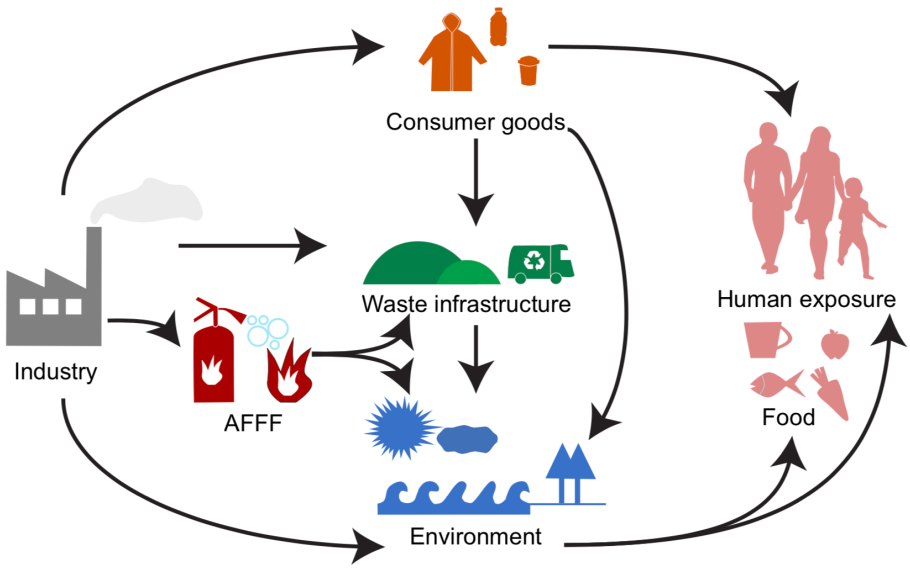

Figure 3. Overview of the pathways of PFAS exposure in the general population. Adapted from Sunderland et al., (2019) [2].

3.1.2 Physiological effects of PFAS exposure

Several epidemiological studies have been carried out both in the general and in highly exposed populations to assess the adverse effects of PFAS exposure in humans. Among these

studies, the C8 Health Project is a survey performed in 69,000 residents in districts of Ohio and West Virginia, where the drinking water had been contaminated with supplies with PFOA. This was attributed to originate from a DuPont plant in the vicinity that manufactured PFOA [155]. Increased levels of PFAS in serum were positively associated with serum lipids [156], kidney disease [157] and pregnancy-induced hypertension [158]. Interestingly, results also stemming from the C8 Health Project have identified a probable positive association between PFOA exposure and testicular and kidney cancers [159], while an inverse association has been found for colorectal cancer [160]. However, these results should be carefully considered as the number of cases in these cohorts remain small and they could be due to chance. Moreover, studies performed on workers exposed to higher PFOS levels have suggested an increased risk of bladder cancer [160,161]. Finally, another study in a small European cohort of exposed workers identified a positive association between PFOA serum concentrations and mortality of liver cancers, diabetes as well as malignant neoplasms of both hematopoietic and lymphatic tissues [162]. Overall, a wide variety of health adverse effects following PFAS exposure have been identified, although the underlying mechanism is still unknown.

3.1.3 Effect of PFAS exposure in immune responses

PFAS exposure from early in life has also been shown to impact the immune system function. For instance, epidemiological studies have reported reduced efficacy of vaccines associated with PFOS exposure, as serum antibody concentrations were inversely associated with PFOS levels in maternal pregnancy serum and in serum in children, which can increase the risk of infections [163,164].

Regarding autoimmune diseases, epidemiological studies have yielded conflicting results in terms of association between PFAS exposure and disease incidence. Analysis of the relation between PFAS and autoimmune diseases in a highly exposed population, identified a positive association between PFOA serum levels and the incidence of ulcerative colitis but not Crohn's disease [165]. This association was also found in a second cohort where the mean PFAS serum levels were comparable to those in the general US population [166]. However, a more recent study performed in a community with high exposure to PFAS in Sweden examined serum PFAS levels and IBD biomarkers levels, but it did not find an association between PFAS exposure and IBD [167]. Differences between cohorts, e.g. the duration of the exposure to PFAS and other environmental factors, as well as in the study design, might affect the interpretation of the results. Therefore, further studies in other populations examining additional biomarkers in samples collected before and after the diagnosis of IBD might help to better understand if there is a causal link, and what could be the potential mechanisms resulting in intestinal inflammation.

Studies in other mammalian models have shed more light on the impact of PFAS exposure on the immune system. Regarding the innate immune system, short-term exposure to PFOA leads to neutropenia, in addition to a general reduction in circulating white blood cells and lymphocytes in mice. Moreover, exposure to PFOA *in vivo* prior to stimulation with LPS

leads to increased *ex vivo* production of proinflammatory cytokines, such as TNF-α and IL-6, in cells isolated from the peritoneal cavity or the bone marrow [168].

Adaptive immunity is also affected by PFAS. *In utero* exposure to PFOS resulted in reduced specific IgM titers following immunization during adulthood, as well as in reduced NK cell activity in mice [169]. Exposure to PFOS in adult mice also led to reduced antigen-specific IgM production following immunization and in alterations in T cell subpopulations in male mice, which showed a 14-fold lower low observed adverse effect level (LOAEL) value than females [170]. Short-term high exposure to PFOS or PFOA through diet resulted in atrophy of the thymus and spleen in mice, while it also led to a reduction in the number of myeloid cells and lymphocytes in the bone marrow [171].

In terms of the immune responses elicited by PFAS, a long-term exposure to PFOS resulted in a shift towards a higher IL-4 production, characteristic of a type 2 cytokine response, and increased antibody titers against a specific antigen following immunization [172]. Specifically in the intestine, Wang et al. (2020) have shown that a PFOS administration in mice for 16 days causes increased expression of *Il1b* and *Il10* in the colon, while also reducing the expression of tight junction proteins and affecting intestinal permeability. This was also accompanied by inflammatory responses and oxidative damage in the liver [173]. Moreover, while PFOS administration prior to *Citrobacter rodentium* infection in mice, a widely used model to study enteropathogenic infections [174], resulted in enhanced pathogen clearance at early stages, continuous PFOS exposure led to chronic inflammation during later stages of infection. In addition, this was associated with decreased mucin production by goblet cells and changes in the microbiota composition at later stages of infection [175]. In this study, PFOS promoted the production of type 3 cytokines such as IL-17 and IL-22 by ILC3s and T_H17 cells through activation of the aryl hydrocarbon receptor (AHR) [175]. However, its effect on myeloid cells, and on early stages of intestinal inflammation remains vastly unclear. In the last manuscript included in this thesis we expanded on this concept and showed that PFOS exposure upon chemically induced colitis resulted in exacerbated intestinal inflammation and epithelial permeability, associated with increased PFOS bioavailability systemically.

3.2 EFFECT OF DIETARY-DERIVED COMPOUNDS IN IBD.

Among the environmental factors that have been associated to play a role in IBD pathogenesis, diet is believed to play a crucial role. Particularly, dietary modifications have been shown to induce dysbiosis in mice, a risk factor in the development of IBD. For instance, mice whose diet changed from a low-fat and plant polysaccharide-rich to a high-fat and high-sugar diet, characteristic of a Western diet, showed rapid changes in the structure of the microbiota [176], as well as increased intestinal permeability and decreased barrier function [177]. Another study showed that a high-fat and high-sugar diet induced an inflammatory environment in the gastrointestinal tract and facilitated colonization by pathogenic bacteria, which was associated with an exacerbated colitis [178].

In a prospective study in humans, increased energy intake from polyunsaturated fatty acids (PUFA) has shown a marginally significant positive association with ulcerative colitis incidence [179]. Particularly, high consumption of linoleic acid, an omega-6 (n-6) PUFA present in red meat and cooking oils, has been positively associated with UC incidence [180]. Conversely, greater long-term intake of n-3 PUFA was associated with a trend of lower UC incidence and the ratio of n-3:n-6 intake inversely associated with disease risk, suggesting a protective effect of the n-3 PUFA [181]. Regarding other macronutrients, significant association between UC or CD incidence and the intake of carbohydrates, protein or total energy intake has not been detected [179,182].

Besides high-fat and high-sugar, a Western diet is also characterized by high intake of red meat, refined grains and processed food, instead of fruits and vegetables, which are rich in fiber [183]. Vegetable consumption and fiber intake were inversely associated with fecal calprotectin levels, a marker of gastrointestinal inflammation [184]. Furthermore, women and children that consumed higher levels of dietary fiber had a reduced risk of developing CD but not UC, with fruit and vegetables being the sources that showed a significant association [185,186]. Although dietary fiber is nondigestible, its protective effects can be partially explained by products of its fermentation by the intestinal microbiota, such as short-chain fatty acids (SCFAs) [187]. SCFAs have anti-inflammatory properties, by supporting the generation of T_{reg} and increasing tolerogenic dendritic cells [188,189], while also promoting the epithelial barrier function by enhancing the assembly of tight junctions [190].

In addition to macronutrients, vitamins and minerals might also play an important role in IBD pathogenesis. For instance, administration of vitamin D in mice undergoing chemically induced colitis led to a milder disease course [191] and a prospective study showed an inverse association between plasma levels of vitamin D and CD incidence in women [192]. Additionally, micronutrients can act as co-factors of enzymes involved in maintaining the epithelial barrier function. For example, prospective studies have found that higher zinc intake was associated with a reduced risk of developing CD but not UC [193], while its supplementation can reduce intestinal permeability in patients with Crohn's disease in remission [194].

Taken together, dietary composition has a major role in shaping immune homeostasis and in maintaining intestinal barrier function in the gastrointestinal tract.

3.2.1 Ligand-activated transcription factors in intestinal homeostasis

Dietary-derived metabolites and xenobiotics can activate ligand-activated transcription factors, hence regulating gene expression directly. Micro- and macronutrients, such as fatty acids, sterols, bile acids, retinoids and vitamins, can be sensed by these transcription factors [195]. Although the expression of these sensors varies between cell types, they are usually expressed by immune and intestinal epithelial cells.

Among the ligand-activated transcription factors, members of the nuclear receptors (NR) sense and translate the environmental cues to gene expression rapidly compared to cell

surface receptors. Among the NRs, some (e.g. AR, ER) are present in the cytoplasm and translocate to the nucleus upon ligand binding. On the other hand, other NRs (e.g. LXR, RAR) are located in the nucleus, bound to DNA on a basal state, inhibiting gene transcription [195]. Upon ligand-dependent activation co-activators are recruited, which leads to gene transcription. Particularly, the liver X receptor (LXR) and the retinoic acid receptor (RAR) are nonsteroid nuclear receptors that bind to DNA response elements as part of a heterodimer with the retinoic X receptor (RXR) [196,197]. Additionally, AHR, which is a ligand-activated transcription factors but does not fit the strict criteria of NR, is located in the cytoplasm and translocates to the nucleus upon ligand-dependent activation, where it binds the AHR nuclear translocator (ARNT) and is recruited to AHR-responsive DNA elements [198].

Metabolites of the amino acid tryptophan as well as those derived from cruciferous vegetables, such as indoles, and xenobiotics can be sensed by AHR [198]. Its activation regulates the differentiation of T_{reg} and T_H17 cells in a ligand-dependent fashion [199], as well as the production of cytokines, such as IL-17 and IL-22 [200]. AHR is also required for the maintenance and function of intestinal RAR-related orphan receptor (ROR)γt^+ ILCs and the control of *Citrobacter rodentium* infection [201], whose clearance is dependent on IL-22 production. Additionally, AHR is essential for the expansion of RORγt^+ lymphoid tissue inducer (LTi) ILCs, and its deficiency results in an absence of cryptopatches, and isolated lymphoid follicles [202]. Finally, AHR is also required for the differentiation of intestinal stem cells and helps to maintain barrier homeostasis [203].

LXR is activated by cholesterol-derived metabolites called oxysterols and has a key role in the regulation of cholesterol metabolism [196]. LXR also controls inflammatory responses, inhibiting the expression of proinflammatory mediators induced by LPS in macrophages and suppressing T_H17 differentiation in mice [204,205]. In addition, LXR deficiency in mice leads to increased susceptibility to colitis and exacerbated disease in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis [205,206]. Moreover, a recent study from our lab has shown that LXR controls the expansion of T_H17 and RORγt^+ T_{reg} in the intestine draining lymph node MLN [207], suggesting LXR mediated regulation of T cells might play a critical role in intestinal inflammation.

Retinoids and metabolites of vitamin A can activate RARs (-α, -β and -γ), which have a broad impact on the immune system development. For instance, supplementation of vitamin A in the diet of pregnant mice results in increased numbers of RORγt^+ LTi ILCs in the embryo, while mice fed with a vitamin A deficient diet through life had secondary lymphoid organs with a reduced size [208]. Additionally, retinoic acid regulates the development of intestinal DC subsets, promoting the generation of anti-inflammatory $Foxp3^+$ T_{reg} in the MLN while inhibiting the differentiation towards proinflammatory T_H17 cells [209-212]. This is particularly important in the GI tract, where balancing the ability to mount an immune response against pathogens while keeping tolerance to food antigens is crucial to maintain homeostasis. Lastly, RA is necessary for the induction of gut homing receptors in T and B cells, and for the presence of IgA-producing B cells in the intestine [213,214].

Dietary retinoids can be absorbed and metabolized by IECs in the small intestine [215], although its impact on these cells remains largely unexplored. Recently, we and others have shown that lack of RARα signaling in IECs led to an altered epithelial composition of secretory cells, with increased numbers of goblet and Paneth cells and a reduction in the number of enteroendocrine cells in the small intestine [1]. Deletion of RARα signaling in IECs also impacted the development of the intestinal immune system, evidenced by a decrease in the number of mononuclear phagocytes and in the number of isolated lymphoid follicles [1]. Additionally, lack of epithelial RARα conferred increased susceptibility to infection in both the small and large intestine, to both *Salmonella* Typhimurium and *Citrobacter rodentium*, respectively [1,216].

Finally, analysis of the role of signaling of another RAR isoform in the epithelium, in this case RARβ, revealed that it is essential for the production of serum amyloid A (SAA) proteins, and in turn, modulating the production of IL-17 by T_H17 cells [217]. In sum, RAR signaling is crucial for the development and function of intestinal immunity.

Taken together, dietary-derived metabolites can be sensed by ligand-activated transcription factors and regulate immune homeostasis by modulating gene expression. Although their impact in immune responses has been widely studied individually, the effect of simultaneous receptor activation is unknown. Recently, we have addressed the effect of combinatorial receptor activation and found that this can lead to synergistic or antagonistic modulation of cytokine expression [4], which can be ultimately used to tailor specific immune responses.

4 ZEBRAFISH AS A RESEARCH MODEL FOR IBD

As indicated above, environmental triggers have a major role in modulating intestinal immune responses. Nonetheless, examining the effect of the thousands of compounds that we are continually exposed to requires a model that allows the testing in a high-throughput manner.

Phenotypic-based screenings performed in cultured cells allow the screening of compounds at high-throughput and analyzing cell-autonomous processes. However, intestinal homeostasis is maintained by a complex network of cell types. Hence, a model to test the impact of environmental triggers in intestinal inflammation should recapitulate the complexity of these biological processes *in vivo*. A vertebrate model that has recently been used in screenings is zebrafish. Assays are often performed in zebrafish larvae, which are optically transparent and allow the imaging of internal organs in an intact organism [5]. Zebrafish have become a relevant model for screenings in drug discovery, considering several advantages in comparison to other vertebrates such as: i) high fecundity, with females laying ~300 embryos per cross; ii) small size in early developmental stages, making it possible to grow embryos in 96- or 384-well plates; iii) external fertilization, allowing an easier genetic modification; iv) reduced cost of husbandry. Additionally, fish can be exposed to many drugs by immersion, which presents an easier administration route compared to those used in other vertebrate models [6] **(Figure 4)**.

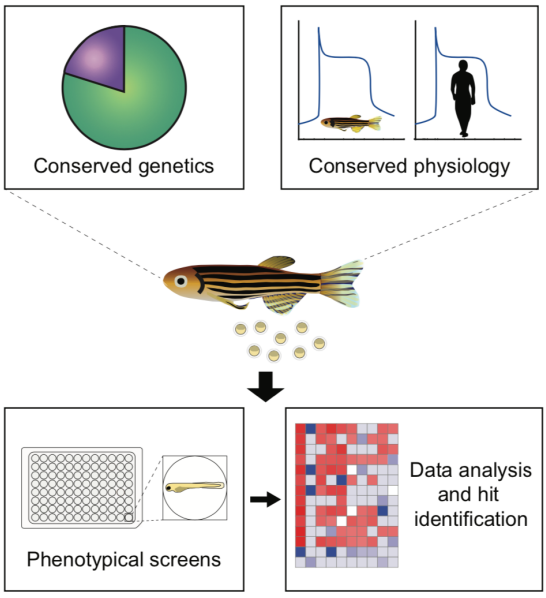

Figure 4. Zebrafish are relevant models for in vivo phenotypical screenings. Around 71% of human genes have an ortholog in zebrafish. Zebrafish possess vertebrate organ systems and share a high degree of genetic of physiology with humans, e.g. in cardiac electrophysiology. Zebrafish larvae can be grown in 96 or 384-well plates, offering the potential of high-throughput screenings. Different readouts based on image analysis using fluorescent reporter lines, in situ hybridization or potentially transcriptomics can be analyzed. Positive hits can be identified and validated in mammalian *in vivo* models. Adapted and modified from MacRae and Peterson, (2015) [5], and Diaz et al., (2019) [6].

Conservation of genetics and physiology between zebrafish and humans further support the use of this model in drug screening. The zebrafish genome has been fully sequenced, and zebrafish orthologues have been identified for 71% of human genes, which increases to 82% when considering disease-causing genes [218]. In terms of physiology, zebrafish contain organ systems found in mammals. Additionally, major biological processes are conserved, which can have a higher similarity to that in humans than rodents, such as the case of the cardiac electrophysiology [5]. Finally, although few compounds that have been identified in zebrafish have been tested in humans, drugs with known function in humans show high conservation of their pharmacological effect in zebrafish [219].

To date, more than 65 screens have been performed in zebrafish, with several disease-relevant compounds having been identified and tested in preclinical models and/or clinical trials. Interestingly, these screens have not only identified new compounds, but also have been useful for identifying novel uses for existing drugs that have already been approved for clinical use [5]. For example, dimethyl prostaglandin E2 (dmPGE2) was found to increase hematopoietic stem cell numbers in a screening performed in zebrafish larvae [220] and expanded hematopoietic stem cell numbers in umbilical cord blood units prior to transplantation in a Phase I clinical trial [221]. In sum, this highlights the use of zebrafish as a relevant model to identify novel compounds with potential therapeutic use.

4.1 THE GI TRACT IN ZEBRAFISH

In contrast to mammals, zebrafish are stomach-less, and the esophagus is directly connected to the intestine, which is a folded tube located in the abdominal cavity [222]. Considering histological features and the presence of different epithelial cell types, the intestine can be divided in three segments. The anterior segment, referred to as the intestinal bulb, has a wider caliber and greater epithelial surface than the posterior intestine. This segment also expresses high levels of digestive enzymes, which is in agreement with its primary role being nutrient absorption. Enteroendocrine cells are absent in the middle segment, where enterocytes express digestive enzymes, suggesting also an involvement in nutrient absorption. The terminal segment is devoid of enterocytes and the epithelial folds are short, which resembles the colon of mammalian vertebrates [222]. Gene expression and transcriptional regulatory mechanisms are highly conserved along the intestine from zebrafish to mammals, suggesting the conservation of the function of IECs [223].

However, there are some differences between the zebrafish intestine and that of mammals, in terms of the epithelial architecture and the cell types present. For instance, the epithelial wall is characterized by large epithelial folds, which are, in proportion, larger than those of mammals. Moreover, proliferating cells in the zebrafish intestine and those expressing the stem cell marker *olfm4* are only located at the base of the epithelial folds, analogous to their location in mammals, although not in crypts, as the typical mammalian crypt-villi architecture is absent [224,225]. The mucosal layer is directly surrounded by the smooth muscle layer in zebrafish, while in mammals an intermediate layer, the submucosa, contains a thick layer of connective tissue embedding blood vessels, lymphatics and enteric nerves [222,223]. Till date,

only three intestinal epithelial cell types have been identified in zebrafish, including enterocytes, enteroendocrine and goblet cells, while M cells, tuft cells and Paneth cells are absent [224]. Finally, although the zebrafish lymphatic system shows similarities to those in higher vertebrates, some structures, such as Peyer's patches and lymph nodes, are absent **(Figure 5)** [226,227].

During embryogenesis, the zebrafish intestine develops into a functional structure able to absorb nutrients by the end of this stage, although it does not fully mature into the adult pattern of epithelial cell proliferation and replacement until 4 weeks later. During the first two weeks post embryogenesis, cells proliferate at the base of the epithelial folds, migrate up during the third week and finally reach the tip fold, where they undergo apoptosis, by the end of the fourth week [222,228]. Moreover, the formation and differentiation of the zebrafish intestinal epithelium is affected by the microbiota, as its presence increases not only the proliferation of epithelial cells but also their differentiation towards goblet and enteroendocrine cells [229,230]. Zebrafish represent an attractive animal model for studying host-pathogen interactions, since they develop in germ-free conditions protected by their chorion and the colonization of the gut by the microbiota takes place after hatching, allowing for a relatively easy generation of germ-free larvae [231].

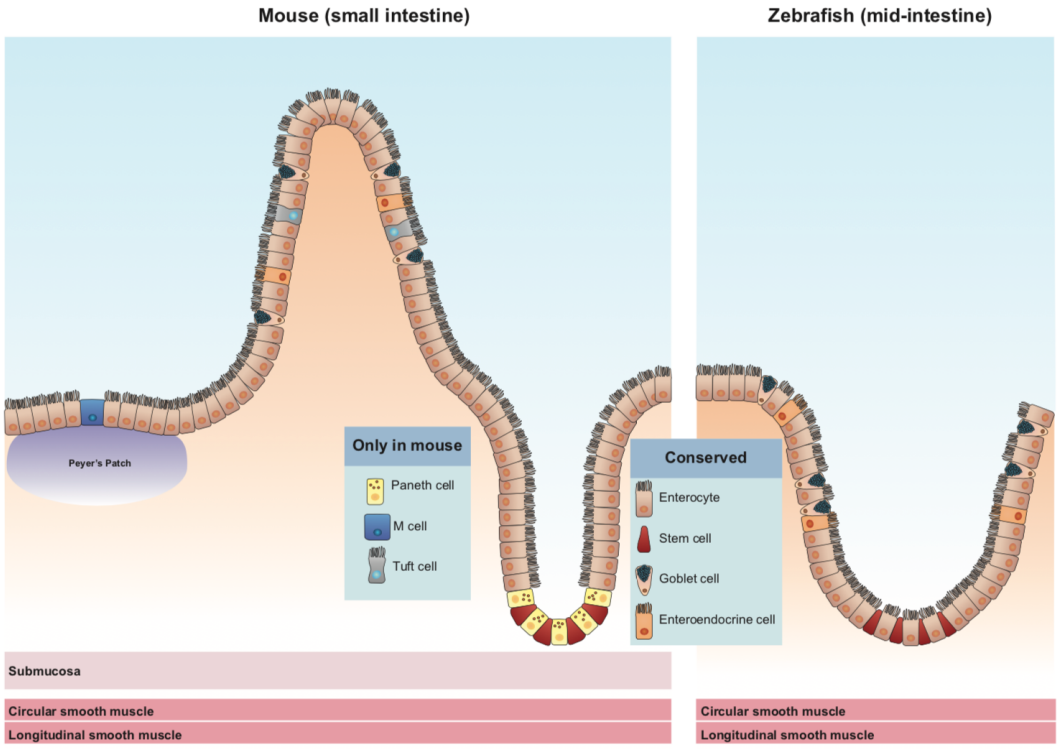

Figure 5. Comparison of the intestine of zebrafish and mouse. The epithelial lining of mouse and zebrafish contain absorptive enterocytes, stem cells, goblet cells and enteroendocrine cells. However, Paneth cells, M cells and tuft cells have only been reported in mice. The architecture of crypt-villus present in mice is not present in zebrafish, where stem cells are located at the bottom of invaginations. Moreover, lymphoid structures, such as lymph nodes and Peyer's patches are absent in zebrafish. Finally, the submucosal layer, located between the lamina propria and the muscle layer is also absent in zebrafish. Adapted and modified from (Diaz et al., 2019) [6].

4.2 THE ZEBRAFISH IMMUNE SYSTEM

The zebrafish immune compartment contains most of the immune cells already described in vertebrates. Innate immune cells are among the first cells to appear during development, with macrophages being observed as early as 24 hours post fertilization (hpf) [232] while granulocytes have been identified at 48 hpf [233,234]. Cells belonging to the dendritic cell lineage in zebrafish have phagocytic capacity and are involved in antigen presentation while also having the classic morphology of their mammalian counterparts [235]. Mast cells have also been observed in mucosal tissues in zebrafish larvae, similar to their location in mammals [236], while eosinophils have been observed in adult fish [233]. Innate lymphocytes, including NK and cells resembling ILCs, have also been recently described in the gastrointestinal tract of adult zebrafish [237,238].

Adaptive immunity is also present in zebrafish. As in mammals, T cell lymphopoiesis takes place in the thymus, and the presumptive thymic rudiment has been identified as early as 48 hpf [239], while it is first colonized by immature lymphoblasts at 65 hpf [240]. The expression of *rag1* and *rag2*, genes involved in the arrangement of the B and T cell receptors, was first observed in the thymus at 92 hpf, which continues to enlarge and acquires its mature structure at 1 month [241], suggesting that a fully functional T cell immunity is not present in larval stages. However, a recent study has shown that there are 2 waves of lymphopoiesis in zebrafish: a transient wave, where only CD4 αβ T cells are generated, lasts until late juvenile stages and is HSC-independent; and a late definitive wave, that takes place from larval stages to adulthood and gives rise to all types of T cells, derived from HSCs [242]. Additionally, γδ T cells have also been identified in zebrafish, being primarily located in mucosal tissues, such as the skin, gills and intestine and promoting humoral immunity [243].

The generation of a *cd4* transgenic reporter in zebrafish has allowed the visualization of the development and characterization of $CD4^+$ T cells in this model. T_{reg}-like cells in zebrafish are enriched in the gut mucosa in zebrafish, similar to mammals, have a gene expression signature similar to those in mammals and its absence leads to chronic inflammation, indicating that they also possess immunosuppressive activity [244,245]. Cells showing increased expression of *gata3* and *il4/il13b*, characteristic of mammalian T_H2 cells, were enriched in the gills and the absence of both *il4/il13a* and *il4/il13b* leads to a proinflammatory state in zebrafish and to an imbalance towards type 1 immune responses [244,246]. However, if T_H1 and T_H17 cells exist in teleost fish and if their function is fully conserved compared to the mammalian counterparts remains to be investigated.

Humoral immune responses, the other arm of adaptive immunity, are also conserved in teleost fish. B cells have been identified at 20 days post fertilization between the dorsal aorta and posterior cardinal vein and in the kidney of zebrafish, while also found in spleen, liver, gills and gut in adults [247]. Three types of immunoglobulins have been described: IgD, IgM, as in mammals, and IgZ/T, which seems to be the functional equivalent of the mammalian IgA, as it is more abundant in the gut mucosa coating luminal bacteria [248-250].

4.3 STUDYING INTESTINAL INFLAMMATION IN ZEBRAFISH

In view on the high degree of genetic and physiologic conservation between zebrafish and mammals and their advantages over other systems described above, it has emerged as a suitable *in vivo* model to study immune responses to a wide variety of environmental compounds in a cost-effective manner. Regarding intestinal immunity, inflammation in the gastrointestinal tract can be driven in zebrafish by exposure to chemicals [251,252], dietary compounds [253,254] and in genetic models [255,256].

Chemically induced models can be used at different developmental stages and do not require genetic manipulation. Additionally, drugs and chemicals that drive inflammation can be dissolved in the aqueous media of the zebrafish larvae, thus greatly facilitating their administration, which is essential for increasing the throughput of drug screenings. Finally, chemically induced models of intestinal inflammation in zebrafish larvae were recently utilized in a screening to test well-characterized drugs and identified novel hits that alleviate intestinal neutrophilic inflammation [257]. Altogether, this underlines the use of zebrafish as an *in vivo* model to examine how environmental factors modulate immune responses.

5 AIMS OF THE THESIS

The general aim of this thesis was to investigate the contribution of environmental factors and genetics in the initiation of intestinal inflammation.

In particular, the specific aims of each study were as follows:

Study I: To characterize how retinoic acid receptor α signaling in intestinal epithelial cells affects intestinal immune homeostasis.

Study II: To examine the cytokine profile induced by combinatorial activation of dietary sensors, such as ligand-activated transcription factors.

Study III: To study the mechanisms by which GPR35 signaling mediates intestinal immune homeostasis.

Study IV: To investigate the immunomodulatory effects of per- and polyfluoroalkyl substances and their impact in intestinal inflammation and epithelial barrier function.

6 MATERIALS AND METHODS

This section briefly describes the main materials and methods used in the four manuscripts included in this thesis. For more details, please refer to the materials and methods section of each manuscript.

6.1 ANIMAL STUDIES (STUDY I-IV)

All mice experiments were approved by the Stockholm regional ethics committee, the Massachusetts General Hospital (Boston, USA) and following the Swiss Federal and Cantonal regulations (Switzerland). All mice were used between 6 and 12 weeks of age, maintained under specific pathogen-free conditions and handled according to guidelines and regulations established by institutional, regional and national authorities.

Zebrafish embryos were obtained from natural spawning of adult zebrafish housed under ethical permits approved by the Stockholm regional ethics committee. Larvae were raised at 28°C and experiments were terminated between 96 and 120 hours post fertilization, a developmental stage at which larvae are not yet considered animals according to European regulations.

Reference numbers for all ethical permits are indicated in each manuscript.

6.2 HUMAN SAMPLES (STUDY III)

The patient samples were collected and stored following the regulations of the Ethics Committee for Northwest and Central Switzerland.

6.3 INTESTINAL INJURY MICE MODELS (STUDY I, III AND IV)

Several models of intestinal inflammation were used in the studies included in this thesis.

In study I, *Citrobacter rodentium* infection was performed using a bioluminescent strain of this bacteria. Sex-matched mice were inoculated by oral gavage with 2 x 109 colony-forming units (CFU) resuspended in PBS. Mice were sacrificed on day 14 post infection.

In study III, colitis was induced by administration of 1.5-2.5% (w/v) dextran sulfate sodium (DSS, MP Biomedicals) in the drinking water ad libitum for 5 days, followed by 2 days of regular water to allow tissue repair.

In study III, mice were challenged with an *E.coli* strain expressing ovalbumin (OVA). Mice were orally gavaged every other day for 21 days with 108 CFU and sacrificed at this timepoint.

In study IV, colitis was chemically induced with 2,4,6-trinitro benzene sulfonic acid (TNBS, Sigma-Aldrich). TNBS diluted in in H2O was mixed in a 1:1 ratio with absolute ethanol, for a final TNBS concentration of 1-2.5%, and administered intrarectally under anesthesia using a plastic feeding needle. Mice were analyzed 1-6 days after colitis induction.

6.4 ANTIBIOTIC TREATMENT OF MICE (STUDY III)

Mice were given an antibiotic cocktail containing Ampicillin, Kanamycin, Gentamicin, Metronidazole, Neomycin and Vancomycin by oral gavage for 10 consecutive days.

6.5 PROCESSING OF TISSUES (STUDY I, III AND IV)

For obtaining single cell suspensions from the colon and small intestine, the intestines were harvested and placed in PBS or Hank's balanced salt solution (HBSS) on ice. Following removal of the mesenteric fat, the segments were opened longitudinally, washed and cut into 1 cm pieces. Dissociation of IECs was performed by incubation at 37°C in 5 mM EDTA and 1mM DTT in buffered solutions under agitation. To analyze the epithelial fraction (Study I), the supernatant was collected, filtered through a 70 μm cell strainer and centrifuged at 500 g, with the pellet containing IECs. To obtain cells from the lamina propria, the tissue pieces were washed and digested in HBSS or RPMI medium containing 0.1 mg/ml DNase I (Roche) and 0.15 mg/ml Liberase TL (Roche) or 0.5 mg/ml Collagenase type VIII (Sigma-Aldrich) under agitation at 37°C. Cells were then washed, filtered through a 70 μm cell strainer and leukocytes were enriched through Percoll gradient (Studies I and IV).

Cell suspensions from the spleen were obtained by mashing the tissues with a syringe plunger through a 70 μm cell strainer.

6.6 FLOW CYTOMETRY AND FLUORESCENT ACTIVATED CELL SORTING (STUDY I, III AND IV)

Single cell suspensions were incubated with anti-CD16/CD32 antibodies (eBioscience) to block non-specific binding and Fixable Viability Dye (eBioscience) at 4°C for 15 min. Cells were then stained at 4°C for 15 minutes with different cocktails of fluorescently labeled antibodies for surface antigens. For intracellular staining, cells were then fixed and permeabilized with fixation and permeabilization buffers (Foxp3 Transcription Factor Staining Buffer Set, eBioscience or Fixation/Permeabilization Solution Kit, BD Biosciences) followed by staining at RT for 20 minutes with fluorescently labeled antibodies against intracellular antigens. Samples were then acquired using FACS Canto II and FACS LSR Fortessa flow cytometers (BD Biosciences). Analysis was performed with FlowJo software (TreeStar).

6.7 HISTOLOGY AND HISTOLOGICAL SCORING (STUDY III AND IV)

Tissue pieces from the colon were collected, fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Sections were stained with H&E and assessed semi-quantitatively by blind investigators or pathologists following established criteria for the corresponding models.

6.8 IMMUNOSTAINING (STUDY I AND III)

Immunohistochemistry: Mouse tissues were fixed in 4% PFA dehydrated in ethanol solutions and embedded in paraffin. Tissues were sectioned at 4-6 μm, de-waxed and rehydrated before

heat-mediated antigen retrieval in citrate buffer or EDTA buffer. Endogenous peroxidase was blocked with BLOXALL Blocking solution (Vector Laboratories). Sections were stained with primary antibodies followed with corresponding secondary antibodies. Additionally, all samples were counterstained with hematoxylin and eosin prior to mounting. For goblet cell staining, slides were incubated in a solution containing 1% Alcian Blue in 3% acetic acid for 5 minutes and counterstained with Nuclear Fast Red solution. Images were quantified in a blind fashion using the ImageJ software. For the analysis of whole small intestines and colons, tissues were prepared as Swiss rolls, placed in cassettes containing OCT compound, and submerged in isopentane cooled with liquid nitrogen. Samples were stored at -80°C until sectioning in 5 μm sections and stained using primary and corresponding secondary antibodies.

Immunofluorescence: Following fixation and dehydration, tissues were embedded in paraffin or prepared for cryo-embedding. Human biopsies were provided in cryoblocks. Six micrometer sections were fixed in PFA and blocked and permeabilized with PBS containing 0.4% Triton X-100 or 0.1% Tween20, and 5% goat serum. Sections were stained with primary and secondary antibodies and a nuclear staining and imaged with a Nikon A1R confocal microscope.

6.9 FLUORESCENCE IN SITU HYBRIDIZATION (STUDY I)

Tissues from the distal ileum were prepared for fixation in Carnoy's fixative, embedded in paraffin and sectioned at 5 μm. Sections were hybridized to a bacterial universal probe against the 16s rRNA gene, conjugated to Alexa488. DAPI was used as a counterstain and tissues were visualized using a CHGR Leika SP5 microscope. Images were taken at 10x magnification and analyzed automatically using the CellProfiler software.

6.10 EXPOSURE TO METABOLITES, POLLUTANTS, ANTIBIOTICS AND TNBS IN ZEBRAFISH LARVAE (STUDY I-IV)

In studies I and II, zebrafish larvae were exposed by immersion to ligands of AHR (FICZ, 100 nM, Syntastic), RAR (retinoic acid, 1 μM, Sigma-Aldrich) and LXR (GW3965, 1 μM, Sigma-Aldrich) from 72 to 108 hpf. All ligands were dissolved in E3 water and renewed every 24 hours.

In study III, zebrafish larvae were stimulated with 10 μM lysophosphatidic acid (LPA; Sigma-Aldrich) or 10 μM Zaprinast by immersion in E3 water from 96 to 120 hpf.

In study III, zebrafish larvae were exposed by immersion from 72 to 120 hours post fertilization (hpf) to an antibiotic cocktail consisting of Ampicillin and Kanamycin. Antibiotics were dissolved in E3 water and the media was replaced every 24 hours.

In studies III and IV, zebrafish larvae were exposed to 50 or 70 μg/ml TNBS (Sigma-Aldrich) from 72 to 120 hpf. TNBS was dissolved in E3 water and replaced every 24 hours.

6.11 INJECTIONS OF LPA AND *VIBRIO ANGUILLARUM* IN ZEBRAFISH LARVAE (STUDY III)

A formaldehyde-inactivated extract of *V. anguillarum* was mixed in a 1:1 ratio with phenol red (Sigma-Aldrich) and diluted 1:3 with PBS. Two nL of this mixture were injected in the swim bladder and intestinal region of 110 hpf anesthetized zebrafish larvae with 0.016% Tricaine MS-222 (Sigma-Aldrich). Larvae were monitored until recovered and analyzed 6 hours after the injection.

Two nL of a mixture of LPA (10 μM) and FITC-Dextran (500 μg/ml) in PBS was injected in the otic vesicle of 110 hpf anesthetized larvae. Larvae were monitored for recovery and macrophage recruitment was analyzed 6 hours post injection.

6.12 ZEBRAFISH LIVE IMAGING AND IMAGE ANALYSIS (STUDY III AND IV)

At the desired timepoint, larvae were washed in E3 medium and anesthetized with 0.016% Tricaine MS-222. Larvae were embedded in 1% low melting point agarose (Sigma-Aldrich) dissolved in E3 water and positioned in Petri dishes for imaging. Imaging of macrophage recruitment was done using a Leica M165FC stereomicroscope (Leica) (study III).

For study IV, epifluorescence was analyzed using a SMZ25 Research Stereo Microscope (Nikon). Images were cropped to include the region of interest and analyzed automatically with the software CellProfiler. Confocal images were taken with a Zeiss LSM800 microscope and the number of neutrophils recruited to the intestine were manually counted. Light-sheet imaging was performed with a Light Sheet Z.1 microscope and 5x air detection objectives.

6.13 WHOLE-MOUNT *IN SITU* HYBRIDIZATION (STUDY III)

A DNA plasmid containing *gpr35b* cDNA was linearized using restriction enzymes to generate the templates for the sense and antisense probes. Sense and antisense digoxygenin (DIG)-labeled RNA probes were transcribed using a DIG-RNA labeling mix, RNase inhibitor and T7 and T3 RNA polymerase, respectively. The DNA template was digested using DNase I and the RNA probes were precipitated and purified using the lithium chloride method.

In situ hybridization was done in whole zebrafish larvae at 72, 96 and 120 hpf. At the corresponding developmental stage, larvae were fixed in paraformaldehyde (PFA) and dehydrated in methanol, followed by depigmentation. Larvae were then rehydrated and digested with proteinase K for a specific time depending on the developmental stage. The reaction was stopped by incubation in PFA, larvae were washed and placed in prehybridization mix. Larvae were then incubated in hybridization mix containing the DIG-labeled RNA probe overnight at 70°C. This was followed by incubation with an anti-DIG antibody conjugated to alkaline phosphatase (AP) and the addition of a chromogenic AP substrate. When the desired intensity signal was observed, the reaction was stopped by the addition of EDTA and larvae were placed in glycerol for mounting and imaging using a brightfield microscope.

6.14 3´-5´-CYCLIC ADENOSINE MONOPHOSPHATE (CAMP) ASSAY (STUDY III)

The *in vitro* screening of GPR35 ligands was performed in a Chinese hamster ovary (CHO)-K1 cell line overexpressing human GPR35 coupled to a G protein that inhibits the accumulation of forskolin-induced cyclic AMP (cAMP), as a response to GPR35 agonists. Cells were treated with 15 μM forskolin and serial dilutions of potential ligands, including 10 μM recombinant CXCL17, 10 μM LPA and 10 mM kynurenic acid. Zaprinast was used as a positive control. cAMP levels were measured by enzyme-fragment complementation (EFC) technology. Two fragments of the β-galactosidase enzyme were used. When free cAMP was present, cAMP labeled with one part of the enzyme was outcompeted to bind to an anti-cAMP antibody, complemented the enzyme complex and cleaved the substrate. This produced a luminescent signal that was analyzed with a microplate reader.

6.15 WHOLE-MOUNT ALCIAN BLUE STAINING IN ZEBRAFISH LARVAE (STUDY I)

Larvae were fixed in PFA, rinsed in acidic ethanol (70% ethanol with 1% hydrochloric acid), and incubated in alcian blue staining solution (0.1% alcian blue in 80% ethanol and 20% glacial acetic acid) for 3 hours at 4°C. Larvae were repeatedly rinsed with acidic ethanol to remove the excess staining and mounted in glycerol for imaging using a Leica M165FC stereomicroscope (Leica). Quantification of positive cells from the middle segment of the intestine to the anus was done manually in a blind manner.

6.16 CHEMICAL ANALYSIS (STUDY IV)

Liver tissues were sectioned and homogenized using a TissueLyser (Qiagen) with a 0.5mm stainless steel bead, followed by vigorous shaking at room temperature and centrifugation. Liver extracts and serum were diluted in a mixture of acetonitrile/water and transferred to a glass insert. The tissue extracts or serum were injected onto the liquid chromatography – mass spectrometer (LC-MS). The samples were analyzed in a Shimadzu UFLC system (Shimadzu Corporation) coupled to a QTRAP5000 (triple quadrupole linear ion trap mass spectrometer) equipped with a TurboIon Spray source (AB Sciex). All samples were analyzed in technical duplicates. Excellent linearity was observed for the calibration standards.

6.17 RNA EXTRACTION AND QUANTITATIVE PCR ANALYSIS (STUDY I-IV)

For analysis of gene expression in cells, mouse or zebrafish tissues, whole zebrafish larvae or human biopsies, RNA was extracted using TRI Reagent (Zymo Research), TRIzol LS reagent (Invitrogen). Alternatively, mice tissues in study I were collected in RNAlater, homogenized and RNA was extracted with the RNeasy kit (Qiagen) according to the manufacturer's protocol. For colonic tissues from DSS-treated mice (Study III), the DSS residues were removed with the Direct-zol RNA MiniPrep kit (Zymo Research). RNA samples were treated with TURBO DNase (Invitrogen, study III) and reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad) or High Capacity cDNA Reverse Transcription (Applied

Biosystems). Analysis of gene expression was performed by quantitative PCR using QuantiNova SYBR Green PCR (Qiagen) or iTaq Universal SYBR Green Supermix (Bio-Rad) kits and run on an ABI ViiA 7 cycler (Applied Biosystems), CFX384 Touch Real-Time PCR (Bio-Rad) or iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Relative quantities of mRNA transcripts were calculated using the ΔΔCt method and *eef1a1l1* (zebrafish), *Hprt*, *Gapdh* or *Actb* (mouse) as housekeeping genes.

6.18 RNA SEQUENCING (STUDY III)

RNA was isolated from sorted colonic GPR35^{+} or GPR35^{-} macrophages from *Gpr35*-tdTomato mice or bone marrow-derived macrophages from WT or *Gpr35*$^{-/-}$ mice. RNA quality and concentration were measured. Indexed cDNA libraries were prepared using commercial kits (Illumina), pooled and sequenced using the NovaSeq 6000 instrument or NextSeq 500 Sequencing System (Illumina).

Reads were aligned to the mouse genome (UCSC version mm10) with STAR (version 2.5.2a). The quality of the reads and alignment was evaluated with the package QuasR in R (version 3.4.2). Differential gene expression analysis was performed using the edgeR package. Genes with logCPM>1 in at least one sample were considered for analysis. Two models were used to test different aspects of genotype and treatment effects. When comparing groups within the same genotype, a nested analysis was performed. For comparisons across genotypes we used a non-nested model with crossed genotype-treatment groups. Both models used different gene dispersion estimates. The glmQLFit and glmQLFTest functions in the edgeR package were used to test the contrasts between models. The resulting P values were false discovery rate adjusted.

6.19 STATISTICAL ANALYSIS (STUDY I-IV)

Statistical analysis was performed using GraphPad Prism Software. All data sets including only 2 groups were analyzed with an Unpaired *t* test. When more than 2 groups were present, data sets were analyzed using one-way ANOVA followed by multiple comparisons tests with Tukey's or Fisher's Least Significant Difference test (α=0.05). Statistically significant outliers were identified with the ROUT method.

7 RESULTS AND DISCUSSION

This thesis aims to investigate the impact of genetic end environmental factors in intestinal inflammation. In **study I** and **study II**, we focused on the role of signaling induced by activation of dietary sensors in maintaining immune homeostasis. While for **study I** we examined its impact on the composition of the intestinal epithelial and immune cell compartments, in **study II**, we investigated how simultaneous activation of multiple receptors modulates cytokine production.

In **study III**, we analyzed the impact of genetic susceptibility on intestinal inflammation and examined how signaling through GPR35, an IBD-risk gene, regulates intestinal immune homeostasis.

In **study IV**, we investigated how exposure to PFAS modulate immune responses during early stages of inflammation and its effect on the intestinal barrier function.

In this section, a brief summary of the main results and a discussion on their implications are presented. The figures presented in this section are shown in full in the corresponding manuscript. More details for each study can be found in the manuscripts attached to this thesis.

7.1 RETINOIC ACID RECEPTOR SIGNALING IN THE INTESTINAL EPITHELIUM SHAPES IMMUNE HOMEOSTASIS AND EPITHELIAL CELL DIFFERENTIATION (STUDY I)

Retinoic acid, a vitamin A metabolite, is sensed by retinoic acid receptors (RARs, -α, -β and -γ), which act as nuclear receptors modulating transcriptional responses [197]. Retinoids have a profound role in the maintenance of immune homeostasis [258], being involved in several processes, such as inducing gut homing of B and T cells [213,214] and regulating the differentiation of intestinal dendritic cell subsets [210]. Although retinoids are absorbed by intestinal epithelial cells in the small intestine, the effect of RAR signaling on these cells was unexplored. Thus, using genetically engineered mice in which RARα signaling was specifically deleted on IECs we investigated the effect of RA activation in the epithelial compartment and how this might affect intestinal homeostasis. In particular, we specifically targeted the RARα isoform within the epithelial compartment by crossing RARα$^{f/f}$ conditional mice with Villin-*cre* mice (RARα$^{\Delta villin}$ mice), which drives expression in intestinal epithelial cells [259]. We then tested if the composition of the epithelial compartment was altered and observed that RARα$^{\Delta villin}$ mice had reduced numbers of enteroendocrine cells and increased numbers of goblet and Paneth cells in the small intestine, compared to controls, by immunohistochemistry. Notably, as the Cre recombinase was only expressed from the upper crypt section to the villi, we cannot exclude that RAR signaling might also modulate crypt progenitors.

Considering that the differentiation of the secretory progenitors might be affected, we wondered if the expression of transcription factors that are necessary for their differentiation

into the different IEC lineages might be affected in the RARα$^{\Delta villin}$ mice. We focused on Kruppel-like factor 4 (KLF4), which has been reported as a regulator of goblet cell differentiation [260] and found increased numbers of KLF4^{+} cells in sections from the small intestine of RARα$^{\Delta villin}$ mice, compared to controls. Additionally, to examine if RAR activation would modulate Klf4 expression *in vivo*, we exposed zebrafish larvae to RA and found decreased *klf4* transcript levels. Altogether, this suggests that RARα signaling modulates *klf4* expression, which might be associated with aberrant goblet cell differentiation.

We also found increased numbers of Paneth cells, which produce the vast majority of antimicrobial peptides (AMPs) in the GI tract. In agreement, we detected increased expression of AMPs produced by Paneth cells, including *Ang4* and *Reg3g* by qPCR. Since AMPs can regulate bacterial colonization, we hypothesized that the microbiota composition could be altered. Indeed, we found a drastic reduction in the content of luminal microbiota by fluorescence *in situ* hybridization in the small intestine of RARα$^{\Delta villin}$ mice.

Since it is well accepted that the microbiota shapes intestinal immune responses and we found that the RARα$^{\Delta villin}$ mice had alterations in the microbiota, we asked if this was also associated with changes in the composition of the immune cell compartment. We found that mononuclear phagocytes, including dendritic cells and macrophages, were reduced in mice where RARα signaling in IECs was impaired. Specifically, we performed the ALDEFLUOR assay [214] and found that intestinal dendritic cells with the ability to metabolize vitamin A into RA, were reduced in RARα$^{\Delta villin}$ mice, in comparison with their control counterparts. In addition, we found decreased frequencies of CD90^{+} lymphocytes and B220^{+} B cells in the colon lamina propria of RARα$^{\Delta villin}$ mice and lack of isolated lymphoid follicles, compared to control. In summary, lack of RARα signaling in IECs results in changes in the composition of the immune cell compartment.

Having shown that RARα deficiency in IECs impaired immune homeostasis, we hypothesized that this could affect immune responses to infection. Infection with *Citrobacter rodentium*, a pathogen with similar properties to those of the human enteropathogenic *Escherichia coli*, leads to inflammation in the distal colon that is usually resolved by immunocompetent mice. We observed that RARα$^{\Delta villin}$ mice had a delayed pathogen clearance, increased bacterial translocation to the spleen and body weight loss. Altogether, RARα signaling in IECs has an important role in the maintenance of intestinal homeostasis.

Here, we found that RARα signaling in IECs regulates the specification of secretory epithelial cells. Considering that it had a different effect on the distinct epithelial cell lineages, we can speculate that RARα could be involved in the differentiation towards each secretory cell type, but once progenitors have been committed to the secretory fate. Among the transcription factors that regulate the specification of secretory cells, we focused on Klf4, whose transcription has been shown to be modulated by RARα [261], and hence, it could be located upstream of Klf4 in the hierarchy of lineage commitment.

Overall, we observed that differences in the secretory epithelial cell differentiation as a result of a lack of RARα epithelial signaling were associated with changes in the composition of the immune cell compartment, which ultimately led to higher susceptibility to pathogen infection **(Figure 6)**. This highlights the importance of adequate sensing of environmental signals, including dietary and microbial products, in the maintenance of immune homeostasis.

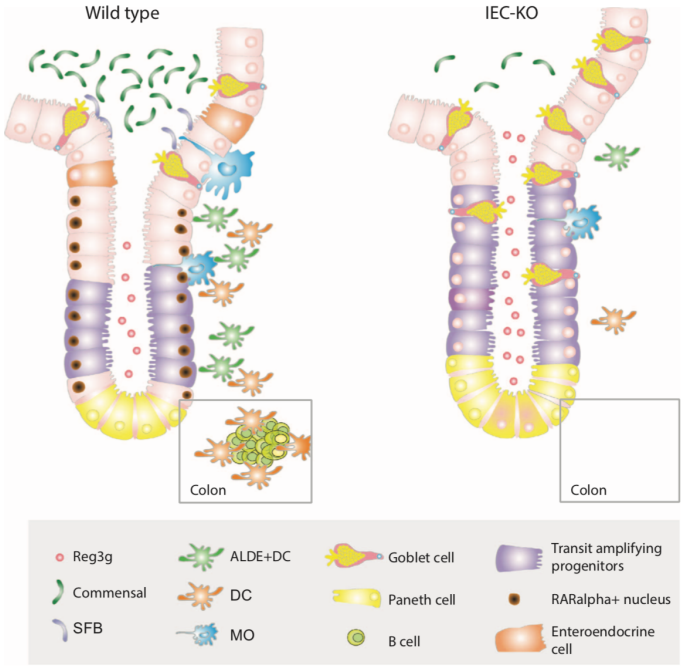

Figure 6. Scheme showing our main findings and the proposed model [1]. Lack of RARα signaling in IEC affected the specification of the secretory lineages of epithelial cells and was associated with alterations in microbiota composition. The development of the intestinal immune system was also affected, as we found lymphoid organs of smaller size and reduced numbers of mononuclear phagocytes. ALDE: Aldefluor, SFB: Segmented filamentous bacteria, DC: dendritic cell, MO: macrophage.

7.2 SIMULTANEOUS ACTIVATION OF LIGAND-ACTIVATED TRANSCRIPTION FACTORS MODULATES CYTOKINE EXPRESSION (STUDY II)

As a barrier organ, the intestinal epithelium is constantly exposed to a plethora of antigens, including dietary-derived metabolites, microbes, pathogens and harmful pollutants. Sensing of these environmental stimuli has a crucial role in the mounting of immune responses and hence, must be tightly controlled. Dietary-derived ligands, which comprise an important class of environmental stimuli, are sensed by ligand-activated transcription factors that are either present in the nucleus already bound to their response elements (e.g. nuclear receptors such as LXR and RAR), or present in the cytoplasm in an unliganded form (e.g. AHR). These receptors, individually, are known to have a profound influence in immune homeostasis [258,262,263]. However, the impact of their combinatorial activation *in vivo* is widely unexplored.

This is particularly important, since tissues are continually exposed to combinations of compounds that might activate or inhibit several receptors simultaneously and we do not understand well the outcome of such combinatorial activation or inhibition. Testing the effect of several compounds simultaneously requires a complex experimental design including

many experimental conditions, and presents several logistical challenges including the administration route and readouts analyzed. Considering this and the high costs associated, it is not possible to perform these experiments in mice. Zebrafish, therefore, provides an excellent model system that can fill this gap for the following reasons. For example, the maintenance cost of zebrafish is substantially lower from that of mice, they have large clutch sizes and allow exposure by immersion. Therefore, we examined the effect of simultaneous activation of nuclear receptors and AHR in modulating immune responses in zebrafish larvae. Particularly, we examined cytokine production, especially those involved in the maintenance of immune homeostasis [264].

We focused on AHR, LXR and RAR, which have been shown to regulate the production of cytokines, especially those relevant in immune mediated disorders [265]. These receptors are conserved in zebrafish [266-270] and we first wondered if exposure to their ligands, would result in their activation. To address this, we analyzed the expression of target genes for each receptor by qPCR and found that exposure to each ligand resulted in increased expression of individual target genes. We next asked if the signaling of each receptor would be affected by simultaneous exposure to other ligands. We found that co-exposure with more than one ligand resulted in increased expression of the corresponding target genes, and to a similar level as when they were individually activated. Thus, indicating that there is no bystander interference upon specific receptor activation.

Further, we examined how the expression of cytokines that regulate mucosal homeostasis [264,271,272] was modulated by receptor activation in our model. We found that RA exposure resulted in increased expression of *il1b*, *il17a/f3* and *il22* and lower transcript levels of *il10*, suggesting a pro-inflammatory cytokine profile. Considering that ligand co-exposure resulted in simultaneous receptor activation, we tested whether treatment with a combination of ligands could result in differential expression of cytokines and found that AHR activation led to a reduction in the transcript levels of *il17a/f3* and *il22* levels induced by RA. Further, LXR activation counteracted the increased transcript levels of *il17a/f3* following RA exposure, while co-activation of LXR and RAR resulted in a further decrease in *il10* expression and increased expression of *tnfa*. In summary, simultaneous activation of ligand-activated transcription factors, such as LXR and RAR, results in a differential cytokine milieu from that of their individual activation **(Figure 7)**. To our knowledge, most of our findings represent the first report of the modulation of cytokine expression in zebrafish larvae by the activation of these receptors.

Previous reports in mouse models have focused on the impact of the signaling of these receptors in lymphocytes, such as the IL-22 production by ILCs [273] and the induction of Th17 responses [274] promoted by RA signaling, which is in agreement with our findings. However, these cells do not exist at this developmental stage in zebrafish larvae. Thus, it is tempting to speculate that although the elicited responses are functionally conserved the cells that respond are different, and this requires further investigation.

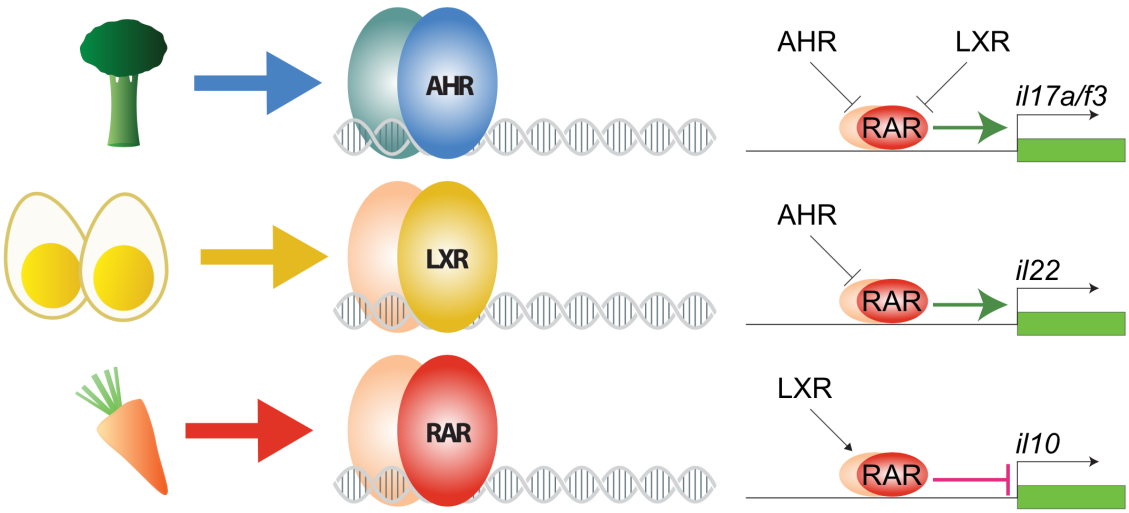

Figure 7. Summary scheme of the modulation of cytokine expression by environmental sensors. Dietary-derived ligands can activate transcription factors, such as AHR and NRs, including LXR and RAR. Combinatorial receptor activation can result in synergistic or antagonistic regulation of immune responses. Modified from [4].

In addition, we found that while co-exposure with different ligands resulted in the induction of the corresponding target genes, without interfering in receptor activity, it resulted in modulation of the cytokine expression. We can speculate that the crosstalk between these receptors might happen further downstream, e.g., binding to DNA response elements or activation of downstream pathways. However, this still remains unclear, and a better understanding of this would aid in the design of therapies considering combinatorial approaches.

An important point to highlight is that the cytokine profile induced by the activation of these receptors is markedly affected by the *in vitro* or animal model and the ligand and concentration used, such as opposite effects in IL-17 production being reported for AHR agonists [199]. Although our results have the potential to be translated to mammals, the conservation of the immune responses should be examined in the corresponding model.

Finally, to our knowledge, this is the first study aimed to understand the impact of the combinatorial activation of ligand-activated transcription factors in immune responses in an *in vivo* context. Considering the advantages of an approach based on a whole organism as opposed to *in vitro* studies, i.e. the complexity of a vertebrate organ system and presence of different cell types, we believe that this could represent a powerful screening platform that could accelerate drug discovery and testing of therapeutic targets.

7.3 ROLE OF GPR35 SIGNALING IN THE MAINTENANCE OF INTESTINAL IMMUNE HOMEOSTASIS (STUDY III)

In the third manuscript included in this thesis we shifted the focus from environmental factors to genetics in the development of intestinal inflammation. Genome-wide association studies (GWAS) have identified more than 200 IBD-risk loci [105], including single-nucleotide polymorphisms in *GPR35* [102,114]. Recently it has been shown that Gpr35 deficiency results in

reduced IEC turnover and worsened chemically induced colitis [119,120] However, the role of GPR35 signaling in the initiation and/or progression of intestinal inflammation and the underlying mechanism is yet to be understood. In this study, we identified lysophosphatidic acid (LPA) as a potential endogenous ligand of GPR35, which controls intestinal inflammation by maintaining TNF-mediated homeostasis, by using a combination of zebrafish and mouse models.

To investigate the role of GPR35 in intestinal homeostasis, we first characterized the expression of its orthologs in zebrafish (*gpr35b*) and mice (*Gpr35*) and found the highest transcript levels in the gastrointestinal tract, similar to what had been described in the Human Protein Atlas [275] (available from http://www.proteinatlas.org), indicating conservation in its expression pattern, from zebrafish to humans.

Considering that G-protein coupled receptors is an IBD-risk locus with expression mostly restricted to the intestine, we hypothesized that GPR35 expression could be modulated by the intestinal milieu. To analyze if Gpr35 expression was dependent on the microbiota, we treated mice and zebrafish with antibiotics and found that its expression was reduced compared to non-treated controls. Moreover, to address if GPR35 expression would be modulated by inflammation, we used several models of intestinal inflammation in zebrafish larvae and mice and consistently found increased transcript levels in the intestine. Finally, to test the clinical relevance of our findings, we also found increased number of $GPR35^{+}$ cells in the lamina propria of biopsies from inflamed areas in patients with ulcerative colitis, compared to non-inflamed regions. Altogether, this indicates that *GPR35* expression is microbiota dependent and increased upon inflammation.

The endogenous ligand of GPR35 that could modulate immune responses in the intestine remains unknown. To address this, we performed an *in vitro* screening of suspected GPR35 ligands and found that LPA would activate this receptor. To test the specificity of LPA as a GPR35 ligand *in vivo*, we generated a *GPR35* mutant zebrafish and mouse lines. We found that LPA treatment induced expression of pro-inflammatory cytokines, such as *Tnf*, in a GPR35-dependent manner. We then hypothesized that production of LPA, which can act as a signaling molecule, could be induced in the context of inflammation. Indeed, we detected increased LPA levels in the supernatant of colonic explants from colitic mice, indicating that this molecule is produced during inflammation.

Considering that LPA production is induced during inflammation and that it modulates cytokine production, we next asked if Gpr35 signaling might affect intestinal inflammation. We observed that *Gpr35*-deficient mice developed exacerbated DSS-induced colitis, in terms of increased body weight loss and increased histological scores. Since *Gpr35* was expressed in $CX3CR1^{+}$ macrophages, and its expression in these cells increased during inflammation, we wondered if the worsened colitis could be due to an intrinsic effect on macrophages. To test this, we crossed conditional $Gpr35^{f/f}$ mice with $Cx3cr1^{CreER}$, where *Gpr35* would be deleted in *Cx3cr1*-expressing cells upon tamoxifen administration. These mice, termed $Gpr35^{\Delta Cx3cr1}$, had an aggravated colitis, compared to control groups.

We then administered LPA to mice undergoing colitis and found that wild-type developed a milder colitis, while *Gpr35*$^{\Delta Cx3cr1}$ did not respond, indicating that the LPA-mediated effect is Gpr35-dependent. *Gpr35*$^{\Delta Cx3cr1}$ mice also had reduced frequencies of TNF-producing macrophages, detected by flow cytometry. Although TNF is mostly known for its pro-inflammatory role, its deficiency results in exacerbated colitis and reduced corticosterone production, which can suppress inflammation [276]. Thus, we hypothesized that TNF administration could ameliorate colitis severity. Accordingly, this treatment resulted in less severe colitis and restored the low levels of corticosterone found in the colon *Gpr35*$^{\Delta Cx3cr1}$ mice. Overall, these results indicate that impaired signaling of Gpr35 in macrophages led to their reduced production of TNF which was associated with decreased corticosterone synthesis and aggravated colitis, which was counteracted by TNF administration.

Finally, to better understand the effect of an IBD-associated variant, we focused on rs3749171, that results in a T108M substitution and represents a hypermorphic allele [119]. By analyzing patients enrolled in the Swiss IBD Cohort Study, we found that the hyperactive GPR35 variant was associated with a higher percentage of patients responding to anti-TNF therapy. We speculate that patients harboring this mutation might have increased TNF production and the enhanced inflammatory response could be ameliorated with TNF blockers.

In this study, we have shown a conservation in the expression pattern of GPR35 from zebrafish to humans at steady state and under environmental stimuli. Further, immune responses mediated by LPA in a GPR35-dependent manner were conserved in zebrafish and mice **(Figure 8)**. Moreover, as the list of ligands for GPR35, remains to be fully identified, we believe that further screenings to characterize their potency and affinity in different contexts and species would aid in the therapeutic targeting of GPR35.

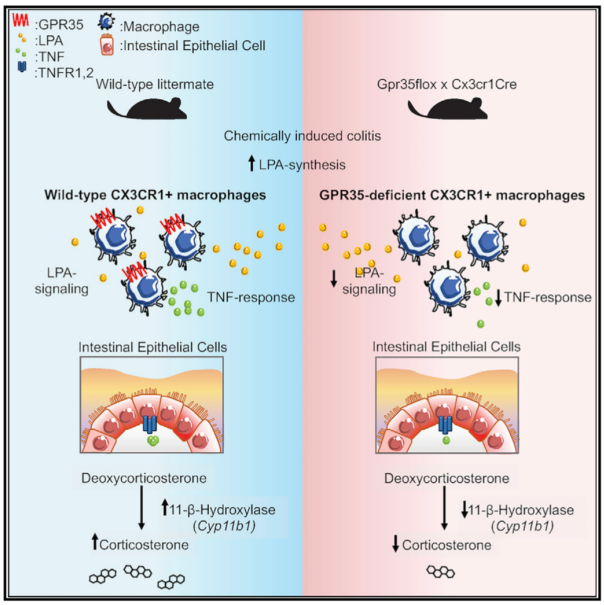

Figure 8. Scheme showing the proposed model of the effect of LPA-mediated GPR35 signaling in the maintenance of intestinal homeostasis [3]. Environmental cues modulated GPR35 expression in zebrafish and mice. GPR35 signaling in macrophages induced their production of TNF and synthesis of corticosterone, which maintained intestinal homeostasis.

7.4 PFOS EXPOSURE EXACERBATES NEUTROPHIL-MEDIATED INTESTINAL INFLAMMATION AND PROMOTES SYSTEMIC T CELL RESPONSES (STUDY IV)

In the last study included in this thesis we examined how exposure to environmental pollutants modulates intestinal inflammation. Among environmental pollutants, per- and polyfluoroalkyl substances (PFAS) represent a class of highly persistent chemicals of anthropogenic origin, that are present worldwide. Several health concerns have been associated to PFAS exposure and effects on the immune system are considered critical for the risk assessment for this class of pollutants [277]. While exposure to PFAS has been positively associated with incidence of ulcerative colitis and to affect clearance of an infection of an intestinal pathogen [165,175], how they affect early stages of intestinal inflammation and epithelial barrier function is largely unclear. To investigate this, we used an established model of chemically induced intestinal inflammation in zebrafish larvae and mice exposed to these pollutants.

Shorter-chain length PFAS are considered to bioaccumulate less than their longer-chain counterparts (such as perfluorooctane sulfonic acid (PFOS)) [139], and have in turn, partially replaced the use of the latter in products. However, little is known about their immunomodulatory properties. To examine this, we exposed zebrafish larvae undergoing TNBS-induced inflammation to several PFAS and found that PFOS exacerbated the expression of *il1b*, a proinflammatory cytokine, in whole larvae, detected by qPCR. We then confirmed, by dissecting the gastrointestinal tract, that the induction of *il1b* expression was intestine-specific. Another hallmark of intestinal inflammation in mammals is increased neutrophil recruitment. By using neutrophil-specific transgenic zebrafish we found increased numbers of neutrophils in the intestine of larvae exposed to PFOS while undergoing colitis. Overall, this indicates that PFOS exacerbates intestinal inflammation in zebrafish larvae.

We then asked if the enhanced neutrophil recruitment to the intestine was recapitulated in mammals, in the corresponding TNBS colitis model in mice. In agreement, we observed that oral PFOS exposure prior and during induction of colitis resulted in increased neutrophil infiltration to the colon, analyzed 3 days after TNBS administration using flow cytometry. This was associated with increased histological score and body weight loss, indicative of a worsened colitis.

Considering that PFOS bioaccumulate in tissues due to limited clearance and that oral exposure remains the main route of intake [145], we tested whether its bioavailability was affected in the context of intestinal inflammation. We detected increased levels of this pollutant in the serum and liver of mice that were exposed to it while undergoing colitis. In view of this, we hypothesized that the function of the intestinal barrier might be affected by PFAS exposure during inflammation. To address this, we performed a FITC-Dextran permeability assay at different timepoints, and found that, within 24 hours of colitis induction, PFOS exposure results in increased permeability compared to TNBS alone.

Further, at day 3 post TNBS administration, mice that were co-exposed to TNBS-PFOS still showed a trend of increased permeability, indicating that PFOS exposure in the context of intestinal inflammation affects intestinal permeability.

Interestingly, there was a similar initial increase in neutrophils 24 hours after colitis induction, and while numbers still remained high at 3 days after TNBS administration in mice that were given PFOS, levels had almost returned to baseline in mice that were not exposed. This suggests that PFOS sustains neutrophils in the colon following a challenge. Neutrophil presence in damaged tissues must be tightly controlled by their timely removal to allow the resolution of the inflammation [278]. We then wondered if the impairment in the epithelial barrier function was affected by the enhanced neutrophil infiltration. To test this, we used a neutrophil depleting antibody and found that permeability decreased to levels similar to those found in mice solely exposed to PFOS. Thus, suggesting that the disruption of the intestinal barrier function is partially affected by the increased neutrophil influx. Altogether, we propose a model where PFOS exposure in the context of intestinal inflammation enhances neutrophil recruitment and leads to an impairment in barrier integrity and translocation of this pollutant into circulation.

Finally, we wondered if the increased PFOS levels systemically, found in the context of intestinal inflammation, could have an effect on the composition of the immune cell compartment in periphery. We then examined the spleen and found that PFOS exposure during colitis resulted in an increase of $CD45^+$ (immune) cells, and in particular, $CD4^+$ T cells. Since this was associated with an increase in neutrophil numbers in the colon of colitic mice exposed to PFOS, we tested if the increase of cellularity in the spleen was neutrophil-dependent. Following neutrophil depletion, we found that both the number of total $CD45^+$ cells and $CD4^+$ T cells were reduced to levels close to the baseline. Overall, we speculate that this neutrophil dependent splenic $CD4^+$ T cell expansion could be partially due to the increased systemic bioavailability of PFOS and/or inflammatory signals resulting from the exacerbated intestinal inflammation.

In summary, by using zebrafish larvae and mice we showed that PFOS exacerbates chemically induced intestinal inflammation. Moreover, our findings indicate that the effects of PFOS exposure during colitis are not only restricted to the intestine, as we also found an expansion of $CD4^+$ T cells in the spleen **(Figure 9)**. Finally, we believe that this study highlights the relevance of the zebrafish as a model to test the immunomodulatory properties of environmental compounds and in the study of mucosal immunity.

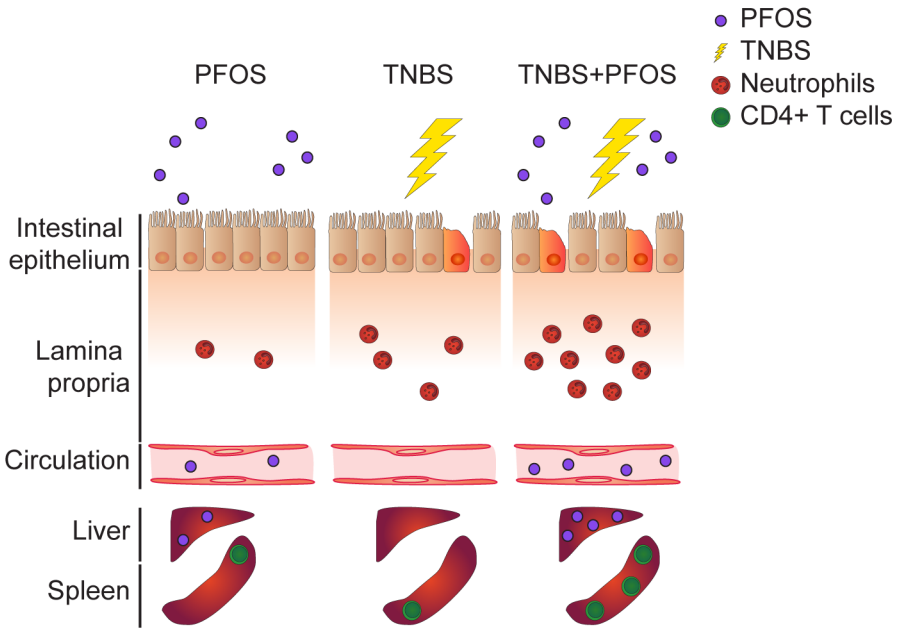

Figure 9. Scheme showing the proposed model. PFOS exposure during intestinal inflammation results in increased neutrophil recruitment to the gastrointestinal tract and intestinal permeability. This is also associated with increased systemic bioavailability of the pollutant and an expansion of T cells in the spleen.

8 CONCLUSIONS

The findings of this thesis have provided insight into how intestinal inflammation is influenced by genetic susceptibility and exposure to environmental elements. However, several questions remain unanswered and should be the focus of future studies.

Particularly, we used zebrafish as a model to investigate how intestinal homeostasis is regulated in different contexts and subsequently validated some of our results in mammals. This highlights the relevance of the zebrafish model in the study of intestinal immunity. In addition, we provided evidence that this model could also be used to examine the interaction between genetic and environmental factors and their impact on intestinal inflammation.

However, many questions remain regarding the composition of the zebrafish immune system and the source of different immune modulators and signaling molecules, such as cytokines. Therefore, a more complete characterization of the zebrafish system, not only during the larval stage but also during adulthood, by using single-cell RNA techniques, for example, would greatly help in the establishment of this model in the immunology field.

In **study I**, we found that epithelial RARα signaling contributes to the specification of epithelial cells and has profound implications in the development and function of the intestinal immune system. This highlights an understudied role of how signals originating from non-immune cells impact intestinal immunity. In this study, RARα^{villin} mice were analyzed at 8 to 12 weeks, and RARα signaling was impaired during their development. Thus, we could speculate that alterations in vitamin A sensing might have a different impact in mice with a mature immune system. A better understanding of the effect of RAR signaling in adult mice (e.g. using a tamoxifen inducible VillinCreERT2) would be essential.

In **study II**, we showed that simultaneous activation of NRs and AHR can induce a distinct cytokine profile. We believe that combinatorial approaches could aid in the design of interventions to tailor specific immune responses. Moreover, since whole larvae are exposed to the ligands, this represents a valuable platform that can be used to examine the response induced in different tissues simultaneously.

In **study III**, we found that GPR35 contributes to the maintenance of intestinal homeostasis and identified LPA as a potential endogenous ligand for GPR35. However, which host cell produces LPA and if this would differ depending on the context still remains to be addressed. Moreover, considering that LPA can be sensed by other LPA receptors, it is possible that other endogenous ligands are activating GPR35 in our system. Finally, further screenings to identify additional GPR35 ligands and a more profound characterization of its function is essential for its possible use as therapeutical target.

In **study IV**, we found how exposure to a globally distributed pollutant affects early stages of intestinal inflammation and affects systemic immune responses. We found increased neutrophil recruitment to the gastrointestinal tract in zebrafish and mice, which emphasizes the relevance of zebrafish larvae to study innate immunity. However, what do these

neutrophils produce and what mediators regulate their recruitment? Answering these questions would be crucial to establish therapies targeting neutrophil-driven intestinal inflammation.

Considering our findings in the context of the wide distribution and persistence of PFAS, it is imperative to regulate and restrict their use and functionally characterize the immunomodulatory properties of shorter-chain length PFAS that are currently substituting the longer-chain length compounds. In this same manner, although beyond the scope of this thesis, the importance of designing novel techniques for the challenging remediation of PFAS are in urgent need.

In summary, by using mice and zebrafish models we have gained greater understanding into the effect of environmental triggers and genetic susceptibility in intestinal inflammation. We believe that incorporating both models can accelerate the identification of novel ligands, test their function *in vivo* and possible therapeutic use.

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