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THÈSE DE DOCTORAT
DE L'UNIVERSITÉ PSL

Préparée à l'Institut Curie
École doctorale Complexité du Vivant ED515

Interleukin-22 : Functional analysis in zebrafish
Interleukine-22 : Analyse fonctionnelle chez le poisson zèbre

Soutenue par

Soraya RABAHI

Le 08/12/2023

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Complexité du vivant

Spécialités

Biologie du développement

Immunologie



Composition du jury :

Marie-Anne, FELIX
Directrice de recherche, École normale supérieure, Paris
Présidente du jury

Shai, BEL
Principal Investigator, The Azrieli Faculty of Medicine, Israel
Rapporteur

Gilles Claude, VANWALLEGHEM
Principal Investigator, DANDRITE, Aarhus University, Denmark
Rapporteur

Claire, WYART
Directrice de recherche, Institut du cerveau, Paris
Examinatrice

Danijela, VIGNJEVIC
Directrice de recherche, Institut Curie, Paris
Examinatrice

Pedro, HERNANDEZ
Principal Investigator, Institut Curie, Paris
Directeur de thèse

A ma famille et à ceux partis trop tôt

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Abbreviation

GI: Gastrointestinal tract

CNS: Central nervous system

ENS: Enteric nervous system

ISCs : Intestinal stem cells

IECs: Intestinal epithelial cells

AMPs: Anti-microbial peptides

TLRs: Toll-like receptors

NODs: Nucleotide-binding oligomerization domain-containing proteins

ILCs: Innate lymphoid cells

ILFs: Isolated lymphoid follicles

VIP: Vasoactive intestinal peptide

TTX: Tetrodotoxin

DCs: Dendritic cells

PRRs: Pattern recognition receptor

LPS: Lipopolysaccharides

TGF: Transforming growth factor

IL: Interleukin

Treg: Regulatory T cells

NK: Natural killer cells

SCFAs: Short chain fatty acids

sIgA: Secretory IgA

IBD: Inflammatory bowel diseases

EECs: Enteroendocrine cells

5-HT: 5-hydrotryptamine or serotonin

EC: Enterochromaffin cells

SST: Somatostatin

CCK: Cholecystokinin

GIP: Glucose-dependent insulinotropic polypeptide

NTS: Neurotensin

GLP: glucagon-like peptides

PYY: Peptide YY

INSL5: Insulin-like peptide 5
5-HTP: 5-hydroxy-l-tryptophan
Tph: Tryptophan hydroxylase
SERT: Serotonin reuptake transporter
5-HIAA: 5-hydroxyindoleacetic acid
TRPA1: Transient receptor potential cation channel
ROS: Reactive oxygen species
DSS: Dextran sodium sulfate
DNBS: 2,4-dinitro-benzenesulfonic acid
VEGF: Vascular Endothelial Growth Factor
IBS: Irritable bowel syndrome
JAK: Janus kinase
TYK: Tyrosine kinase
STAT: Signal transducer and activator of transcription
IL-22BP: IL-22 binding protein
IL22RA1: IL-22 receptor chain alpha 1
Th: T helper
LTi: Lymphoid tissue inducer cells
Ahr: Aryl hydrocarbon receptor
NCR: Natural cytotoxicity receptor
HDAC: Histone deacetylase
C3aR: Complement component 3a receptor 1
MNP: Mononuclear phagocytic cells
ARNT: Aryl hydrocarbon receptor nuclear translocator
I3A: Indole-3-Aldehyde
RARs: Retinoic Acid Receptors
IFNs: Interferons
MCMV: Cytomegalovirus
ISGs: Interferon stimulated genes
HFD: High fat diet
Hpf: Hours post fertilization
Dpf: Days post fertilization
LREs: Lysosome-rich enterocytes
Fxr: Farnesoid X receptor

GF: Germ-free

NLRs: NOD-like receptors

NITRs: Novel immune-type receptors

MHC: Major histocompatibility complex

AID: Activation-induced cytidine deaminase

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INTRODUCTION

Chapter I. Intestinal physiology and immunity

I. The intestine and its functions

1.1 Overview of intestinal structure

In all organisms, the gastrointestinal tract (GI), is a complex and essential organ that serves the primary functions of absorbing nutrients and water, and excreting waste products of digestion, while safeguarding the body from harmful substances and pathogens. In mammals, the GI is divided into two main sections: the small intestine (duodenum, jejunum, and ileum) and the large intestine (colon and rectum), separated by the caecum¹.

The small intestine is the longest section of the gut and is the primary site for digestion and nutrient absorption. Its lining consists of epithelial cells with an extensive surface area, essential for efficient nutrient uptake². Coordinated muscular segments, both longitudinal and circular, work together to mix and propel the contents within the gut³.

The large intestine is shorter than the small intestine, but it is still an important organ for digestion and absorption¹. The large intestine is where the water is absorbed from the remaining food, and it is also where some of the nutrients that were not absorbed in the small intestine are digested by bacteria located in the lumen (Figure 1).

Nerve cells are organized into two systems: the central nervous system (CNS) and the enteric nervous system (ENS). The CNS is located in the brain and spinal cord, while the ENS is located in the myenteric plexus of the GI tract itself (Figure 1). Often referred to as the "second brain"⁴, the ENS can function independently, controlling digestive enzyme and hormone secretion, gut motility, food movement, and nutrient absorption.

In addition to the ENS, the muscular layers in the digestive tract, driven by smooth muscles (Figure 1) are forming electrical and mechanical connections to control gut motility⁵. These contractions are regulated by the myenteric and submucosal plexuses, which interact with the enteric nervous system to influence gut motility and patterns of muscle activity⁵.

The GI tract also plays a key role in the immunity of the organism. The gut contains a very large number of immune cells located mostly in the lamina propria that protect against infections and regulate inflammation⁶ (Figure 1).

Another key player in gut health is the microbiota, it is composed of billions of microorganisms living in the gut. It helps to protect the gut from infections, and it also produces short-chain fatty acids that are essential for colon cell health^{6,7}.

Overall, the gut is a highly specialized organ that is essential for the health of the entire body. The mechanisms regulating its function are not yet fully understood and remain a critical area of ongoing research. *In this first chapter, we will take a closer look at the different compartments of the gut and their interconnectedness. I will illustrate how these compartments communicate with each other and how this communication influences gut health.*

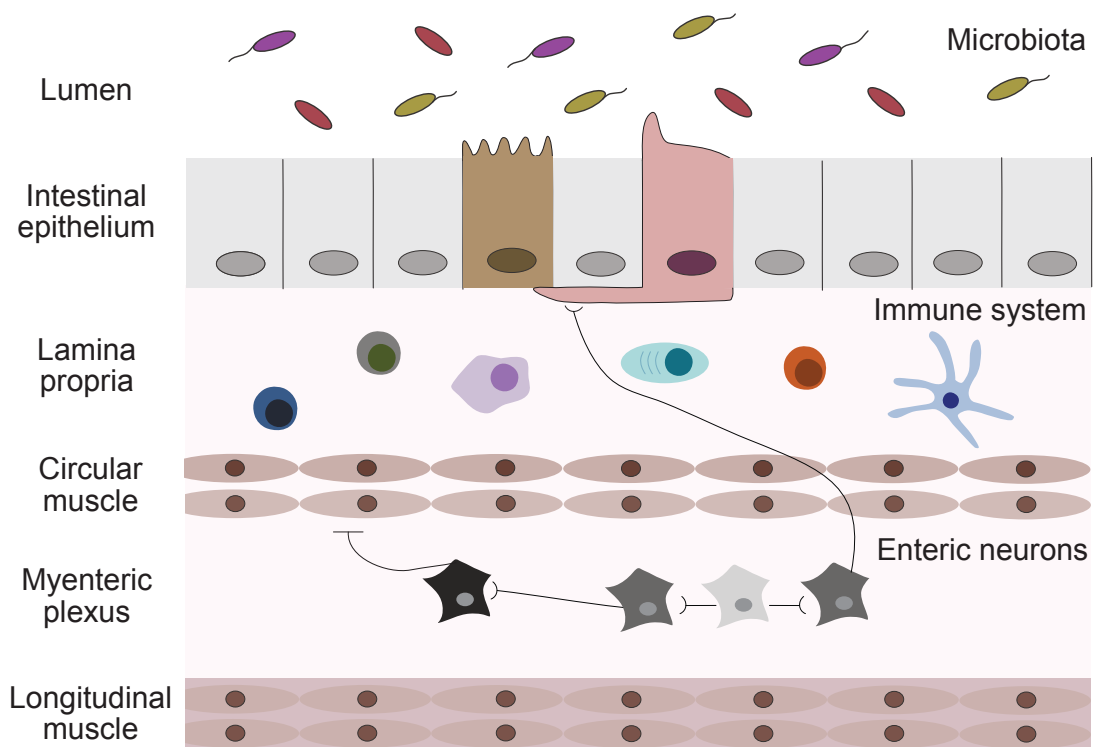


Figure 1. Mammalian intestinal architecture

Simplified schematic showing the general architecture of the intestine and its main compartments: the lumen, which contains billions of microorganisms, commonly known as the microbiota; the intestinal epithelium, which contains several different subtypes that ensure proper intestinal physiology. Some of them are able to communicate with enteric neurons; the lamina propria, which contains immune cells that protect the gut from infection and maintain tissue homeostasis; the muscles: circular and longitudinal, separated by the myenteric plexus, which contains enteric neurons. All these compartments are critical players in gut physiology and homeostasis. *Adapted from Wallace et al, 2005.*

1.2 The intestinal epithelium and its role in immunity

The gut mucosa acts as a physical barrier, separating the gut lumen from the host internal environment⁸. This barrier primarily consists of the mucus layer, the epithelial layer, and the underlying lamina propria. In mammals, the epithelium forms a monolayer mainly composed of enterocytes, along with various other cell types, such as enteroendocrine cells, goblet cells, Paneth cells, tuft cells, M cells, and immune cells.

The organization of the epithelial layer includes crypts containing progenitor cells and Paneth cells, as well as villi harboring differentiated cell types. Paneth cells and intestinal stem cells (ISCs) reside at the crypt bottom, while transit-amplifying cells, actively dividing, populate the rest of the crypt⁹ (Figure 2A). During the renewal process, intestinal epithelial cells (IECs) continuously migrate from the crypt bottom to the tip of the villi, where apoptosis occurs, leading to shedding of cells into the intestinal lumen⁹.

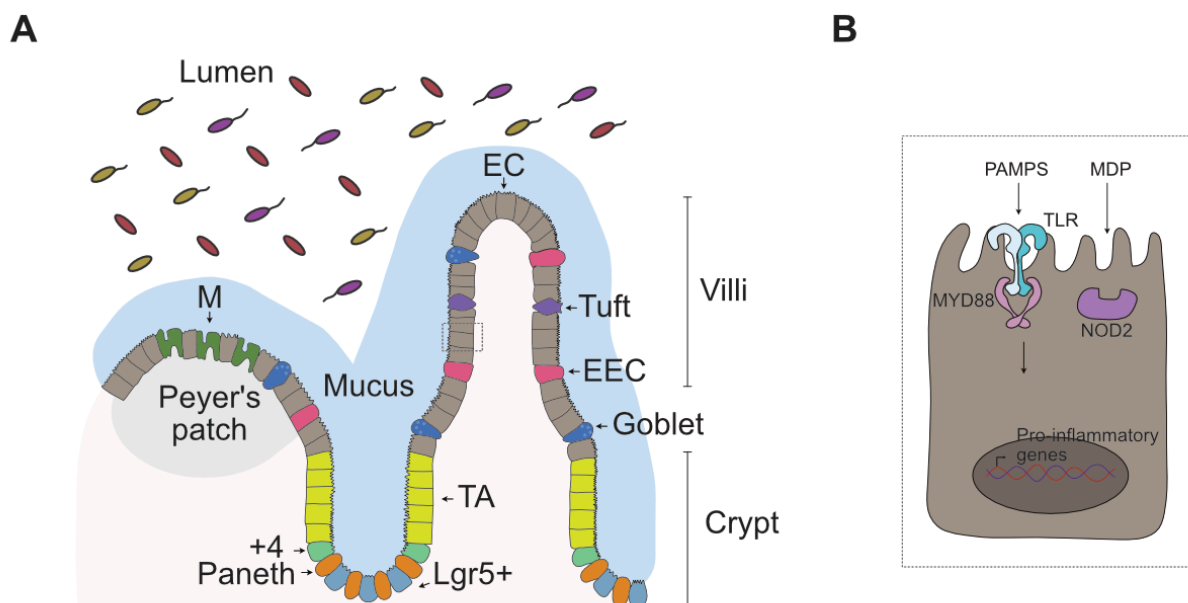


Figure 2. Cell type diversity in the intestinal epithelium and their immune function

A. LGR5⁺ stem cells located in the crypts differentiate into various types of intestinal epithelial cells. They then migrate to the tip of the villi where they are shed. Paneth cells are an exception: they migrate to the inside of the crypts. M cells transport luminal antigens to Peyer's patches, lymphoid follicles associated with intestinal tissue that allow a rapid immune response. The epithelial layer is covered by mucus, which protects it from bacteria. **B.** Recognition of PAMPs and MDP by TLRs or NOD2 expressed by IECs induces the expression of pro-inflammatory genes. *Adapted from Ferguson and Foley, 2021*

In humans, the formation of the intestinal barrier structure is completed by the end of the first trimester¹⁰. However, maturation and functional development of the intestinal barrier continue during the post-natal period and are influenced by factors like feeding mode and diet¹¹. In fact, the epithelial structure continues to change with crypts beginning to form around postnatal day 7. One example of changes in IECs from neonates to adult comes from Toll-like receptor 5 (TLR5), which detects bacterial flagellin and is present throughout the intestinal epithelium of neonatal mice and is then mostly restricted to Paneth cells at weaning/adulthood¹². Some differences can also be seen at the epithelium composition level with Paneth cell usually appearing within the first two weeks¹³.

In the past, the intestinal epithelial layer was believed to act as a strict barrier, preventing the immune system from being activated by the diverse contents within the intestine. However, studies have revealed the fundamental role of the gut epithelium in the maintenance of intestinal immune balance, in addition to the absorption of nutrients¹³.

IECs form two main types of selective barriers to protect the gut mucosa from commensal microbes and pathogens: physical and chemical barriers. Physical barriers include the mucus layer, the glycocalyx on the microvilli of absorptive intestinal epithelial cells, and the tight cell junctions between these cells. These barriers physically avoid the invasion of the mucosa by intestinal microorganisms. Goblet cells are known produce the mucous layer. In the large intestine, where there is a high concentration of intestinal bacteria, the number of goblet cells is significantly higher than in the small intestine, leading to a dense mucus layer. The inner mucus layer, containing polymerized gel-forming mucin MUC2, further hinders microorganisms from invading the intestinal epithelia¹⁴. Indeed, in *Muc2*-deficient mice, the colonic epithelial is fully invaded by bacteria¹⁴. Chemical barriers play a critical role in the segregation of intestinal bacteria and epithelial cells, particularly in the small intestine, where goblet cells are less abundant. These barriers include anti-microbial peptides (AMPs) such as defensins and the regenerating islet-derived 3 (Reg3) family of proteins produced by intestinal epithelial cells, particularly Paneth cells^{15,16}.

In addition to forming a barrier, IECs can sense bacteria from the microbiota or pathogens by expressing pattern recognition receptors such as Toll-like receptors (TLRs), and nucleotide-binding oligomerization domain-containing proteins (NODs)

(Figure 2B). For example, the production of anti-microbial molecules by Paneth cells is regulated by TLR4/MyD88 signaling and NOD2 signaling, both driven by gut microorganisms^{15,16}. In mice lacking NOD2, which recognizes conserved structures in bacterial peptidoglycans, the expression of defensins is substantially reduced, leading to high susceptibility to infections by *Listeria monocytogenes*¹⁷. These anti-microbial molecules target both pathogenic and commensal bacteria, thus also shaping the composition of the intestinal microbiota.

In summary, the diverse functions of different epithelial cell types in the intestine contribute significantly to intestinal immunity and to the maintenance of a balanced microenvironment within the gut.

In addition of forming a barrier, IECs also mediate the crosstalk between gut microbes and the host immunity. Indeed, IECs play a significant role in modulating host immune responses through the secretion of cytokines and chemokines. For example, when stimulated by flagellin proteins from Gram-negative bacteria, the TLR5 signaling pathway induces the production of IL-8 by epithelial cells which in turn recruits neutrophils to the lamina propria, helping to the immune defense against bacterial pathogens^{18,19}.

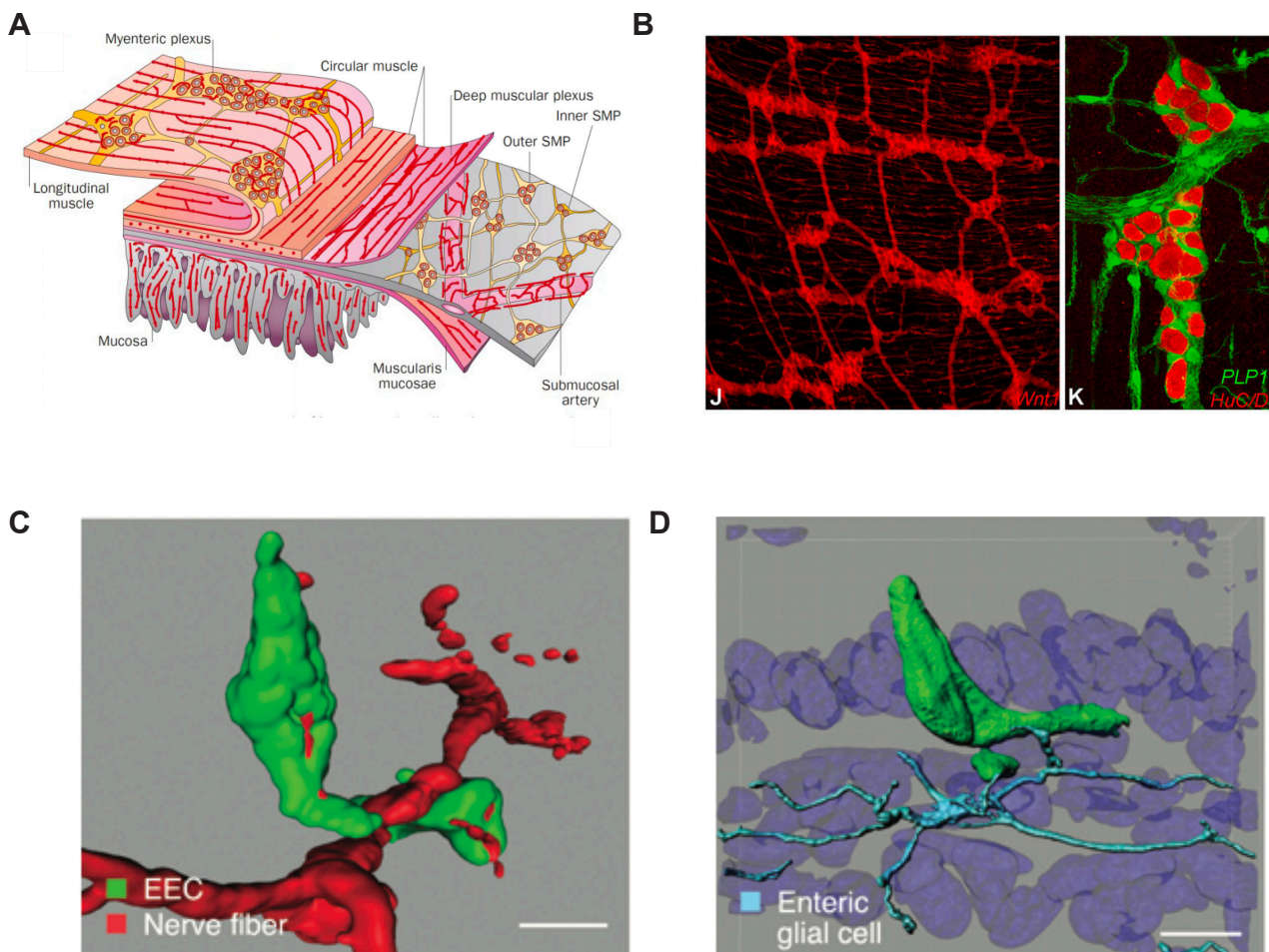
Recent studies have also highlighted the contribution of tuft cells, a type of taste-chemosensory epithelial cell, in the elimination of parasites. Tuft cells produce IL-25, which activates ILC2 (innate lymphoid cells type 2) to secrete IL-13. This process induces Th2 responses, ultimately enhancing the differentiation of both tuft and goblet cells^{20–22}. Such responses are beneficial in mounting a strong immune defense against parasitic infections.

On the other hand, M cells cover isolated lymphoid follicles (ILFs) and Peyer's patches and are specialized in antigen uptake and delivery from the lumen to dendritic cells and T cells through a process called transcytosis²³. This process plays a significant role in initiating immune responses and can also be exploited by certain pathogens to breach the epithelial barrier²⁴.

Altogether, IECs can sense signals from commensal and pathogenic microbes as well as from immune cells, enabling the latter to coordinate the immune response through the secretion of cytokines and chemokines.

1.3 The enteric nervous system

The ENS is a highly intricate network of neurons extending from the esophagus to the anal sphincter. In rodents, it comprises over 80-100 million neurons, while in humans, this number increases to 400-600 million, equivalent to that found in the spinal cord²⁵. Additionally, the ENS includes a significant population of enteric glia, outnumbering neurons by about 5 times, along with a network of nerve fibers that communicate with and project to effector tissues. Studies indicate that enteric glial cells play an essential role in supporting the survival and function of ENS neurons²⁶. Furthermore, emerging evidence suggests that enteric glial cells possess multiple immune functions, potentially contributing to the immune homeostasis of the gut. Interestingly, the ENS is often referred to as the "second brain" due to its size, complexity and autonomous functionality⁴.



(Figure legend below)

Figure 3. Organization of the enteric nervous system and its contact with EECs
A. The enteric nervous system has two main plexi: the myenteric plexus, which is located between the longitudinal and circular muscle layers, and the submucosal plexus, which has outer and inner components. Nerve fibers connect the ganglia in each plexus and also form plexi that innervate the longitudinal muscle, circular muscle, muscularis mucosae, intrinsic arteries, and mucosa. SMP stands for submucosal plexus. *Data from Furness et al, 2012* **B.** Photomicrographs showing enteric neurons from the myenteric plexus (labeled in red) (left panel) and enteric ganglia with glia labeled in green and neurons in red (right panel). *Data from Nagy and Goldstein, 2017* **C.** 3D reconstruction of a confocal z-stack shows an EEC in the gut epithelium extending a neuropod to connect with an underlying nerve fiber and **D.** Enteric glia underneath the epithelium extend processes to contact the neuropod of an EEC. *Data C and D from Bohórquez and Liddle, 2015.*

ENS neurons are organized into two main ganglionated plexi: the myenteric plexus located between the longitudinal and circular muscle layers, and the submucosal plexus found between the submucosal matrix and the external circular smooth muscle layer. Enteric glia populations are distributed within the plexi and mucosa²⁷ (Figure 3A and B). ENS neurons can be classified as motor neurons, intrinsic sensory (primary afferent) neurons, and interneurons²⁵. They interact not only with each other but also with other cell types like immune cells, enterocytes, and enteroendocrine cells (Figure 3C and D), allowing them to control various gut functions such as gut motility.

Although the sympathetic and parasympathetic autonomic nervous systems provide external innervation to the GI tract and can modulate ENS activity, the ENS is capable of independent function without input from the brain or spinal cord. It plays a central role in coordinating various digestive processes, such as motility, enzyme supply, absorption, fluid exchange, storage, and excretion²⁵. In fact, the absence of enteric nerves in patients with Hirschsprung's disease who have a dysfunctional colon demonstrates the crucial role of the ENS in intestinal motility²⁸.

Moreover, the ENS is involved in epithelial barrier function, nociception, and immune responses. For example, it has been reported that the neuropeptide VIP (Vasoactive Intestinal Peptide) could trigger²⁹ or inhibit³⁰ the production of the cytokine IL-22 which in turn contributes to intestinal integrity.

During development, the migration, differentiation, and organization of enteric neurons predominantly occur during early *in-utero* life. Following birth, the ENS continues to be shaped by microbial colonization and the development of the immune system⁷.

1.4 The interplay between the intestinal musculature and the enteric nervous system

Gastrointestinal motility arises from the coordinated contractions of the muscular layers of the digestive tract. In most of the gastrointestinal system, smooth muscles are arranged in either circular or longitudinal muscle bundles. These smooth muscle cells form electrical and mechanical connections between cells, enabling the synchronization of contractions. The process of excitation-contraction coupling involves the entry of calcium ions (Ca^{2+}) through ion channels in the cell membrane, leading to an increase in intracellular Ca^{2+} levels³¹. The binding of Ca^{2+} to calmodulin activates myosin light chain kinase, initiating the cycling of cross-bridges. To relax the muscles, myosin phosphatase dephosphorylates myosin, and the activity of the phosphatase is regulated by a process called Ca^{2+} sensitization³¹.

Gastrointestinal smooth muscle exhibits “autonomous” behavior, generating spontaneous electrical activity known as slow waves that does not depend on nerve input³². These slow waves originate from interstitial cells of Cajal, which are electrically coupled to smooth muscle cells⁵.

Smooth muscle cells acting autonomously would not produce purposeful movements, but by establishing electrical and mechanical connections with neighboring cells, they form a syncytium that enables coordinated contractions involving many cells. Achieving gastrointestinal motility patterns and orderly progression of luminal contents, however, involves greater complexity. Many regulatory elements, including motor neurons, hormones, paracrine agents, and inflammatory mediators, layer upon myogenic activity to generate expected contractile behaviors⁵. For example, the patterns of contractile activity in gastrointestinal muscles are determined by signals from enteric motor neurons that innervate both smooth muscle and interstitial cells³³.

The intestinal muscle layers control movements like segmentation and peristalsis. Segmentation involves circular muscle movements that mix the food bolus, while peristalsis uses the longitudinal muscles to propel the bolus forward⁵. Regulation of these muscle layers is orchestrated by the myenteric and submucosal plexuses, which communicate with the vagus nerve, and contribute to the "gut-brain axis".

Early motility patterns in embryonic mice³⁴, zebrafish³⁵ and chicken³⁶ have been identified as myogenic, functioning independently of neural inhibitors and persisting in the absence of the enteric nervous system or interstitial cells of Cajal. These initial movements are driven by calcium waves that propagate through circular smooth muscle networks and exhibit calcium wave-like properties³⁷. The transition from myogenic to neurally controlled motility is a major developmental milestone. In mice, for example, the first movements controlled by neurons, appear in the duodenum around embryonic day 18.5³⁴ while zebrafish motility start depending on neuronal activity around 5-7 days post fertilization³⁵. These movements are sensitive to tetrodotoxin (TTX), a neurotoxin that blocks neural activity, indicating their dependence on neural input.

In summary, the orchestrated interplay of smooth muscle cells, enteric neurons and regulatory mechanisms harmoniously controls gastrointestinal motility, ensuring the precise coordination of movements essential for digestion and nutrient absorption.

1.5 The gut immune system

The immune system, which is a complex network of cells, tissues, and organs, is best known for its role in defending the body against invading pathogens and disease. It is divided into two primary categories: the innate immune system and the adaptive immune system. Nevertheless, most immune cells are derived from hematopoietic stem cells located in the bone marrow. The innate immune system acts as the first line of defense, quickly recognizing common molecular patterns in invading pathogens and responding against them. On the other hand, the adaptive immune system mounts a specific and strong response by recognizing antigens expressed in pathogens, leading to immunological memory for future encounters.

The immune system carefully balances tolerance and immunological responsiveness. The innate immune system is responsible for initiating adaptive immune responses. Innate immune cells such as dendritic cells (DCs), macrophages or neutrophils have a continuous surveillance mechanism, detecting microbial antigens through various pattern recognition receptors (PRRs)³⁸. TLRs are one type of PRR that recognize conserved molecular motifs on microorganisms, such as lipopolysaccharide (LPS) from gram-negative bacteria *via* TLR4, or flagellin *via* TLR5. Additionally, NOD

receptors 1 and 2 in the lamina propria recognize peptidoglycan, a major component of bacterial cell walls. The physiological activation of these receptors is crucial for maintaining colonic homeostasis^{39,40}.

The adaptive immune response is mainly mediated by T helper cells, which are specialized in recognizing and responding to different types of microorganisms and pathogens⁴¹. Th1 is the primary producer of IFN γ which is important for fighting intracellular pathogens such as viruses and bacteria, Th2 has been associated with IL-4, IL-13, IL-10 and transforming growth factor (TGF) β , and Th17 with IL-17 and IL-22⁴². Th17 cells are typically induced by a healthy gut microbiota to maintain the gut integrity⁴³. In the context of strong inflammation and leaky gut, both Th17 and Th1 are likely recruited. On the other hand, type 2 polarization mostly occurs in response to parasites infection⁴⁴. To prevent harmful immune reactions, regulatory T cells (Treg) play a crucial role in suppressing immune responses⁴⁵.

The intestinal immune system is the body's largest collection of immune cells, providing defense against environmental threats that may harm the epithelial layer. The intestine hosts various immune cell types, such as macrophages, dendritic cells, T cells, B cells, natural killer (NK) cells, and recently discovered innate lymphoid cells (ILCs). The latter are organized in structures like Peyer's patches, isolated lymphoid follicles, and cryptopatches, as well as scattered throughout the epithelium and lamina propria. ILCs have recently emerged as significant producers of cytokines that regulate mucosal homeostasis, and they play critical roles in early immune responses⁴⁶.

Unlike T and B cells, which require recombination machinery to express high-affinity antigen receptors and expand to fight pathogens, ILCs rapidly respond to invading pathogens by secreting cytokines without the need for expansion⁴⁶. They are particularly numerous at mucosal surfaces, where they continuously encounter commensal microbes as well as potential pathogens⁴⁶.

The gut microbiota is critically involved in the shaping of immune development during early postnatal life. Indeed, in absence of microbiota, lymphoid tissues remain immature and the number of lymphocytes in the gut is dramatically reduced⁴⁷. Even more striking is the need of microbial presence to induce the development of isolated lymphoid follicles³⁹, which constitute B cell reservoirs crucial for IgA production⁴⁸. Interestingly, the effects of gut microbes on immune maturation depend upon the

nature of the bacterial communities. For instance, segmented filamentous bacteria, which have been shown to predominantly reside within the mucus layer in close proximity to the epithelium, are potent activators of gut Th cells including Th17 cells and likely play a unique role in the postnatal maturation of gut immune functions^{6,49,50}.

Overall, the immune system interactions with the gut microbiota and the intestinal immune system are intricate and essential for maintaining health and defending against infections.

1.6 The gut microbiota : “friends or foe” ?

The microbiota was during a long time thought of as a collection of harmful bacteria causing diseases. However, recent research has unveiled a different perspective, revealing that the microbiota also holds a crucial role in maintaining overall health.

One of the first mentions of the “germ theory of disease” dates back to 36 BC, when Marcus Terentius Varro, a Roman scholar, wrote that “certain minute creatures, which cannot be seen by the eyes, which float in the air and enter the body through the mouth and nose, and there cause serious diseases”⁵¹. This theory was later validated by pioneering scientists Robert Koch and Louis Pasteur in the late 19th century, and it led to the development of hygiene and vaccination⁵².

However, in the early 20th century, Ilya Metchnikoff proposed that bacteria are not always harmful. He suggested that lactic acid-producing bacteria, such as those found in yogurt, can actually prolong life by inhibiting the growth of “bad” bacteria. This led to the development of probiotics, which are live microorganisms that are intended to provide health benefits when consumed.

Today, we know that the microbiota is essential for a healthy gut. It helps to digest food, absorb nutrients, and fight off infection. The composition of the microbiota varies from person to person, but it is generally stable over time. However, certain factors, such as diet, antibiotics, and stress, can disrupt the microbiota and lead to health problems.

Despite considerable progress, there is still much to explore and comprehend about the microbiota. By deepening our understanding of its functions, we can develop innovative ways to enhance our health and prevent diseases in the future. The study of the microbiota continues to hold immense promise in shaping the future of medicine and healthcare.

1.6.1 Composition of the intestinal microbiota

The human gut is home to an astonishing 10^{13} bacteria, which is comparable to the number of cells in our body⁵³, and close to 1000 distinct species without taking into account archaea, fungi, and viruses. In this vast microbial world, the human gut stands as a remarkable example of the intricate relationship between our body and the diverse microbial communities that inhabit it.

The most represented bacterial phyla in the human gut are Firmicutes and Bacteroidetes. Firmicutes are a diverse group of bacteria that includes *Lactobacillus*, *Clostridium*, and *Enterococcus*⁵⁴. Bacteroidetes are also a diverse group of bacteria that includes *Bacteroides* and *Prevotella*, for example. Other phyla present in the gut microbiota include Actinobacteria (*Bifidobacteria*), Proteobacteria (such as *Escherichia coli*), Fusobacteria, Verrucomicrobia, and Cyanobacteria⁵⁴. The composition of the microbiota varies depending on its specific location along the GI tract⁵⁵ but also differs according to gender^{56,57,58}. For example, women tend to have a higher abundance of *Bifidobacteria* in their gut microbiota than men⁵⁹.

In mammals, the gut microbiota is acquired during birth and its initial composition relies on the delivery mode⁶⁰. The early colonization plays a crucial role in building a healthy immune system development. Numerous factors contribute to the development of microbiota, such as gestational age, antibiotic usage, breastfeeding, and exposure to family members and pets⁶¹. In the first weeks, the gut microbiota exhibits low diversity and stability. However, at three years, the microbiota composition resembles that of an adult-like profile⁶².

Although it has been traditionally believed that the intrauterine environment and fetus are sterile until birth, there is emerging evidence of bacterial presence in the intrauterine environment⁶³. This suggests that these bacteria might influence the child microbiota even before birth. In addition, recent studies have shown that metabolites from the microbiota of the mother can reach fetal tissues and influence their developmental status⁶⁴. Thus, the microbiota constantly interacts with the host to modulate numerous physiological processes from early life through adulthood.

1.6.2 Host-microbiota interactions

Traditionally, fitness has been defined as the ability of an organism to survive and reproduce in a particular environment. However, selection can also operate at the group level, where the fitness of the group is more important than the fitness of the individual.

One example of selection at the group level is the evolution of the gut microbiota. These bacteria provide a number of benefits to the host, such as improved digestion, protection from infection, and regulation of the immune system. This can lead to the evolution of genes that promote the growth of beneficial bacteria in the gut, even if these genes reduce the fitness of the individual. In other words, the host and its microbiota are often referred to as a holobiont⁶⁵, where the fitness of the group is more important than the fitness of the individual. As a result, the co-evolution process culminates in the most favorable alliance between the host and its microbiota, as shown below.

In the gut, microbiota helps to digest food and absorb nutrients. It also produces short-chain fatty acids (SCFAs) such as butyrate, acetate, propionate, which are important for energy metabolism and gut health^{66,67}. Additionally, butyrate have also been implicated in the development and function of Tregs. SCFAs activate G-protein coupled receptors expressed by the IECs and regulate Tregs by increasing the acetylation of the Foxp3 locus^{68,69}.

Studies have shown that germ-free mice have a lower intestinal surface area⁷⁰, thinner villi⁷¹, increased cell cycle time⁷², and impaired peristalsis⁷³. This is because the gut microbiota helps to maintain the structure and function of the gastrointestinal tract. For example, *Bacteroides thetaiotaomicron* is reported to induce expression of the small proline-rich protein 2A (sprr2A), which is required for maintenance of desmosomes at the epithelial villus⁷⁴. Another mechanism that maintains the tight junctions is by TLR2 mediated signaling that is stimulated by the microbial cell wall peptidoglycan⁷⁵. In addition, the microbiota has also been shown to contribute to the development of the intestinal microvasculature by inducing the transcription factor angiogenin-3⁷⁶. This is further supported by a significant reduction of villus capillary network in germ-free mice, which in turn may affect the digestion and absorption of

nutrients. Interestingly, the microbiota has also been shown to be involved in intestinal epithelial cell differentiation⁷⁷.

The gut microbiota also trains the immune system and protects against infection. Germ-free mice have underdeveloped gut-associated lymphoid tissues, such as Peyer's patches and cryptopatches^{78,79}. They also have reduced antibody production and diversity⁸⁰, as well as lower levels of lamina propria CD4+ T cells such as Th1 and Th17 and regulatory T cells⁴⁹. The gut microbiota also induces maturation of the systemic immune system. In germ-free mice, as well as in antibiotic-treated mice, antigen-presenting cells are unable to produce type I interferons, which are required for NK cell priming and consequent protection against systemic viral infections⁸¹. Altogether, these findings highlight that the gut microbiota is essential for the normal development and function of the immune system.

Another study described *Bacteroides thetaiotaomicron* to increase the efficiency of lipid hydrolysis by upregulating the expression of a colipase that is required by pancreatic lipase for lipid digestion⁸². This suggests that the microbiota may play a role in lipid metabolism and may help to protect against obesity and diabetes. Indeed, it has been reported that the microbiota can protect from diabetes type I in non-obese diabetic mice model⁸³.

The gut microbiota has developed several strategies to control the growth of pathogenic strains, and one of these mechanisms is the stimulation of local immunoglobulins. In particular, Gram-negative bacteria such as *Bacteroides* can activate intestinal DCs, which in turn induce plasma cells in the intestinal mucosa to produce secretory IgA (sIgA). This sIgA then coats the gut microbiota, preventing it from adhering to the intestinal wall and causing infections⁸⁴.

The composition of the gut microbiota is influenced by a number of factors, including diet, genetics and the environment. A healthy gut microbiota is characterized by a diverse range of bacteria. However, disturbances in the gut microbiota, such as those caused by antibiotic use or poor diet, can lead to a range of health problems.

1.6.3 Microbiota dysbiosis

The “hygiene hypothesis”, initially proposed by Strachan in 1989 suggests that decreased exposure to infectious agents and changes in the gut microbiota during infancy could lead to altered immune regulatory networks, contributing to the rise of allergic diseases in developed countries⁸⁵. Studies have revealed differences in the faecal microbiota composition of infants who develop allergic diseases compared to those who do not. For instance, a reduced frequency of *Lactobacillus* and *Bifidobacterium spp.* has been associated with the onset of allergies⁸⁶. Prophylactic approaches using probiotics have been explored, with some studies indicating a reduced incidence of eczema and food-specific IgE in high-risk children with prenatal and postnatal pre- and probiotic supplementation⁸⁷.

The role of the gut microbiota is not limited to allergies but extends to inflammatory bowel diseases (IBD). Dysregulated immune responses to the gut microbiota and subsequent inflammation are believed to contribute to IBD, with various studies showing dysbiosis in patients with these conditions⁸⁸. Different scenarios have been proposed, including the role of pro-inflammatory bacteria⁸⁹, reduced frequency of beneficial bacteria like *Faecalibacterium prausnitzii*⁹⁰, and global changes in microbiota composition. Indeed, a reduced bacterial diversity with less Firmicutes and/or Bacteroides and an overgrowth of Proteobacteria have been associated with intestinal inflammation^{90–92}.

The intestinal microbiota potential involvement in systemic autoimmunity has garnered attention. Changes in gut flora have been observed in the early phases of rheumatoid arthritis⁹³, but direct links between dysbiosis and disease development remain elusive. Studies using germ-free mice have provided some insights, with varying results. In some models, the microbiota triggered autoimmunity^{94,95}, whereas in others, it had a protective role^{83,96}. For example, the microbiota promoted disease in certain arthritis models by inducing uncontrolled Th17 cell responses⁹⁴, while it demonstrated protective effects in collagen-induced arthritis and diabetes development in MYD88-deficient non-obese diabetic mice^{83,96}. The microbiota role in these scenarios is likely linked to its influence on Th17 cell responses and TLR signaling pathways.

The gut microbiome plays a multifaceted and dynamic role in regulating immunity and disease progression. To fully comprehend its mechanisms and potential therapeutic applications, more extensive studies are necessary. *Enteroendocrine cells (EECs) are a crucial component of this intricate gut ecosystem, alongside the microbiome and will be described in the next section.*

II. Enteroendocrine cells

The intestinal epithelium, microbiota, and gut immune system cooperate to maintain gut homeostasis. Enteroendocrine cells have an important function by detecting microbiota compounds and releasing hormones that regulate various processes, such as digestion, absorption, and immunity.

2.1 Role of enteroendocrine cells in the regulation of the digestive system

EECs are a diverse group of cells in the gastrointestinal tract that play crucial roles in hormone secretion and mediating communication between the gut and the enteric nervous system. They were initially thought to originate from the neural crest⁹⁷ but it is now accepted that they arise from intestinal stem cells and the term "neuroendocrine cells" has been dropped.

EECs are traditionally classified based on the principal hormone they produce (Figure 4). Some EECs, like serotonin (5-Hydroxytryptamine, or 5-HT) producing enterochromaffin cells (EC cells), are distributed along the entire length of the gut. Others, such as those in the stomach, produce specific hormones like histamine, somatostatin (SST), gastrin, and ghrelin⁹⁸⁻¹⁰⁰. In the duodenum, EECs predominantly produce glucose-dependent insulinotropic polypeptide (GIP), cholecystokinin (CCK), and secretin. As we move distally in the gut, the types of EECs change, with N-cells producing neurotensin (NTS), and L-cells producing glucagon-like peptides 1 and 2 (GLP-1 and GLP-2), peptide YY (PYY), and insulin-like peptide 5 (INSL5)^{99,101}. Hormone expression is tightly regulated by cues received by EECs, which indeed express several sensory receptors on their surface on the luminal side of the intestine, including taste, amino acid, and free fatty acid receptors⁹⁸.

| Subtype | Location | Peptides/Hormones expressed | Function |
|-----------------------------------|----------------------------|----------------------------------|--|
| D cell | Stomach and duodenum | Somatostatin (SST) | Inhibit gastrin release |
| Enterochromaffin cells | All along the GI tract | Serotonin (5-HT) | Gut motility, visceral hypersensitivity, nausea, lipolysis, immunity |
| Enterochromaffin cells-like cells | Stomach | Histamine | Increase gastric acid |
| G cell | Stomach | Gastrin | Stimulate acid secretion |
| I cell | Upper small intestine | Cholecystokinin (CCK) | Promote digestion, promote nutrient absorption and satiety, delay gastric emptying and motility |
| K cell | Duodenum and upper jejunum | Gastric inhibitory peptide (GIP) | Stimulate insulin secretion, promote lipid uptake and storage |
| L cell | Jejunum, ileum and colon | Peptide YY (PYY) | Digestion, nutrient absorption, food intake, increase satiety and maintain fluid homeostasis |
| | | Glucagon-like peptide-1 (GLP-1) | Digestion, nutrient absorption, food intake, bile acids metabolism, gut motility, insulin and glucagon secretion |
| | | Glucagon-like peptide-1 (GLP-2) | Maintain homeostasis of intestinal mucosa upon injury, and growth of the small intestine |
| | | Oxyntomodulin (OXM) | Body weight homeostasis |
| | | Insulin-like peptide 5 (INSL5) | Promote food intake and glucose production |
| M cell | Duodenum and jejunum | Motilin | Increase motility and appetite |
| N cell | Jejunum, ileum and colon | Neurotensin (NTS) | Reduce motility, and regulate insulin secretion |
| S cell | Duodenum and jejunum | Secretin (SCT) | Reduce motility, gastric acid and appetite |
| X/A cell | Stomach and duodenum | Ghrelin | Increase appetite, fat storage, inhibit insulin and increase motility as well as gastric acid |

Figure 4. Summary of mammalian enteroendocrine cells subtypes in the gut
Adapted from Xingting Guo et al, 2021

Many EECs are "open type" cells with microvilli-covered surfaces that directly contact the luminal content. However, in the gastric corpus, a significant proportion of EECs, including EC cells, are "closed type" cells that do not sense the gut lumen^{100,102} (Figure 5). Instead, they interact with neighboring cells, enabling paracrine regulation of hormone secretion. Recent 3D reconstructions of EECs producing PYY and CCK revealed a basolaterally located process called a neuropod, extending towards the enteric nervous system and other glial cells enabling uni- or bidirectional communication between them¹⁰³.

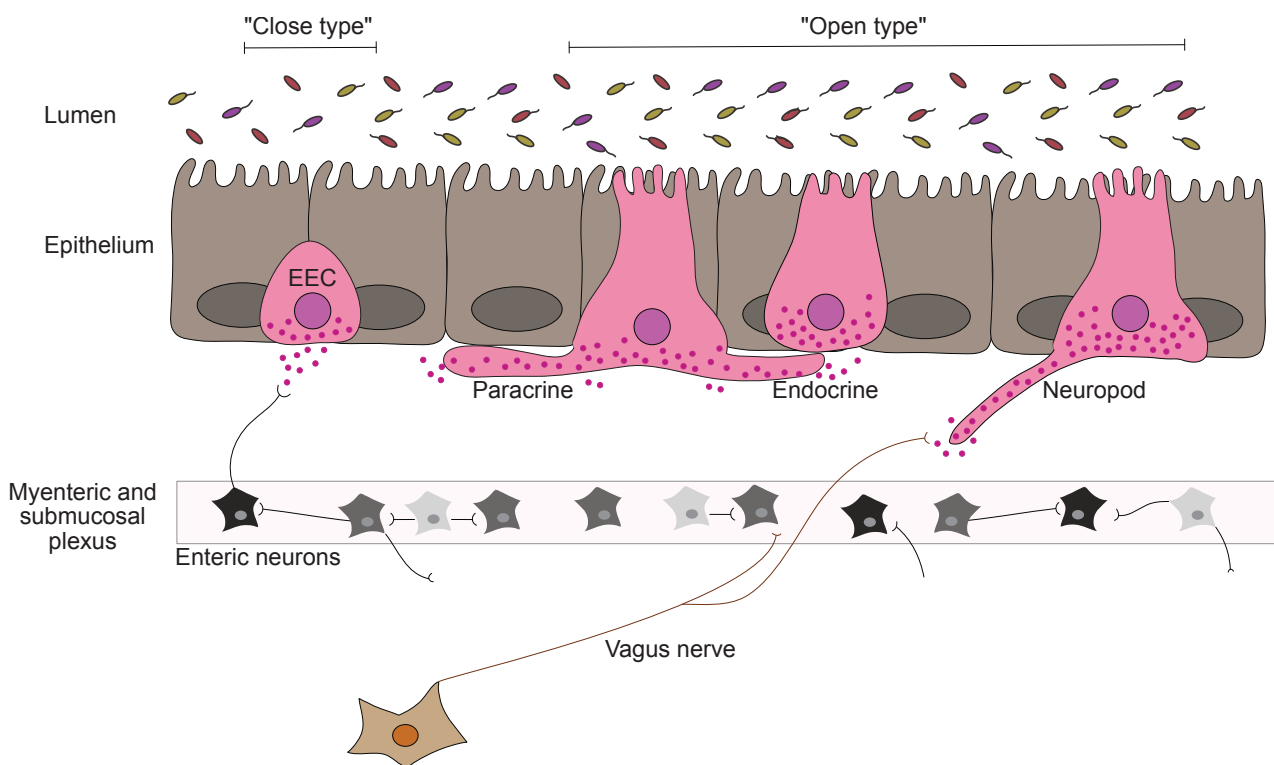


Figure 5. The different enteroendocrine cell types and their mode of action

EECs can be classified into two types based on their morphology: open type and closed type. Open type EECs have microvilli at their apical surface and are located in the villi of the gut epithelium. Closed type EECs are also located in the villi, but they do not have microvilli. Open type EECs sense the contents of the gut lumen and secrete hormones into the circulatory system (endocrine signaling) or to adjacent cells (paracrine signaling). Some EECs also have a basal cytoplasmic process called a neuropod, which can form synaptic connections with vagus nerves (direct neural signaling). *Adapted from Xingting Guo et al, 2021.*

The microbiota and its metabolites can have a significant impact on various pathways of EECs, influencing their cell numbers, gene expression, and hormone production. Germ-free mice studies have showed that these mice exhibit elevated numbers and altered transcriptomic profiles of colonic L cells, along with increased plasma GLP-1 levels^{104,105}. Bacterial colonization in these mice rapidly reduces L cell numbers through pathways dependent on nutrient availability. For instance, colonization with SCFA-producing bacteria or a high-fat diet can suppress EECs¹⁰⁵, or increase the number of "closed" cells¹⁰⁶. On the other hand, *in vitro* mouse models have shown that SCFAs increase GLP-1-positive cell numbers¹⁰⁷.

EEC-secreted peptides and hormones play many important roles in the gut, including^{25,108,109}:

- Coordinating the gut response to ingested nutrients.
- Inducing gastrointestinal, pancreatic, and biliary secretions.
- Modulating GI motility to facilitate digestion and absorption.
- Playing roles in tissue growth, repair, and increasing the intestinal barrier function through the activation of local and neuronal pathways, including the brain-gut axis.
- Triggering a protective response to potentially harmful substances in the gut lumen.
- Regulating food intake.

Several hormones play essential roles in regulating food intake through various signaling pathways. Some hormones act directly on the hypothalamus, while others influence food intake through afferent vagal nerves. Ghrelin is a key hormone promoting food intake, and recent research has highlighted that INSL5 exerts similar effects¹⁰¹. On the contrary, CCK has been demonstrated to decrease food intake, and the administration of analogs or resistant forms has led to reduced food intake in both mice and humans^{110,111}. Additionally, CCK also plays a role in regulating PYY activity on food intake by increasing the expression of its receptor on vagal afferent neurons¹¹². Throughout the gastrointestinal tract, food undergoes a complex process, with the need to eliminate toxins and non-digestible elements. After initial digestion in the proximal part, nutrients must be absorbed in the small intestine, necessitating a decrease in transit time. Gastric emptying is a crucial step that allows further processing of nutrients in the duodenum. Ghrelin, serotonin, and motilin promote gastric emptying, while postprandial hormones like CCK, GLP-1, and PYY slow down this process⁹⁸.

In addition to regulation of food intake, EECs can coordinate a protective response upon potentially harmful substances in the gut lumen. This response aims to avoid or reject the threat by delaying gastric emptying, increasing intestinal secretion, and inducing vomiting for example. The activation of vagal afferents and neurons is involved in these protective response^{113–115}. This can be illustrated by the rotavirus-

encoded enterotoxin, a major cause of gastroenteritis in children, which likely induces vomiting through its action on EC cells, leading to the release of 5-HT. Through vagal activation, this serotonin release activates vomiting¹¹⁶. These interactions between EECs, luminal content, and the nervous system exemplify the intricate bidirectional communication between the gut and the brain. This communication plays a pivotal role in regulating gut function and coordinating responses to various environmental stimuli.

Compounds targeting EECs functions hold promising therapeutic potential. For instance, a GLP-1 receptor agonist is currently used to treat type 2 diabetes, as it effectively stimulates insulin secretion from pancreatic β -cells¹¹⁷. Furthermore, in patients who have undergone Roux-en-Y gastric bypass surgery, GLP-1 and PYY3-36 secretion have beneficial effects in reducing food intake. This could offer additional therapeutic possibilities to treat obesity¹¹⁸.

2.2 Serotonin and its functions in gut physiology

The term "enteramine" was first used by the Italian pharmacologist Vittorio Erspamer in 1937 to refer to 5-hydroxytryptamine (5-HT), a substance that he extracted from the rabbit gastric mucosa¹¹⁹. In 1948, Page and Rapport isolated a compound from bovine serum that had vasoconstrictor properties. They named this compound serotonin, after the Greek word for "seizing"¹²⁰. The structure of serotonin was later identified as 5-HT by Rapport's group. Vittorio Erspamer, who had previously discovered 5-HT in the gut, demonstrated that enteramine, the bioactive amine he had named, was in fact 5-HT.

There are several reasons why the term "enteramine" might be preferable to "serotonin" when referring to 5-HT. Indeed, Erspamer was the first to discover 5-HT, and he named it after its location in the gut. However, the name "serotonin" became more widely used, likely because it was made available to researchers by Upjohn Pharmaceuticals under that name¹²¹.

Erspamer hypothesized that because 5-HT was highly produced in the gastrointestinal tract of vertebrates from fish to frogs to primates, it must play an important role in gut function. Interestingly, his hypothesis turned out to be correct. We now know that 5-HT is predominantly produced in the gut, not in the brain. In fact, about 95% of 5-HT is found in the gut, mainly in enterochromaffin cells. Only about 5%

of 5-HT is found in the brain which has led to a renewed interest in the role of 5-HT in gut function.

EC cells synthesize, store, and release serotonin in the intestinal mucosa. EC cells are located throughout the gut, but they are most abundant in the stomach and small intestine. The synthesis of 5-HT in EC cells involves two steps (Figure 6). First, the amino acid tryptophan is converted to 5-hydroxy-l-tryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH). There are two isoforms of the rate-limiting TPH enzymes: TPH1 is mainly expressed in EC while TPH2 is present in CNS and enteric neurons. Then, 5-HTP is decarboxylated to 5-HT by the enzyme l-amino acid decarboxylase. After being released from EC cells, 5-HT goes into the bloodstream, surrounding tissues, and the gut lumen. Once released, 5-HT is taken up by adjacent epithelial cells through the serotonin reuptake transporter (SERT) and subsequently metabolized to 5-hydroxyindoleacetic acid (5-HIAA). Additionally, SERT facilitates the uptake of 5-HT by platelets in the circulation, further regulating its levels and distribution throughout the body.

The release of 5-HT from EC cells is regulated by a variety of stimuli, including mechanical and chemical cues. For example, the presence of food in the gut can trigger the release of 5-HT, which in turn can stimulate peristalsis and secretion.

5-HT released from EC cells acts on a variety of receptors in the gut, including 5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄, and 5-HT₇ receptors. These receptors are located on smooth muscle cells, enteric neurons, enterocytes, and immune cells (Figure 6). The activation of these receptors can have a variety of effects on gut function, including peristalsis, secretion, pain perception, and nausea.

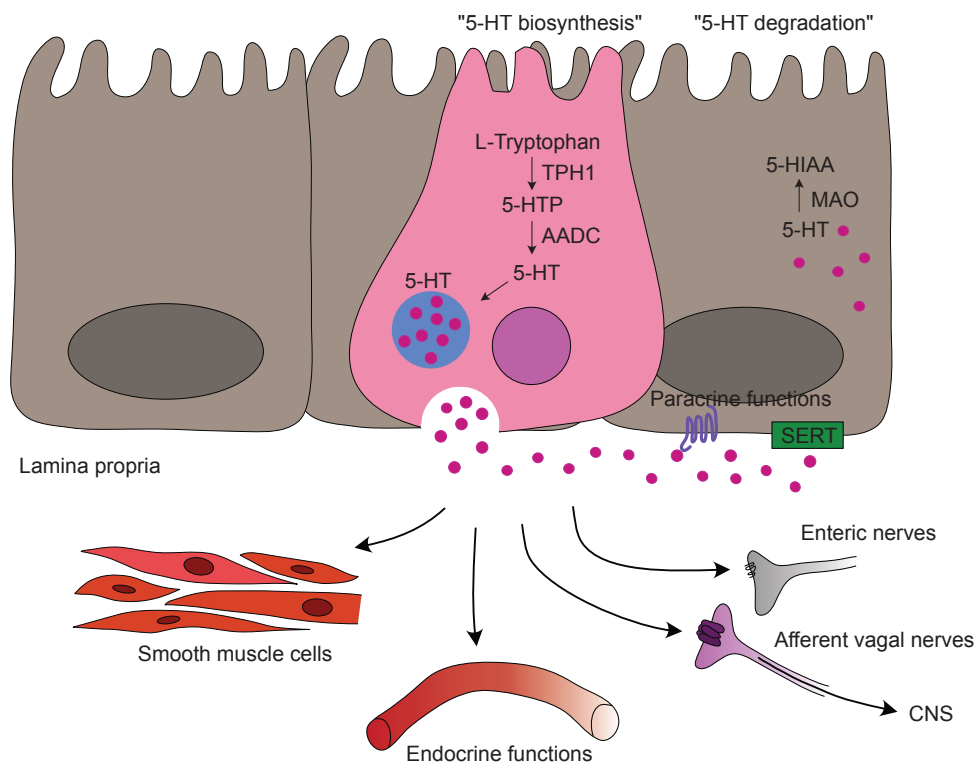


Figure 6. 5-HT synthesis, degradation and target cells

Serotonin is synthesized from L-tryptophan in two steps: Tryptophan hydroxylase (TrpH) hydroxylates L-tryptophan to form 5-hydroxytryptophan (5-HTP). Aromatic amino acid decarboxylase (AAAD) decarboxylates 5-HTP to produce serotonin. Serotonin can also be degraded by the MAO enzyme in IECs. It can act on nearby cells (paracrine signaling), enteric nerves, and afferent vagal nerves, which communicate directly with the central nervous system. Serotonin can also act throughout the body by traveling through blood vessels (systemic signaling).

2.2.1 5-HT in gut motility

The concept that 5-HT initiates peristalsis was proposed by Edith Bülbring and colleagues in the late 1950s. Their experiments showed that 5-HT is released in response to mucosal pressure and can restore peristalsis when halted pharmacologically^{122,123}. Several studies have demonstrated that mucosal stimulation results in 5-HT release, activating the ascending contractile and descending relaxant limbs of the peristaltic reflex^{124–126}. These observations have been strongly supported by both *ex vivo* and *in vivo* studies showing a slower gut motility when 5-HTR3 and/or 5-HTR4 were antagonized^{126–129}. Interestingly, by using a *Tph1*-deficient mice, Heredia et al, have shown that mucosal 5-HT plays a role in the generation of peristaltic reflexes¹³⁰.

5-HT exerts its effects through various pathways, stimulating both intrinsic and extrinsic nervous systems, and inducing physiological and pathophysiological responses, including GI contractions. Additionally, 5-HT is involved in smooth muscle contraction and relaxation, with roles in vagal pathways and mucosal sensory transduction. Transient receptor potential cation channel (TRPA1) causes an influx of calcium ions into enterochromaffin cells, which triggers the release of 5-HT in response to tryptophan metabolites, promoting intestinal motility¹³¹ through activation of cholinergic enteric nerves (Figure 7). Interestingly, 5-HT release upon TRPA1 activation can also bind to 5-HTR3 receptors on smooth muscle cells, which causes them to contract¹³² and control gut motility (Figure 7).

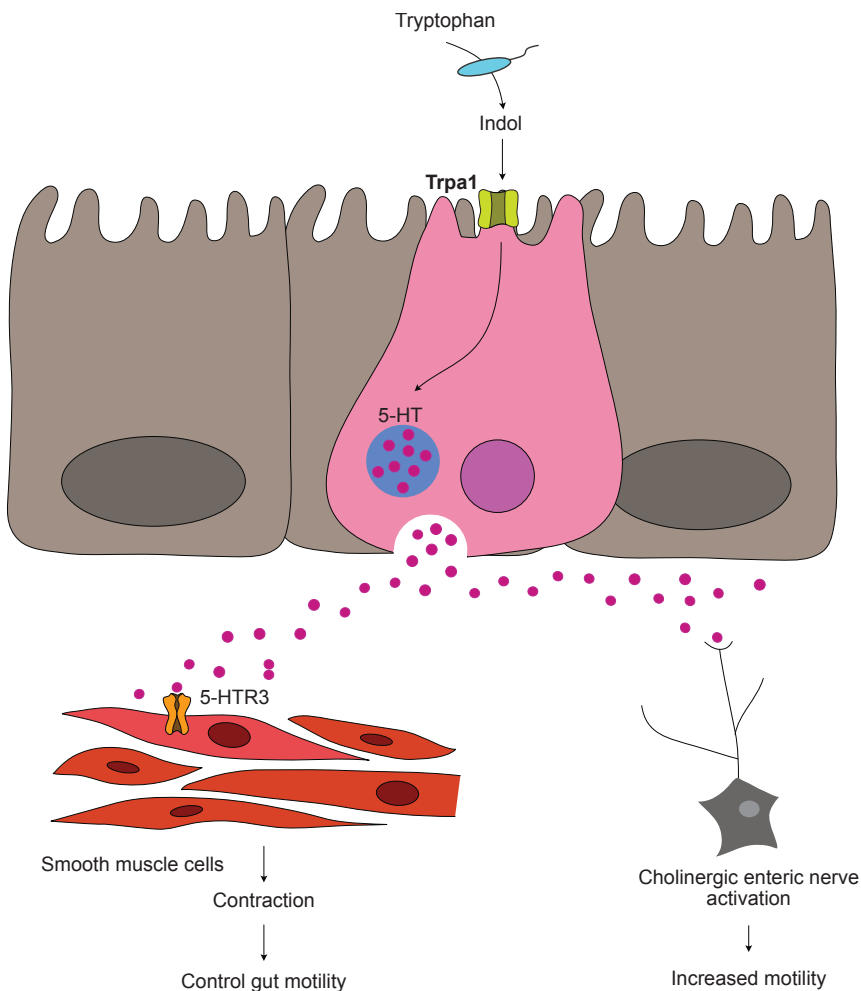


Figure 7. 5-HT induced upon Trpa1 activation controls gut motility by targeting different cell types

Bacterial tryptophan metabolites induce 5-HT secretion through Trpa1 activation on EECs. This activates enteric nerves and increases gut motility. Another mechanism is through 5-HTR3 activation by 5-HT expressed on smooth muscle cells, which induces their contraction and regulates gut motility. Adapted from Ye et al, 2020 and Nozawa, K., et al 2009.

2.2.2 5-HT in immunity

5-HTRs are found on the majority of immune cells in both humans and rodents. During acute inflammation, platelets play a crucial role in ensuring serotonin release since they store 5-HT synthesized by EC cells in the gut¹³³. This leads to the recruitment of innate immune cells, such as immature DCs, monocytes, mast cells, neutrophils and eosinophils to the site of inflammation^{134–136}. In addition to innate immune cells, serotonin can also target lymphocytes¹³⁷ and overall influence cytokines production. Indeed, Dürk et al, have shown that serotonin played a role in modulating the release of specific cytokines in LPS-stimulated human blood monocytes¹³⁸. They observed that serotonin influenced the secretion of IL-1 β , IL-6, IL-8/CXCL8, IL-12p40, and TNF- α . The upregulation of IL-1 β , IL-6, and IL-8/CXCL8 secretion was found to be mediated through the 5-HTR3 receptor. On the other hand, activation of the 5-HTR4 and 5-HTR7 receptors resulted in an increase in LPS-induced release of IL-1 β , IL-6, IL-8, and IL-12p40. Interestingly, the activation of the same receptors showed an inhibitory effect on LPS-induced TNF- α release. Thus, serotonin exerts its modulatory effects on cytokine release through different 5-HT receptor subtypes depending on the specific cytokine involved. Another example illustrating the role of serotonin in cytokine production comes from the work of Idzko et al. In their study, they demonstrated that the activation of 5-HTR3, 5-HTR4, and 5-HTR7 on dendritic cells led to the production of IL-1 β and IL-8.

5-HT has also been shown to regulate the innate immune response of colon epithelial cells by inducing transient reactive oxygen species (ROS) production through NADPH oxidase 2. This leads to increased inflammatory cytokines and adhesion molecules, reduced E-cadherin, and disrupted epithelial barrier¹⁴⁰.

Additionally, 5-HT suppressed interactions between monocytes and NK cells¹⁴¹ leading to dysregulated functions of these cells characterized by an increased cytotoxicity and IFN- γ production^{142,143}. Interestingly, 5-HT has also been shown to affect directly NK cells by promoting their proliferation and by protecting them against oxidative damage¹³³.

Regarding its effect on the adaptive immune system, 5-HT is involved in macrophages ability to activate T cells¹³⁶. It also enhances pro-inflammatory T cell responses through 5-HTR7 on DCs¹⁴⁴. This effect is achieved through 5-HTR7, which

promotes the proliferation and activation of T cells¹⁴⁵. In addition to T cells, 5-HT has been described to influence the proliferation and early phase activation of B cells¹³⁷.

These findings indicate that besides its physiological functions, 5-HT also plays a crucial and modulatory role in immune responses during gut inflammation. These insights highlight the multifaceted nature of serotonin and its significant impact on the immune system regulation.

2.2.3 5-HT and intestinal disorders

Crohn's disease and ulcerative colitis are the principal types of IBD. Both conditions share common features, such as intestinal dysbiosis, compromised barrier function, and a similar set of genetic risk factors¹⁴⁶. However, they can differ in terms of the location and histological appearance of intestinal inflammation, as well as specific risk factors and comorbidities. The prevalence of these cases is estimated at 250 per 100,000 individuals in industrialized countries, and their incidence is steadily increasing in emerging countries, highlighting the role of the environment in their initiation¹⁴⁷.

IBD develops when the mucosal immune response becomes dysregulated in genetically susceptible individuals exposed to commensal bacteria and/or other environmental triggers. This inflammatory process weakens and damages the epithelial barrier, leading to further microbial translocation, which in turn amplifies the immune response. Current treatment options for IBD are mainly focused on anti-inflammatory approaches with limited efficacy, and a significant portion of active IBD patients do not respond adequately to these drugs. Therefore, there is a need for therapeutic agents that can promote mucosal healing and enhance the function of the intestinal barrier, which may ultimately improve the effectiveness of IBD treatment¹⁴⁷.

A change in 5-HT producing cells number in the gut has been associated with gut inflammation¹⁴⁸. Interestingly, several studies have shown a change in 5-HT content in animal model having colitis and in IBD patients^{149,150}, in addition to an increase in EC cell number in Crohn disease and ulcerative colitis patients^{151,152}. Interestingly, in a study that used *Tph1*^{-/-} mice to simulate a reduction of 5-HT in the gut, they observed a decreased severity of colitis in the mutant mice following induction of colitis using dextran sodium sulfate (DSS) and 2,4-dinitro-benzenesulfonic acid

(DNBS)¹⁵³. This supports the notion that reduced 5-HT levels in the gut have a protective effect against colitis development in these experimental models. These findings are corroborated by other studies showing that deleting the serotonin reuptake transporter leads to an increased severity in intestinal inflammation models^{154,155}. DCs have been identified as a key player in the inflammatory response induced by 5-HT¹⁴⁴. Interestingly, the 5-HTR7 is expressed on DCs, and when this receptor is blocked, experimentally induced colitis is alleviated¹⁴⁵. This suggests that serotonin acts on dendritic cells through 5-HTR7 to promote inflammation.

Conversely, it has been recently demonstrated that 5-HT could also have anti-inflammatory properties in the intestine. Indeed, administrating 5-HTR4 receptor antagonist worsen the colitis severity in DSS-treated mice. In addition, the histological damage in the colon of 5-HTR4-deficient mice is more severe than in wild-type. This 5-HTR4 protective effect was associated with epithelial proliferation, improved wound healing, resistance to oxidative stress-induced apoptosis and increased motility¹⁵⁶. Thus, these observations suggest that serotonin may target several different cell types during gut inflammation and that its function varies depending on the targeted receptor. This suggestion is further reinforced by the discovery that the absence of 5-HTR4 results in the development of inflammatory scores in the tissue compared to wild-type littermate. Based on these findings, it is implied that a 5-HT4 agonist with limited luminal activity could potentially offer an effective treatment approach for IBD.

Taken together, these studies show the complexity of 5-HT, demonstrating its dual pro- and anti-inflammatory roles, and highlight that the role of 5-HT in inflammation is not yet fully understood. However, it is clear that it is an important mediator of inflammation and may be a target for the development of new treatments for inflammatory diseases.

Angiogenesis, the process of forming new blood vessels, is a natural occurrence during development and wound healing. However, in certain diseases like IBD, it can become problematic. Indeed, it can increase the permeability of the gut lining, allowing harmful substances and bacteria to pass from the gut into the bloodstream. In addition, uncontrolled angiogenesis can contribute to the growth of tumors within the gut.

5-HT signaling is known to significantly impact angiogenesis¹⁵⁷. Indeed, in a thrombotic tumor environment, platelet aggregation leads to the significant release of 5-HT stored within platelets. This release has been proposed to promote the sprouting

of new blood vessels from pre-existing vessels by influencing matrix metalloproteinases expression in tumor-infiltrating macrophages, thereby affecting the production of circulating angiostatin, an endogenous angiogenesis inhibitor¹⁵⁸. 5-HT has been identified as an activator of Src/PI3K/AKT/mTOR/p70S6K phosphorylation signaling, similarly to VEGF (Vascular Endothelial Growth Factor). This suggests that 5-HT-induced angiogenesis might be a contributing factor to the resistance observed in VEGF-targeting anti-angiogenic therapy for cancer treatment¹⁵⁷.

As previously mentioned, IBD is associated with elevated levels of 5-HT in the gut, however it is also characterized by the formation of new blood vessels which have been shown to exacerbates the inflammatory response^{159,160} suggesting that 5-HT-dependent angiogenesis might have a crucial role in exacerbating the gut inflammatory response. However, further characterization is needed to fully understand the role of 5-HT in angiogenesis in the context of IBD.

5-HT has also been involved in irritable bowel syndrome (IBS). IBS is characterized by chronic abdominal pain, discomfort, bloating, and changes in bowel habits, such as diarrhea, constipation, or alternating between the two. IBS is considered a functional gastrointestinal disorder, meaning that it is not associated with any structural abnormalities but rather results from dysregulation in the gut-brain axis and abnormal intestinal motility. The exact cause of IBS is unknown, but it is thought to be due to a combination of factors, including genetics, the gut microbiome, and stress. IBS is not life threatening, but it can be very disruptive to daily life.

Interestingly, increased 5-HT level have been observed in diarrhea predominant IBS whereas decreased level of 5-HT are found in constipation-predominant IBS. Several studies in both humans and animal models have reported the association of IBS symptoms with the number of EC cells, the level of 5-HT in the gut, *Tph1* mRNA expression levels, as well as the expression of SERT in mucosal biopsies^{161,162}. Interestingly, Malinen et al, analyzed the microbiota composition of IBS patients and found that IBS severity was associated with the presence of *Ruminococcus torques*-like¹⁶³. We could hypothesize that the microbiota may also impact the function of 5-HT, influencing its levels and signaling within the intestinal mucosa, and subsequently contributing to intestinal diseases. So far, the etiology of IBS is still unclear, however these studies suggests a role of 5-HT in the pathophysiology of this syndrome.

Chapter 2 : Interleukin-22

III. Interleukin-22 : cellular sources and regulation

Cytokines are soluble messengers that mediate communication between cells. Among these small secreted proteins, interleukin-22 (IL-22) has been shown to have critical functions in the gut. IL-22, which was initially named IL-10-related T cell-derived inducible factor¹⁶⁴, was discovered in 2000 when researchers conducted genome analysis and identified it thanks to its predicted structural homology to IL-10^{165,166}. IL-22 is one of the best studied members of the IL-10-related cytokine family, which also includes IL-19, IL-20, IL-24, and IL-26. *The extensive body of data acquired from studying IL-22 will be summarized and discussed below.*

3.1 IL-22 gene and protein

The human *IL22* gene divided in 5 exons is located on chromosome 12q15 in close proximity to the *IL26* and *IFN-γ* genes. The *IL22* gene encodes a protein of 179 amino acids, sharing 79% homology with the corresponding gene in mice¹⁶⁶. Following the removal of the predicted 33-amino-acid signal peptide, this cytokine is released as a protein with a length of 146 amino acids^{165,167}. The structure of IL-22 (expressed in *Escherichia coli* and *Drosophila melanogaster*) has been analyzed through crystallization and X-ray diffraction^{167,168}. IL-22 exhibits a bundle-like structure comprising six α -helices labeled from A to F, along with a small N-terminal helix and connecting loops^{167,169,170}. Although the biologically active form of IL-22 appears to be a monomer, dimers and tetramers have also been observed^{167,171,172}.

3.2 IL-22 Receptors

3.2.1 IL-22 membrane receptor and IL-22-induced signaling pathways

The IL-22 receptor is a transmembrane complex composed of two heterodimeric subunits, IL-22R1 and IL-10R2^{165,173,174}. This complex is able to activate many different signaling cascades. The gene encoding for IL-22R1 is present on chromosome 1p36.11, whereas the gene encoding for IL-10R2 is located on chromosome 21q22.11¹⁷⁵. A high binding affinity has been reported between IL-22 and

IL-22R1 (KD=20nM), however a very low affinity has been shown for IL-10R2. Interestingly, when IL-22 binds IL22R1, a conformational change of IL-22 takes place which causes an increase in the affinity of IL-22 to bind IL-10R2. This allows the complex IL-22/IL22RA1 to bind IL-10R2, thereby enabling downstream signaling^{176,177}.

Binding of IL-22 to its receptor complex leads to the phosphorylation of Janus kinases (JAKs) and Tyrosine Kinases (TYK), specifically JAK1 and Tyk2 (Figure 8). It triggers the phosphorylation and activation of the transcription factor Signal Transducer and Activator of Transcription 3 (STAT-3) which will then translocate into the nucleus and induce the transcription of several genes. Although STAT-3 appears to be the main mediator of IL-22 signaling, phosphorylation of STAT-1 and STAT-5 has also been reported¹⁷⁸. In addition, other signaling pathways have been shown to be activated by IL-22 such as Akt and several MAPKs^{179–181}.

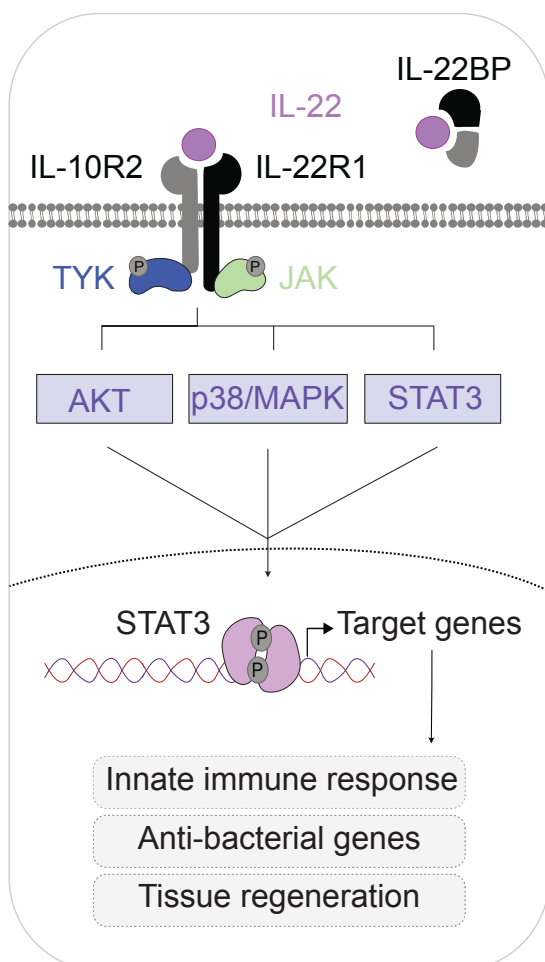


Figure 8. IL-22 signaling

The IL-22 receptor consists of two chains: IL-10R2 and IL-22R1. When IL-22 binds to its receptor, JAK1 (Janus kinase 1) and TYK2 (tyrosine kinase 2) are phosphorylated. These phosphorylated enzymes then further phosphorylate STAT3 (signal transducer and activator of transcription), leading to their dimerisation and subsequent translocation to the nucleus, where they induce the expression of several genes involved in innate immune responses and tissue regeneration. In addition to JAK/STAT signalling, IL-22 also triggers the PI3K/AKT (phosphoinositide 3-kinases/protein kinase B) and MAPK (mitogen-activated protein kinase) pathways through the involvement of JAK and TYK2 molecules. IL-22 also has an inhibitor called IL-22 binding protein (IL-22BP). IL-22BP binds to IL-22 much more strongly than the IL-22 receptor (IL-22R). In the presence of IL-22BP, most IL-22 will bind to IL-22BP instead of the IL-22R. This prevents IL-22 from binding to the IL-22R and causing changes in epithelial cells. Adapted from Arshad et al, 2020.

Unlike most cytokines targeting hematopoietic cells, IL-22 exerts its main effects on non-hematopoietic epithelial cells and fibroblasts in a wide range of tissues. Indeed, IL-10R2, is widely expressed throughout the human body. However, IL-22R1 expression is restricted to the lung, liver, kidney, thymus, pancreas, gastrointestinal tract, skin, and synovium^{178,182,183}. This IL-22R1 expression pattern indicates that IL-22 serves as a crucial communication channel between the immune system and specialized cell types such as epithelial cells in these tissues.

3.2.2 IL-22 soluble receptor (IL-22BP)

IL-22 binding protein (IL-22BP), the soluble form of the IL-22 receptor 1 (IL-22R1) receptor, is a crucial regulator of IL-22 signaling. The gene encoding for IL-22BP, *IL22RA2* in humans and *Il22ra2* in mice, is located at chromosome 6 in humans and 10 in mice^{184–187}. IL-22BP exhibits a sequence homology of 34% with the extracellular domain of IL-22 receptor^{184,187}. IL-22BP inhibit the binding of IL-22 to IL-22R1 by occupying the same binding site¹⁸⁸. Interestingly, IL-22 exhibits a significantly higher affinity for IL-22BP, with a binding affinity that is over 1000 times greater than its affinity for the IL-22 transmembrane specific receptor chain (KD=1pM)^{189,190}. Unlike IL-22, IL-22BP is expressed constitutively and mostly in dendritic cells and macrophages^{191,192}. IL-22BP exhibits high levels of expression in lymph nodes and the spleen and shows a substantial expression in the gastrointestinal tract in comparison with other tissues such as the thymus or the liver¹⁹¹.

3.2 IL-22 cellular sources in the intestine

3.2.1 Adaptive sources

Initially, the production of IL-22 in humans was attributed to T CD4 lymphocytes or T helper (Th) cells. More specifically, Th cells, which develop in the presence of IL-12 and whose differentiation is regulated by the transcription factor T-bet, were the first cell type identified to produce IL-22¹⁸³. Th1 cells have been estimated to account for up to 35% of all IL-22-producing CD4+ T cells in human peripheral blood¹⁹³. While murine Th1 cells also express IL-22, the expression is comparatively lower. With the discovery of Th17 cells, IL-22 became closely associated with these cells. Indeed, in mice, the primary source of IL-22 production in T cells is attributed to Th17 cells, which

also produce IL-17 and express the transcription factor ROR γ t, crucial for their development^{194–196}. Among the cytokines that promote the development of Th17 cells, IL-6, IL-1 β and IL-23 drive IL-22 production, whereas transforming growth factor (TGF- β) inhibits it. In humans, a distinct group of cells known as Th22 cells has been discovered, which exclusively produce IL-22 without IL-17¹⁹⁷. Interestingly, these cells do not express ROR γ t or T-bet, but they have similar dependencies on IL-6 in addition to TNF- α for IL-22 production. Additionally, vitamin D further enhances the production of IL-22 in these cells. Other reports have also shown that IL-22 can be secreted by natural killer T cells, gdT cells, and CD8⁺ T cells upon activation, particularly in the presence of IL-23¹⁸³ (Figure 9).

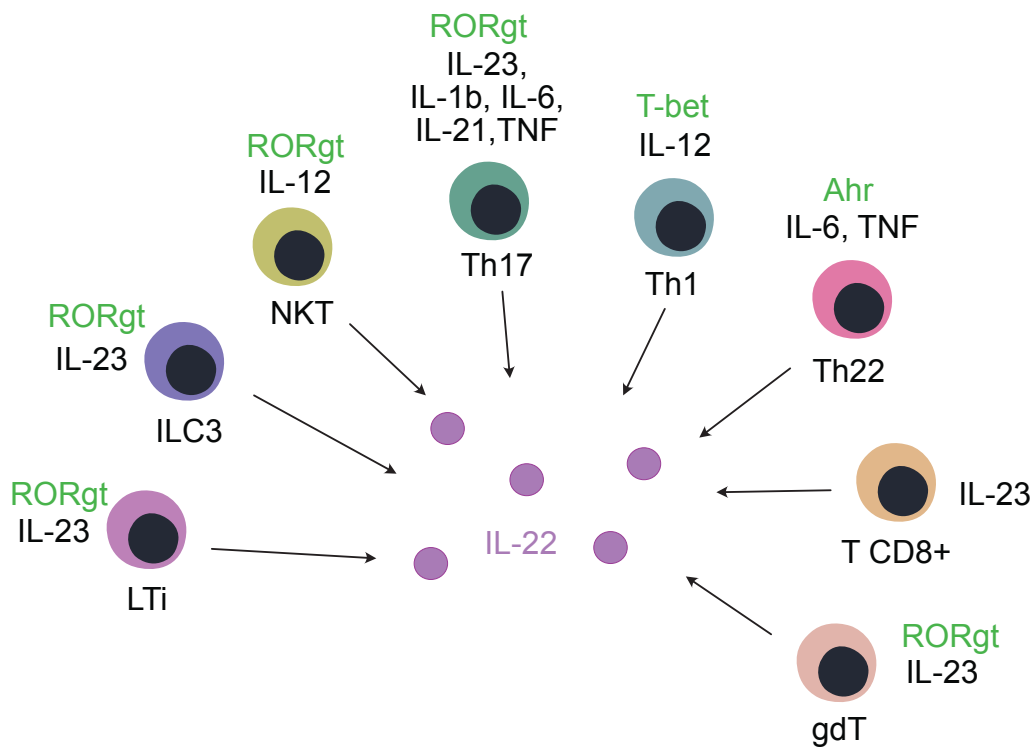


Figure 9. IL-22 innate and adaptive cell sources

IL-22 is produced by many immune cells, including T helper cells (Th1, Th17, Th22, and CD8⁺ Th cells), natural killer T cells (NKT cells), and innate lymphoid cells (ILCs) as well as LTi cells. The cytokines that induce IL-22 expression are shown in black in the figure, and the transcription factors required for the development of these cell types are written in green. *Adapted from Arshad et al, 2020.*

3.2.2 Innate sources

The significance of a non-T-cell origin for IL-22 was initially evidenced in a *Citrobacter rodentium* infection model. In this model, both *Rag2*-deficient mice (lacking functional T cells) and wild-type mice exhibited comparable IL-22 production and maintained normal host defense during the early stage of the infection¹⁹⁸. Interestingly, IL-22 was detected by immunostaining on CD11c⁺ cells which is expressed in dendritic cells but can also be upregulated ILCs¹⁹⁹.

The identification of ILCs has greatly transformed our comprehension of innate immunity and the interactions of immune cells with non-hematopoietic cells at epithelial barrier sites. ILCs play a crucial role in regulating immune homeostasis, responding to injuries or infections, and facilitating tissue repair. They possess a lymphoid ontogeny but lack the rearranged T or B cell receptor of adaptive T cells. They have no cytotoxicity and they appear to function mainly by producing cytokines. ILCs mirror the subsets of T cells in terms of transcription factors and cytokine expression. Indeed, NK cells resemble Th1 cells as they express IFN- γ , ILC2 produce the Th2 cytokines IL-13 and IL-5, and ILC3 are dependent of RORCyt for their development and produce IL-17 and IL-22. Furthermore, ILCs are regulated by the transcription factors AhR (Aryl hydrocarbon receptor), Notch, Runx3, and the presence of IL-7 in the environment²⁰⁰. ILC3 cells are found in the lamina propria of the intestine, as well as in the skin, spleen, and lungs under certain inflammatory conditions²⁰¹. ILC3 are activated and release cytokines in response of IL-1B and IL-23 (Figure 9).

Type 3 ILCs can be divided into two subgroups: lymphoid tissue-inducer (LTi) cells and ILC3 cells. LTi cells were the first type 3 ILCs identified in the late 1990s²⁰². These cells play a crucial role in the organogenesis of secondary lymphoid structures during fetal development, such as Peyer's patches and lymph nodes, as evidenced by their absence in mice deficient in RORyt or Id2^{203,204}. In adults, another group of cells were named LTi-like cells. Although they are phenotypically similar to embryonic LTi cells, they seem to have other functions. For example, they have been shown to be involved in the restoration of lymphoid tissues, such as spleen restoration after viral infection²⁰⁵. Besides the well-studied role for LTi cells during embryonic lymph node formation, their role in the adult still require further investigations. Interestingly, it is worth noting that LTi cells represent the major source of IL-22 production during the

fetal period in humans²⁰⁶ and in mice²⁰⁷ (Figure 9). The role of this high level of IL-22 during fetal development is not yet fully understood. It has been suggested that it may reflect the involvement of LTi cells in a pro-inflammatory program initiated by the expression of ROR γ t, which is necessary for their function during the genesis of secondary lymphoid organs²⁰⁸. After birth, LTi cells are no longer involved in organogenesis but continue to produce IL-22 and IL-17A, thereby exerting their role in tissue remodeling and protection at barrier sites²⁰⁹.

ILC3 cells are phenotypically distinguished from LTi cells by the expression of the NKp46 cytotoxicity receptor, hence their designation as NCR+ (natural cytotoxicity receptor) ILC3 cells¹⁹⁹. These cells exclusively produce IL-22 (Figure 9). More recently, another population has been identified in both humans and mice, characterized by the absence of NKp46 expression and their low frequency in homeostasis. These cells are referred to as NCR- ILC3 cells and produce IL-17A and IFN- γ in addition to IL-22. They significantly increase in numbers during inflammatory episodes like for example in patients with Crohn's disease²¹⁰.

Based on the similarities between ILC3 and T cells expressing IL-22, we could wonder whether they may have redundant roles. Interestingly, it has been recently shown that in *C. rodentium* infection models in mice, ILC3 predominantly produce IL-22 during the initial wave of bacterial colonization and spread, while Th22 cells appear to be indispensable to increase and sustain STAT-3 activation in order to prevent bacterial invasion of intestinal crypt²¹¹. This strongly suggests a non-redundant role of the IL-22-producing cells in intestinal anti-bacterial defense.

In humans, monocytes, dendritic cells, and non-hematopoietic tissue cells do not produce IL-22. However, it has been reported that *in vitro*-cultured alveolar macrophages, when stimulated with IL-23 and peptidoglycan (a bacterial cell wall component), can produce IL-22²¹². Furthermore, mast cells have been identified as IL-22 producers in patients with psoriasis²¹³. Finally, neutrophils have been reported to express IL-22 in cases of acute colitis²¹⁴.

3.4 Regulation of IL-22 expression

IL-22 production by ILC3 in the intestine is highly dependent on signals present in their environment²¹⁵. These signals, primarily derived from bacteria, dietary factors, and cell-to-cell contact, directly and indirectly regulate the production of IL-22.

3.4.1 Direct regulation by bacteria

Gut microbiota plays a critical role in IL-22 production in the intestine, as demonstrated by the observation that germ-free mice exhibit impaired IL-22 production²¹⁶. Interestingly, microbial products can directly act on ILC3 cells. Indeed, it has been shown that NCR+ ILC3 cells and human LTI cells express high levels of innate immunity receptors TLR1/2 and TLR2/6²¹⁷. These TLRs can respond to products present typically in the cell wall of Gram-positive bacteria and induces IL-22 production in these cells. However, mouse ILC3 cells do not express these innate immunity receptors.

In a recent study, they found that colonization of antibiotic-treated neonatal mice with *Clostridia* bacteria leads to the induction of IL-22 production by ILCs and CD4+ T cells²¹⁸. Interestingly, *Clostridia* bacteria produce SCFAs²¹⁹, which are the major metabolic products of gut microbiota derived from dietary fiber. SCFAs, such as butyrate promote IL-22 production in CD4+ T cells and ILCs by inhibiting histone deacetylase (HDAC) and activating the GPR41 receptor, but not GPR43 and GPR109a receptors. Administration of butyrate to mice resulted in reduced gut inflammation during enteric infection due to the increased production of IL-22²²⁰.

Surprisingly, it has also been reported that butyrate produced by commensal bacteria in the ileum contribute to reducing the numbers of NCR+ ILC3 cells and their IL-22 production in Peyer's patches. Indeed, inhibiting the butyrate receptor GPR109a, which is highly expressed in ILC3 cells, restored the production of IL-17A and IL-22 by ILC3, which were diminished in the presence of butyrate alone²²¹. These findings suggest that butyrate, and more broadly SCFAs, may regulate the expression of IL-22 in a location-specific manner, in addition to the receptors involved in its detection.

Finally, ILC3 cells can respond to other fundamental innate immune factors, such as the complement system. A subset of ILC3 cells appears to be sensitive to the

complement cascade through the expression of C3aR (Complement component 3a receptor 1)²²². Then, it has been reported that the complement factor P, an activator of the alternative complement pathway, directly binds to NKp46, a receptor expressed on human and mouse ILC3²²³. Altogether, these data suggest that NCR⁺ ILC3 cells can detect pathogen infections through interactions with complement system proteins.

3.4.2 Indirect regulation by bacteria

Mice treatment with flagellin – the main protein composing bacterial flagella – which is known to be a TLR5 ligand activates the splenic and mucosal production of IL-22 in a CD103⁺ dendritic cell-dependent manner²²⁴. Once activated, DCs produce IL-1 β , IL-6, and IL-23, which are necessary to enhance IL-22 production by IL-23R-expressing ILC3 cells²²⁵. In the intestine, it has been clearly established that IL-23 produced by CX3CR1⁺ mononuclear phagocytic cells (MNPs) is essential for inducing IL-22 production in ILC3 cells^{226,227}. However, studies on ILC3 cells from IL-23-deficient mice have shown that IL-23 is not required for their constitutive IL-22 production²⁰⁷. It goes in line with IL-23 being a potent inducer of IL-22 production in ILC3 cells under inflammatory conditions in the intestine and in other tissues such as the skin²²⁸.

It is interesting to note that ILC3 cells constitutively produce high levels of IL-22 in the mouse intestine before weaning, and this production gradually decreases after bacterial colonization of the intestine in adulthood²⁰⁷. After weaning, IL-25 produced by epithelial cells in response to gut-colonizing bacteria acts on the transcription factor ROR γ t to suppress IL-22 production. This effect is likely indirect, as ILC3 cells do not express the IL-25 receptor called IL-17BR. However, CD11c⁺ dendritic cells in the intestine strongly express IL-17BR, and when cultured with ILC3 cells in the presence of IL-25, it significantly decreases IL-22 production²⁰⁷. This mechanism seems necessary to establish a balance between the gut flora and the mucosal immune system. After weaning, CX3CR1⁺ mononuclear phagocytes are known to produce IL-23 in response to microbial colonization²²⁹, this suggests that IL-23 could be meant to counteract IL-22 inhibition in situations where the actions of IL-22 are required.

In addition to dendritic cells, IL-22 expression has also been described to be regulated by glial cells. Indeed, Ibiza et al, showed that enteric ILC3 cells express RET,

a receptor known to recognize neurotrophic factors²³⁰. Its ablation reduced IL-22 production, leading to dysbiosis and increased susceptibility to intestinal infection and inflammation. More specifically, they showed that glial cells sense microenvironmental cues in a MYD88-dependent manner to control neurotrophic factors and thus innate IL-22 production²³⁰.

3.4.3 Regulation by diet / Aryl hydrocarbon receptor (Ahr)

AhR is necessary for the postnatal maintenance and expansion of ILC3 cells and plays a critical role in the production of IL-22. AhR is located in the cytoplasm, bound to the protein Hsp90, until the binding of a specific ligand induces a conformational change that leads to the exchange of Hsp90 with the nuclear translocation component ARNT (Aryl hydrocarbon Receptor Nuclear Translocator)²³¹. Potential AhR ligands could be derived synthetically from exogenous sources such as halogenated and polycyclic aromatic hydrocarbons, or from dietary sources such as tryptophan degradation derivatives and flavonoids²³². In addition to these ligands, cyclic AMP and Ca^{2+} can activate AhR²³³. It has been reported that microbial-derived AhR ligands may not be required for the development of ILC3 cells, although they may be important for IL-22 transcription²³⁴. In their study, Zelante et al. demonstrated that the stimulation of AhR by a tryptophan metabolite, Indole-3-Aldehyde (I3A), produced by *Lactobacillus*, increased IL-22 production by NCR+ ILC3 cells, thus promoting resistance to *Candida albicans* infection²³⁵. Indeed, AhR acts synergistically with ROR γ t to activate *Il22* gene expression²³⁴. Furthermore, tryptophan metabolites are transmitted during breastfeeding, and administration of I3A to pregnant mice was sufficient to increase the number of NCR+ ILC3 cells in the offspring²³⁶, suggesting that the maternal microbiota can promote the development and function of ILC3 cells. Finally, an important role of vitamin A in the induction of IL-22 production by ILC3 cells has been identified. Retinoic acid, derived from the degradation of vitamin A by gut bacteria, can activate Retinoic Acid Receptors (RARs), which can directly interact with the *Il22* locus²³⁷. Moreover, it has been shown that a vitamin A-deficient diet decreases the number of NCR+/- ILC3 cells and LT α i cells, as well as their production of IL-22 and IL-17A²³⁸.

IV. IL-22 Functions

4.1 IL-22 protective role against pathogenic bacteria

The high bacterial load in contact with the epithelium requires enhanced defenses and justifies the existence of a well-developed and constantly active mucosal immune system. IL-22 plays a central role in anti-bacterial responses as several studies have shown that this cytokine induces anti-microbial peptide production by intestinal epithelial cells during pathogenic infections. Indeed, IL-22 has been identified as a crucial factor in the initial defense mechanisms against *Citrobacter rodentium*. During infection, IL-23 is necessary to trigger the production of IL-22 which will activate the production of anti-bacterial proteins from the Reg family by intestinal epithelial cells¹⁹⁸. This has also been supported by the study of Behsen et al, in which they demonstrated that IL-22-deficient mice show a defect in Reg3 β and Reg3 γ production in the colon²³⁹. Reg3 proteins have been shown to exhibit bactericidal activity against Gram-negative bacteria by interacting with peptidoglycan and are thus essential for maintaining mucosal protection and preventing bacterial translocation^{240,241}. Interestingly, IL-22-mediated anti-microbial peptide induction has been also found to be protective against *Salmonella typhimurium*, *Helicobacter pylori* and *Klebsiella pneumoniae* infections^{242,243,244}. Moreover, IL-22 provides protection via the STAT-3 signaling pathway by promoting the production of other anti-microbial peptides such as BD-2, BD-3, S100A7-9 and lipocalin-2^{178,195,245}.

In addition to the anti-microbial proteins, IL-22 has been shown to stimulate the expression of several mucins in a STAT-3-dependent manner (such as mucins 1, 3, 10 and 13), to prevent the physical penetration of bacteria and colon epithelial cells²⁴⁶. Thus, IL-22 plays a vital role in the host's ability to fight bacterial infections.

However, when IL-22 attempts to protect the body against pathogenic microorganisms, some can exploit these mechanisms to facilitate their colonization of the intestine, as is the case with *Salmonella*. Indeed, *Salmonella* takes advantage of IL-22 induction to enhance its colonization in the inflamed intestine by suppressing commensal *Enterobacteriaceae*, which are susceptible to anti-microbial proteins²³⁹. These findings highlight the complexity of IL-22 effects on pathogen colonization resistance, which are influenced by bacterial stimuli and microbiota composition.

4.2 IL-22 role in controlling microbiota composition

IL-22 roles during infections have been well studied, however, its ability to induce anti-microbial peptide expression also plays a crucial role in shaping microbiota composition. Indeed, *Il22^{-/-}* mice showed dysbiosis due to an alteration of Reg3 β and Reg3 γ expression and were more susceptible to colitis²⁴⁷. Supporting these data, it has been demonstrated that injection of an anti-IL-22 blocking antibody in germ-free mice colonized with a human microbiota changes the composition of the gut microbiota²⁴⁸. Thus, IL-22 seems to shape both mice and human microbiota.

Interestingly, a recent study revealed the critical role of IL-22 signaling in the maturation of Paneth cells, which are known for their key anti-microbial functions. Gaudino et al. observed that Paneth cell-specific *Il22ra1^{-/-}* mice displayed reduced expression of key Paneth cell markers such as Mmp7, Lyz1, and some α -defensins which was accompanied by a decline in the production of anti-microbial peptides, leading to dysbiosis²⁴⁹. This data suggest that in addition of controlling directly anti-microbial production by enterocytes, IL-22 signaling also plays a critical role in the development of highly specialized epithelial cells types secreting anti-microbial peptides. It emphasizes the broad importance of IL-22 signaling in mucosal anti-bacterial defense mechanisms.

Additionally, IL-22 has also been shown to modulate microbiota composition indirectly by promoting the growth of commensals. Indeed, Zenewick et al, showed that *Il22^{-/-}* mice have low *Lactobacillus* and *Bacteroides* abundance and a high pathogenic bacteria abundance including *Helicobacter* species²⁴⁷. Interestingly, the dysbiotic flora of IL-22-deficient mice is transmissible to co-housed wild-type mice and can promote their susceptibility to developing colitis. Several studies have unraveled different mechanisms by which IL-22 could modulate microbiota composition in addition to anti-microbial peptide regulation. Indeed, in the humanized microbiota mouse model mentioned above, Nagao-Kitamoto et al, showed that IL-22 can modulate the glycosylation of host N-linked glycans which will then promote the growth of bacteria consuming succinate such as *Phascolarctobacterium spp*, to compete and inhibit the growth of *Clostridium difficile*²⁴⁸. Another example has been provided by the work of Pham et al, in which they showed that IL-22 induces the production of the fucosyltransferase Fut2. Fut2 promotes intestinal fucosylation which is crucial for

shaping microbiota by favoring beneficial commensals and preventing the colonization by opportunistic bacteria such as *Enterococcus faecalis*²⁵⁰.

IL-22, in addition to maintaining a healthy microbiota composition, avoids overactivation of the gut immune system through ILC function. Indeed, Sonnenberg et al, showed that in absence of ILCs, *Alcaligenes* species were disseminating and inducing systemic inflammation in mice, which was prevented by administration of IL-22²⁵¹. Overall, these studies highlight the importance of the IL-22/ILCs axis in ensuring a healthy microbiota composition and thus protect the intestine against the colonization and dissemination of pathogenic bacteria which could lead to inflammatory diseases.

4.3 IL-22 protective role in viral infections

The type III interferons (IFNs), also called lambda (λ) interferons, not only share a receptor chain with IL-22 but also exert their actions on the same cells. Specifically, the expression of IFNLR1, is predominantly limited to epithelial cells, making these cells susceptible to IFN stimulation²⁵². Given the strong association between IL-22 and these anti-viral proteins, it has been hypothesized that IL-22 may also play a protective role against viral infections. Indeed, some studies found IL-22 induction in mice infected with Cytomegalovirus (MCMV)²⁵³ or in patients with hepatitis B²⁵⁴ supporting the idea that IL-22 would play a role during viral infections.

Although, Wolk et al, showed that IL-22 does not induce direct anti-viral responses in the epithelium as IFN λ or IL-29²⁵⁵ do, several studies suggested a beneficial role of IL-22 in limiting tissue damage due to viral infections. For example, it is known that HIV infection disrupt the gut barrier, impairing epithelial integrity. Th22 cells are depleted during the infection resulting in severely reduced IL-22 production in the gut. However, when exogenous IL-22 was introduced, it counteracted HIV-induced epithelial damage indicating the crucial role of IL-22 in HIV-mediated mucosal immunopathogenesis²⁵⁶. A protective role of IL-22 was also reported in hepatic hepatitis B virus infection in mice and humans²⁵⁴.

Interestingly, Hernandez et al, have shown that in addition of its tissue protective functions, IL-22 produced by ILC3 can amplify IFN- λ signaling to control rotavirus infection in mice²⁵⁷. Surprisingly, IL-22 did not exert its function through STAT-3, but instead acted in synergy with IFN- λ to induce an efficient antiviral state in intestinal epithelial cells through STAT-1 and activation of interferon stimulated genes (ISGs).

Collectively, such data identify IL-22 as an important factor limiting mucosal viral infection and epithelial damage.

4.4 IL-22 roles in intestinal epithelial regeneration

IL-22 has been suggested to play a crucial role in the healing and regenerative processes of the epithelial barrier during inflammatory episodes. It has the ability to trigger the expression of anti-apoptotic genes and promote epithelial cell proliferation and differentiation^{198,245}. More precisely, IL-22 can directly target intestinal stem cells as its specific receptor chain is abundantly expressed in this epithelial lineage. Indeed, Zwarycz et al, have shown that increasing IL-22 resulted in more Ki67+ intestinal epithelial cells²⁵⁸. Mechanistically, IL-22 signaling through STAT-3²⁵⁹ induces pro-survival genes expression such as *MCL1*, *Hsp70* or *BCL* to promote epithelial cells survival upon radiation or epithelial damage^{259,260}. Supporting these data, other studies have shown that lacking or inhibiting IL-22 results in a reduced number of Lgr5+ intestinal stem cells and in impaired gut epithelial regeneration during inflammation or injuries^{260,261}.

IL-22 has also been shown to be required to activate the DNA damage response in the intestinal epithelium. Indeed, Gronke et al, have elegantly demonstrated that the specific removal of the IL-22-specific receptor chain in colonic epithelial stem cells resulted in the suppression of apoptosis triggered by the DNA damage response. Consequently, stem cells lacking IL-22 signaling exhibited a higher accumulation of mutations, and increased the likelihood of developing colon cancer²⁶². Interestingly, they showed that phytochemicals known as glucosinolates, found in high quantities within cruciferous vegetables, can produce metabolites that have the potential to damage DNA. However, they also have the ability to activate AhR signaling, which in turn stimulates the production of IL-22²⁶². This intriguing evidence implies that this biological mechanism might have evolved to enable the safe consumption of diets containing DNA-damaging agents. Overall, these studies highlight the crucial role of IL-22 in tissue safeguarding the intestinal epithelium against potential cancerous transformations caused by DNA damage.

4.5 IL-22 role in intestinal physiology

Given the diverse functions of IL-22 in the gut and its close interactions with both intestinal epithelial cells and the microbiota, it raises the question of whether IL-22 may be involved in additional physiological processes within the intestine. Researchers hypothesized that IL-22 could regulate metabolic functions. They first showed that obese mice exhibited impaired IL-22 production and adaptive antibody responses. To assess the potential role of IL-22 in obesity, they used mice lacking the IL-22 receptor and fed them with a high-fat diet (HFD) or used a genetically obese leptin-receptor deficient mice²⁶³. They observed more severe features of the metabolic syndrome, including increased body weight, glucose intolerance, and insulin resistance, compared to wild-type. However, when obese mice were treated with exogenous IL-22-Fc, beneficial effects were observed. Treated mice exhibited reduced body weight and fat mass, along with lower blood glucose levels in both fed and fasting conditions. Furthermore, they showed improved glucose tolerance and reduced insulin resistance compared to untreated controls. The treatment with IL-22-Fc also had other positive effects. It enhanced mucosal immunity and downregulated chronic inflammation²⁶³. Surprisingly, this treatment led to increased levels of peptide YY, a hormone associated with reduced food intake, which consequently resulted in a decrease in food consumption by the treated animals²⁶³. These findings highlight the potential of IL-22-Fc as a therapeutic agent in managing metabolic syndrome-related conditions in obesity. More recently, Mao et al. demonstrated that in addition of shaping microbiota, the continuous production of IL-22 by ILC3 cells, independently of adaptive CD4+ T-cell activity, has also a significant impact on host lipid metabolism. Indeed, this persistent IL-22 production leads to a reduction in lipid transporter expression, resulting in impaired host lipid metabolism²⁶⁴.

In addition, IL-22 through STAT-3, has been shown to also affect the circadian circuitry in intestinal epithelial cells. Indeed, it was shown activation of STAT-3 by IL-22-produced ILC3 cells inhibited the expression of the circadian clock transcriptional suppressor REV-ERB α . Because REV-ERB α represses the circadian transcription factor NFIL3, it led to a dysregulated lipid metabolic program in enterocytes and promoted lipid uptake into intestinal epithelial cells²⁶⁵. Interestingly, it was also shown that ILC3 cells themselves highly express some key circadian clock genes such as

REV-ERBa leading to circadian oscillations in cytokine expression such as IL-17 and IL-22²⁶⁶. They found that REV-ERBa regulates ILC3 development and functions due to its roles in the regulation of ROR γ t²⁶⁶. The regulatory loop between the circadian clock and ILCs could lead to dysregulated secretion of IL-22. It was hypothesized that circadian fluctuations may impair the ability of ILC3 cells to maintain balanced interactions with nutrients and commensal bacteria, potentially impacting gut homeostasis. Further research is needed to find out which intestinal functions are affected by daily fluctuations in ILC3 cells.

To summarize, these findings indicate that signals from the commensal microbiota and the environment play a crucial role in promoting both barrier integrity and lipid metabolism in an ILC3 and IL-22-dependent manner.

4.6 IL-22 role in intestinal immunopathology

4.6.1 *Inflammatory bowel diseases (IBD)*

The discovery of a polymorphism affecting the gene encoding IL-23R²⁶⁷, which is the receptor for IL-23, a cytokine inducing IL-22 expression by ILCs, has led to suspicion of a potential role of IL-22 in the pathophysiology of IBD. Moreover, the abundant production of IL-22 in the intestine, and its protective functions on the intestinal epithelium, has further strengthened this hypothesis. In both types of inflammatory bowel disease, the inflamed intestine exhibits an increased presence of IL-22 producing cells. However, patients with Crohn's disease tend to have higher numbers of IL-22 producing T helper cells compared to those with ulcerative colitis²⁶⁸. In addition, levels of IL-22 are increased in the blood of patients with Crohn's disease and correlate with disease severity¹⁹⁰.

Sugimoto et al, showed for the first time the protective effect of IL-22 produced by Th2 cells in ulcerative colitis-like intestinal inflammation in mice²⁴⁶. In addition, inhibition of IL-22 activity during DSS-induced colitis in mice resulted in increased tissue damage. A similar conclusion was drawn from *IL22*-deficient or *IL22ra1*-deficient mouse models in which DSS-induced colitis or T-cell induced colitis was exacerbated²⁶¹. Another study also showed that treating mice with IL-22 during inflammation limits epithelial permeability, which should limit bacterial translocation²⁶⁹.

These protective functions of IL-22 during inflammation have been linked with : (1) an increase of anti-microbial peptides production which will likely modulate microbiota composition²⁴⁷, (2) an increase of mucus-associated molecules and the restitution of mucus-producing cells, notably goblet cells²⁴⁶, (3) an increased proliferation of intestinal epithelial cells allowing tissue regeneration upon inflammation²⁵⁹. Although IL-22 levels are elevated in patients with IBD, barrier dysfunction and disease continue to persist. One plausible explanation for the insufficient healing effect of IL-22 is the concurrent up-regulation of its antagonist, IL-22BP²⁷⁰, which counteracts the potentially protective impact that IL-22 could exert.

Additionally, the involvement of IL-22 in inflammation seems to be more complex. In fact, Eken et al. demonstrated that neutralizing IL-22 improves colitis induced by the injection of an anti-CD40 antibody²⁷¹, while Reyes et al. observed a similar effect in colitis induced by the administration of DNBS²⁷². These findings suggest a potential pathogenic role for IL-22 in these models of inflammation.

Since no animal model fully replicates all aspects of human IBD, obtaining clinical data will be essential to understand the role of IL-22 in human intestinal disease. In this context, UTTR1147A, an IL-22Fc IgG4 fusion protein was developed to activate IL-22 signaling. The Phase I of the clinical trial demonstrated acceptable safety, pharmacokinetics and IL-22R activation when administered to human volunteers²⁷³. The Phase II is undergoing, they are currently testing patients with ulcerative colitis and Crohn's disease (NCT03558152). This clinical trial holds promise in shedding light on the therapeutic potential of IL-22 in IBD treatment. However, if systemic delivery is not sufficient, it may be necessary to use gut-restricted IL-22 activators.

4.6.2 Cancer

Around 20% of patients with IBD develop colorectal cancer²⁷⁴. The rapid turnover of the intestinal epithelium due to inflammatory episodes exposes colonic stem cells to a notable risk of malignant transformation²⁷⁵. Given the proliferative, survival, and regenerative effects exerted by IL-22 on epithelial cells in the gut, researchers quickly established a link between this cytokine and tumorigenesis. Going in line with this hypothesis, elevated expression of IL-22 has been observed in

colorectal adenocarcinomas. In addition, this hint has been strongly supported by a study showing that a single nucleotide polymorphism in the *IL22* gene locus is associated with a 50% increase in colon cancer incidence²⁷⁶.

It is interesting to note that STAT-3, the major downstream signaling molecule of IL-22, has been well described as an oncogene^{275,277}. Indeed, intestinal epithelial cells lacking specifically *Stat3* developed less tumors than wild-type mice in AOM/DSS model of colorectal cancer²⁷⁸. However, IL-22 itself does not seem to promote cancer formation as mice constantly overproducing IL-22 in the liver did not have an increased of spontaneous tumors formation²⁷⁹. However, *IL22*^{-/-} mice developed a noteworthy increase in tumor formation in the AOM/DSS model of colon cancer compared to controls. This indicates that the expression of IL-22 serves as a protective barrier against tumor development²⁸⁰. Huber et al, have analyzed IL-22 and IL-22BP behavior and role during different phases of cancer development. Interestingly, IL-22BP expression was decreased during active colitis to let IL-22 play its protective function but it increased again during the recovery phase when IL-22 is strongly produced²⁸⁰. IL-22BP role is crucial in cancer formation as *IL22bp*^{-/-} mice had an increased tumor burden confirming that a non-controlled IL-22 production in this model is an important driver of carcinogenesis. More precisely, IL-22 demonstrates a dual nature in facilitating carcinogenesis. IL-22 anti-inflammatory and anti-microbial effects protect against cancer, while its ability to induce epithelial cell proliferation could, in contrast, promote tumor growth²⁸⁰. A decrease in the expression of this cytokine lead to impaired tissue repair, thereby prolonging the inflammatory process and ultimately contributing to carcinogenesis. Conversely, increased IL-22 expression can promote extended tissue regeneration and also stimulate the development of colon cancer.

Apart from colon cancer, IL-22 also influences other intestinal cancers. A SNP in the *IL22* locus has been associated with a significant increase in the risk of developing gastric cancer²⁸¹. Furthermore, elevated levels of IL-22R1 and IL-22 expression were detected in gastric cancer biopsies when compared to healthy control tissue. Several other studies have indicated a higher abundance of IL-22-producing T cells within gastric tumors, and this observation correlates with poor patient survival²⁸². Taken together, these findings suggest that precise regulation and controlled release of IL-22 are essential for efficient wound healing without the risk of progression to cancer.

V. Using the zebrafish to study interleukin-22

5.1 Introduction to the zebrafish model

The zebrafish was introduced in the early 1900s and quickly became popular. Its use in 1970s by the geneticist George Streisinger to investigate the development of the vertebrate nervous system marked the beginning of its widespread adoption as a model organism in scientific research, and its applications have since been continually expanding. Today, the zebrafish serves as an invaluable tool in various fields, contributing significantly to our understanding of biology and human health. Indeed, the zebrafish possess several advantages that make it an excellent vertebrate model. It undergoes external fertilization with a relatively short generation time, exhibits transparency during the embryonic stage, is cost-effective to maintain and allow easy microbiota manipulation. But what really made the zebrafish a household name in the scientific community was the introduction of a user-friendly and ever-expanding genetic toolkit as well as its genome sequencing. The zebrafish genome sequencing project started in 2001 at the "Wellcome Trust Sanger Institute". The first genome assembly named GRCz9 identified 26,000 protein-coding genes. When compared to the human reference sequence, at least 70% of human genes have an ortholog in the zebrafish, making it significant for translating studies to potential applications in humans²⁸³.

The zebrafish genome consists of 25 chromosomes, collectively containing about 1.7 billion base pairs of DNA, approximately half the size of the human genome. Around 320-350 million years ago, a whole-genome duplication event occurred in teleosts, resulting in the generation of homologous genes. Subsequently, evolution led to chromosomal rearrangements and divergence of gene sequences through the accumulation of mutations. The resulting paralogs often acquired different functions, which can be either complementary or novel²⁸⁴.

5.2 Zebrafish development

5.2.1 General aspects

Zebrafish embryonic development starts shortly after fertilization, and it progresses rapidly compared to mice. The zygote stage lasts 3/4 of an hour in zebrafish and one day in mice. During the epiboly stage (4 hpf to 10 hpf), the blastoderm forms, constituting a thin layer of cells that migrate and spread to cover the yolk cells. In mice, the blastula stage (E4.5) forms the blastocyst, consisting of an inner cell mass surrounded by the trophectoderm and a cavity²⁸⁵.

Gastrulation occurs between E6.5 and E7.5 in mice, during which the single-layer blastoderm reorganizes and differentiates into three germ layers: ectoderm, mesoderm, and endoderm²⁸⁶. In zebrafish, this period lasts five hours (5 to 10 hpf) and involves morphogenetic movements, including spreading and thinning of cell layers during epiboly, internalization of mesodermal progenitor cells, and convergent extension along the anterior-posterior axis of the body²⁸⁷.

Following gastrulation, organogenesis takes place, leading to the formation of organs from the three germ layers established during gastrulation. This phase is characterized by crucial cellular interactions and inductive cells guiding the fate of other cell groups to form new tissues. Cell layers from the germ layers enable the development of organs through bending or condensing and generating structures that later rearrange.

Segmentation starts between 10 and 24 hpf²⁸⁸. Organs become visible, body movement starts, and cell differentiation occurs. Embryo length rapidly increases, and somites serve as a development index. Sensory tissues form, and motoneurons play a role in behavioral reflexes. During the second day, pharyngula stage begins²⁸⁵. The head compacts, fins develop, and the circulatory system forms with a beating heart.

Finally, hatching occurs between 48 and 72 hours²⁸⁵. The fish grows, most organs develop, and fins, jaws, and gills form quickly. Most of our experiment are carried after 5 days, when zebrafish larvae are still transparent and organs main developmental steps are over.

5.2.2 Intestinal Development in Zebrafish

The zebrafish digestive tract undergoes a sequential developmental process with specific stages. It starts with the formation of the gut tube, which consists of polarized epithelial cells expressing apical alkaline phosphatase and beta-actin, as well as basolateral cadherin²⁸⁹. This stage occurs during mid-somite stages²⁹⁰ (Figure 10A). Following the gut tube formation, the pharynx and esophagus develop within the endoderm anterior to the gut, leading to the visible rudimentary primary organs of the fish. During this stage, body movement starts, and cells undergo morphological differentiation. Between 34 hpf and 120 hpf, the intestine experiences a dramatic increase in size, and histological sections reveal the appearance of epithelial cells during this period²⁸⁹. The epithelium consistently remains a single layer of cells without stratification (Figure 10C).

At 76 hpf, intestinal folds have developed in the anterior and middle intestinal regions and peristalsis started²⁸⁹. Proliferating cells mainly localize at the base of the folds, suggesting that progenitor cell specification begins at the larval stages²⁸⁹. The mouth opens around 74-98 hpf when microbiota colonization of the gut start taking place²⁹¹. The presence of the gut microbiota has been shown to influence the rate of cell proliferation (Figure 10B). Indeed, larvae raised in the presence of normal commensal bacteria exhibited higher proliferation rates compared to germ-free larvae²⁸⁹.

Differentiated cell types within the intestine, such as enterocytes, goblet cells, and enteroendocrine cells, first appear at 74 hpf, and their distribution within the intestine is already established by this stage. Interestingly, secretory cell determination is also promoted by the microbiota in a Myd88-dependent manner²⁹². At 5 dpf, most of the zebrafish digestive tract is fully functional²⁹⁰, comprising the mouth, pharynx, esophagus, intestinal bulb, intestine, and anal opening. Notably, extensive folding is observed in the anterior intestine, while the posterior regions lack folds at this stage²⁹³. Between 6 to 8 dpf, cell proliferation starts to decrease²⁹⁴. As the larval zebrafish continues to age, folding persists, but the folds become shorter towards the caudal end²⁹⁵. Additionally, the lumen widens at the anterior end and gradually narrows towards the posterior region²⁸⁹ (Figure 10C).

Neural crest-derived enteric neurons and smooth muscle progenitors appear around 32 to 74 hpf (Figure 10D). Enteric neurons migrate into the intestine at 32 hpf and reach the posterior part by 66 hpf²⁹⁶, their differentiation is initiated around 72 hpf^{297,298}. Their number and distribution increase significantly between 96 hpf and 120 hpf, along with the development of complex axonal projections. Smooth muscle differentiation is observed at 74 hpf, with the formation of a discontinuous layer of circularly aligned smooth muscle cells²⁸⁹.

From 20 to 33 dpf, the zebrafish intestine undergoes a metamorphosis characterized by the development of deeper epithelial folds and intestinal looping. As a result, the adult intestine has a more complex architecture than the larval intestine, resembling an S-shape²⁸⁹.

Overall, the zebrafish digestive tract develops through distinct stages involving cell proliferation, epithelial maturation, and the differentiation of smooth muscle and enteric neurons, while the presence of gut microbiota also plays a role in influencing intestinal development.

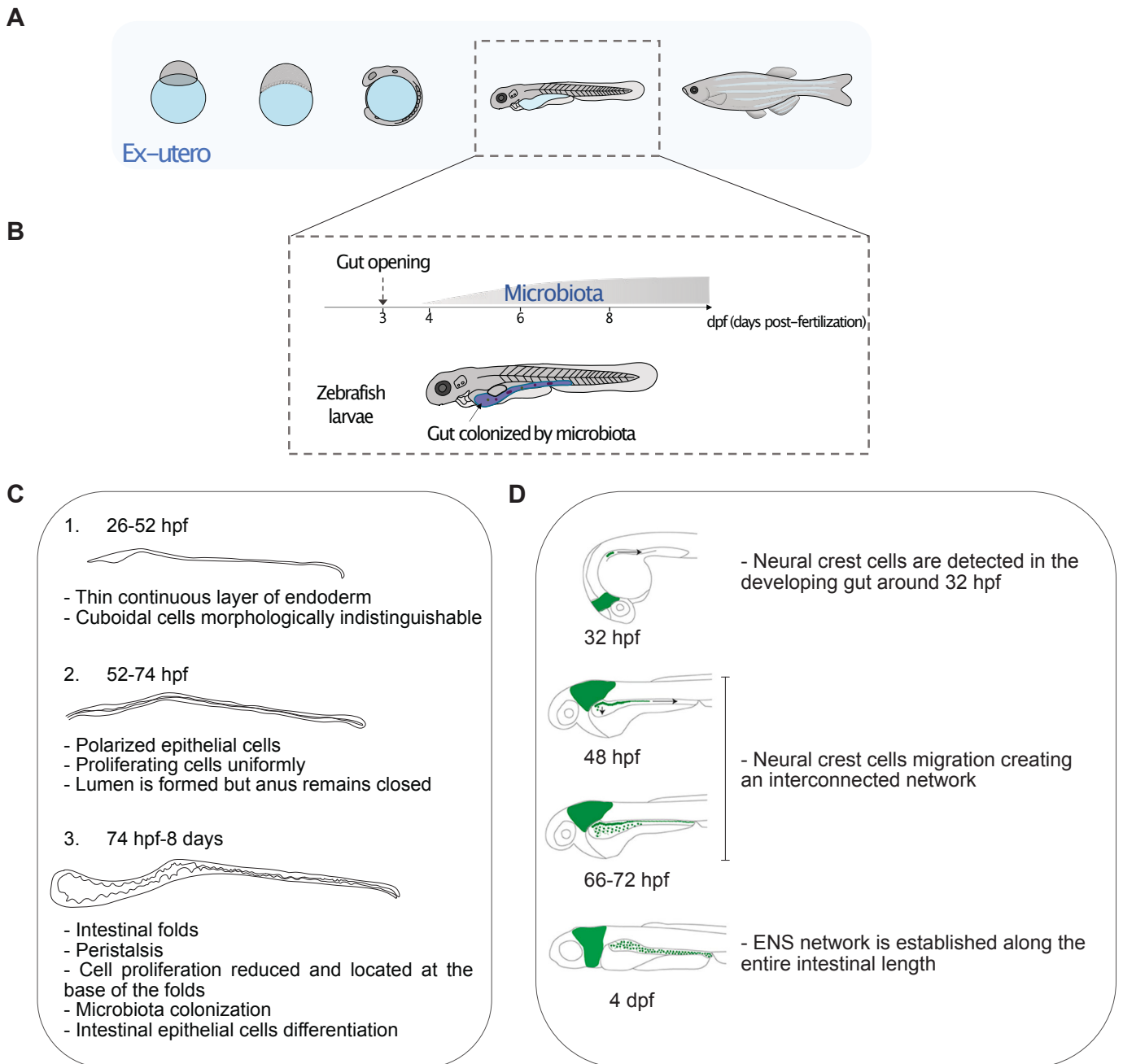


Figure 10. Stages of zebrafish intestinal development

A. Scheme of *ex-utero* zebrafish development, from left to right: one-cell stage, blastula (2 hpf), segmentation (around 16 hpf), larvae (5 dpf), and adult (3 months). **B.** Drawing of the gut opening at 3 dpf, with microbial colonization starting at 4 dpf and becoming more diverse and abundant over time. **C.** Diagram showing the different developmental stages of the zebrafish gut, with the main physiological processes indicated. **D.** Scheme of enteric nervous system development and migration into the gut. Adapted from *Kuil et al, 2021* and *Annie N.Y. Ng et al, 2005*.

5.2.3 Comparative analysis of the digestive system between Zebrafish and Mammals

Zebrafish belongs to the group of stomach-less fish and their digestive tract is divided into different sections: the mouth, the esophagus, three gut segments (anterior, middle, and posterior) and the anus (Figure 11A). Even if this model lack stomach, the anterior intestinal bulb is functionally comparable to the mammalian stomach. The nutrient absorption takes place mostly in the anterior part due to a high presence of digestive enzymes and longer folds and then gradually diminishes towards the posterior gut segments. The middle and posterior gut segments are responsible for ion transport, water reabsorption, and certain immune functions²⁸⁹. The zebrafish lack Peyer's patches, the submucosa layer and muscularis mucosa^{289,299} (Figure 11B). In addition, the intestinal epithelium is organized into folds that resemble the villi of the mammalian small intestine but lack crypt structures (Figure 11C).

The intestines of zebrafish and mammals exhibit remarkable homology in their development, structure, and biological functions. Wang et al, did a transcriptomic analysis of the zebrafish gut and compared it to mice. The mouse gut is anatomically divided into nine sections: mouth, esophagus, stomach, duodenum, jejunum, ileum, cecum, large intestine, rectum, and anus, while transcriptomic analysis divided the zebrafish gut into three morphologically distinct segments (herein named S1-S5, S6 and S7)²⁹⁹.

Segment S1-S5 resembles the mammalian small intestine as it expresses villin (*vil1*), fatty acid-binding protein 2 (*fabp2*), apolipoprotein 1(*apoa1*), and apolipoprotein 4 (*apoa4*), which are all involved in protein and lipid absorption. Segments S6 and S7 express aquaporin 3 (*aq3p*) and cofilin1 (*cff1*), which are molecular features of the mammalian large intestine and are involved in water absorption²⁹⁹. This indicates functional similarities with mammalian small and large intestines.

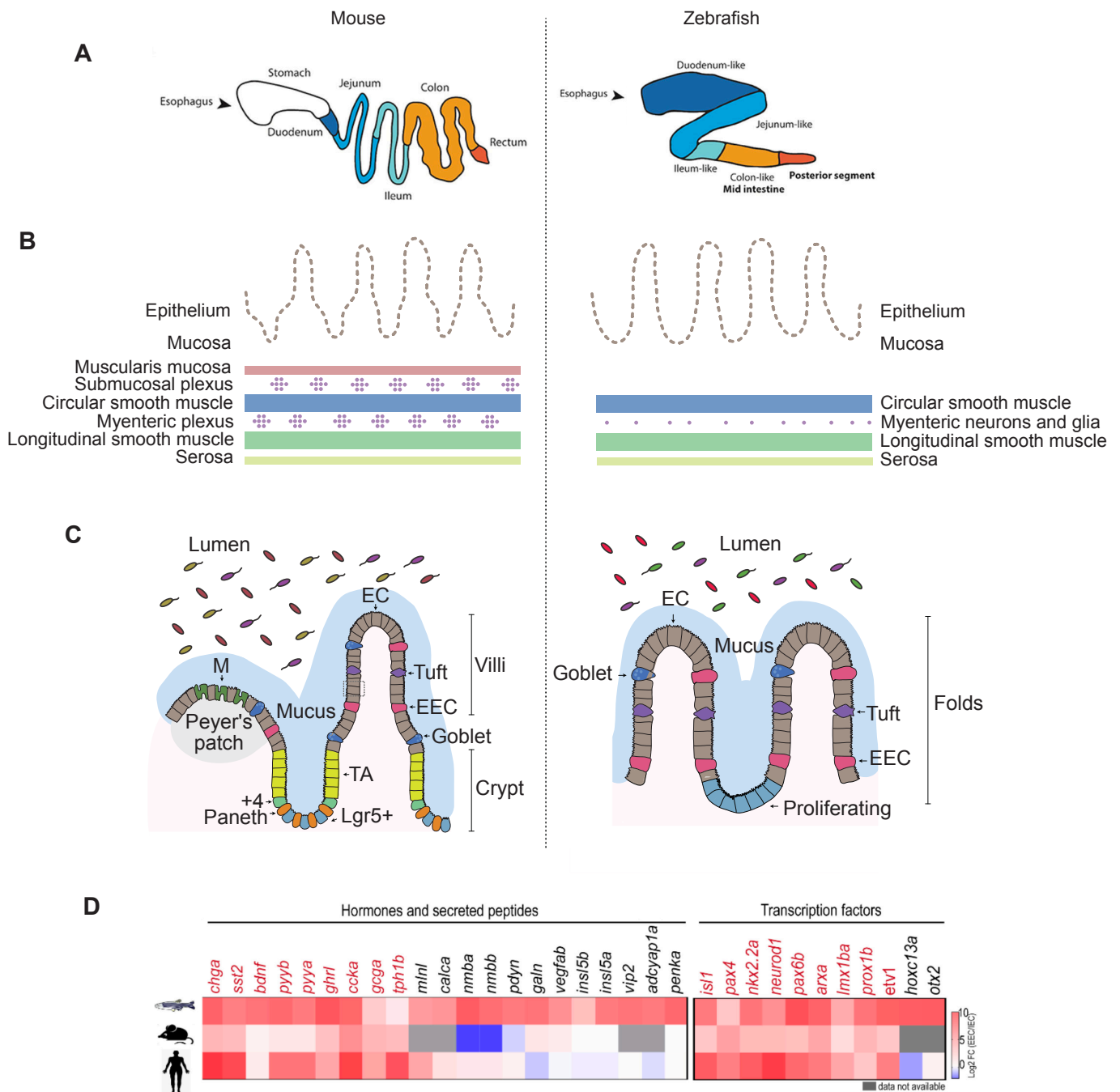


Figure 11. Comparison of the zebrafish and mammalian gastrointestinal tract
A. Digestive system of mammals and zebrafish. Mammals have a four-part digestive system: esophagus, stomach, small intestine, and large intestine. Zebrafish have a three-part digestive system: rostral intestinal bulb, mid intestine, and posterior segment. *Figure from Kuil et al, 2021*
B. Drawing of the layers present in the GI tract of mammals and zebrafish. Zebrafish lack the muscularis mucosa and the submucosal plexus; enteric neurons are not arranged in ganglia but as individual cells. *Adapted from Kuil et al, 2021.*
C. Structure of the zebrafish and mouse small intestine. The zebrafish gut is organized into folds, while the mouse small intestine has crypts at the base and villi at the top. Zebrafish lack Paneth cells, however most other IECs as well as their function are conserved. *Adapted from Ferguson et al, 2021*
D. Heatmap showing the conservation of EECs markers and function (hormones, peptides and transcription factors) between human, mouse and zebrafish. *Data from Ye et al, 2021.*

Regarding intestinal epithelial cells types, they are mostly conserved in zebrafish. Indeed, single-cell RNA-sequencing of the zebrafish gut revealed conservation of absorptive enterocytes, goblet cells (including those that resemble mammalian tuft cells), enteroendocrine cells, secretory precursors and ionocytes (including those that resemble mammalian BEST4/OTOP2 cells)³⁰⁰ (Figure 11C). In addition, most EECs subtypes, including those expressing serotonin or PYY are also conserved¹³¹ (Figure 11D). However, zebrafish lack Paneth cells. Interestingly, it has been shown that enterocytes, enteroendocrine cells, and goblet cells in zebrafish have conserved functions in nutrient absorption, hormone production, and mucus secretion respectively⁷⁷. For example, Ye et al, have shown that tryptophan catabolites derived from intestinal bacteria can activate the Trpa1 receptor in enteroendocrine cells and regulate gut motility. This activation leads to the secretion of serotonin, which in turn activates enteric and vagal neurons in zebrafish and in mammals¹³¹.

In addition, Lickwar et al, performed transcriptomic analysis combined with chromatin accessibility assessment and showed that the gene expression levels, transcriptional start sites, and regulatory regions of intestinal epithelial cells are conserved between zebrafish, stickleback, mouse, and human³⁰¹. This suggests that many aspects of IECs physiology are also conserved between zebrafish and mammals.

Interestingly, Lysosome-Rich Enterocytes (LREs) are highly endocytic vacuolated cells that are found in the ileum of suckling mammals and the mid-intestine of zebrafish. Zebrafish LREs share the same morphology, marker expression, and activity as mice. However, unlike mammals, where these cells are replaced by mature enterocyte at weaning, LREs still remain in adult zebrafish²⁹⁹. This is likely because zebrafish have no stomach, making their luminal environment similar to suckling mammals. Studies show that LREs preferentially internalize dietary proteins via fluid-phase and receptor-mediated endocytosis and digest them intracellularly both in zebrafish and suckling mice³⁰². Thus, these data indicate a highly conserved cellular mechanism of protein absorption between mammals and zebrafish.

Another example has been provided by the research conducted by Wen et al. in which they studied bile salt metabolism and the involvement of the bile salt-binding transcription factor farnesoid X receptor (Fxr) in zebrafish. Their findings revealed an

evolutionary conservation of essential components within the bile salt-Fxr signaling axis between zebrafish and mammals. They have notably shown that the anterior intestine of zebrafish is specialized for bile salt recovery, similar to the mammalian ileum³⁰⁰.

Finally, the ENS is a vast network of neurons and glial cells that is essential for intestinal physiology in both mammals and zebrafish. The ENS modulates gut peristalsis, water balance, hormone expression, and absorption. In zebrafish, the ENS is relatively simpler, comprising a single myenteric layer of neurons, glia, and other cell types, such as interstitial cells of Cajal, in contrast to the more complex organization found in mammals with two plexuses (myenteric and submucosal), each with interconnected ganglia²⁸⁹ (Figure 11B).

To summarize, the zebrafish has highly conserved gene expression and biological functions in the gut in comparison to mammals, highlighting its potential as a powerful alternative model for studying microbiota and intestinal physiological processes, with potential translational value to humans.

5.3 The zebrafish microbiota

The intestinal gene expression, structure, and function in fish are similar to those in humans, but in a less complex manner. At 4 dpf, the mouth and anus of zebrafish larvae open, allowing microbes to enter into their digestive tract. This transition marks a key point at which zebrafish larvae become permissive to microbial colonization. The microbial presence within the gastrointestinal tract of fish is estimated to range from 10^7 to 10^{11} bacteria/g of intestinal content, compared to 10^8 bacteria/g in mice and 10^{13} bacteria/g in humans^{53,303}.

Although the molecular mechanisms underlying responses to microorganisms are conserved, there are striking differences in the composition of gut microbiota between zebrafish and humans. In the human gut, the dominant microbial phyla consist of *Bacteroidetes* and *Firmicutes*, whereas in the zebrafish gut, *Proteobacteria* are the majority. The zebrafish intestinal microbiome is composed of *Proteobacteria*, *Fusobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Verrucomicrobia*^{303,304}.

This divergence in microbial composition can be attributed to genetic variations, environmental conditions, and dietary distinctions among species.

The gut microbiota of zebrafish undergoes significant changes during development, particularly at the phylum level. The γ -proteobacteria class is the most abundant bacterial class throughout development, but there are distinct shifts within the α -proteobacteria and β -proteobacteria classes during specific developmental phases. When zebrafish transitions into adulthood, these proportions decline, while δ -proteobacteria increase during aging³⁰⁵. Studies using 16S rRNA gene sequencing have shown that members of the Proteobacteria phylum maintain their dominance in the zebrafish gut microbiota across developmental time points, followed by an expansion of *Firmicutes* and *Fusobacteria* in later adulthood^{306,307}.

In zebrafish, intestinal microbes have been shown to promote epithelial differentiation and proliferation similarly than in mammals^{291,294}. For example, in germ-free (GF) zebrafish larvae, the intestine exhibits an incomplete development and impaired functionality, a phenomenon that can be reversed by transplanting bacterial communities, thereby highlighting the critical role of gut microbiota in shaping intestinal epithelial cell fate and overall gut development²⁹¹.

Beyond developmental implications, intestinal microbes significantly influence the immune status of the host by activating key receptors such as TLRs and TNF receptors^{308,309}. Commensal microbe recognition is notably regulated through the TLR/MyD88 signaling pathway, as validated by Galindo-Villegas et al³¹⁰. This recognition triggers a series of responses, including neutrophil recruitment, upregulation of inflammation-related and antiviral genes, ultimately decreasing larvae susceptibility to viral infections³¹⁰.

The far-reaching impact of gut microbiota extends to host metabolism regulation. SCFAs can enhance insulin sensitivity and promote glucose homeostasis in mammals through GLP-1 secretion as well as engagement of SCFA receptors FFAR2-3 on L-cells^{311,312}. Similarly, the interplay between the gut microbiota and glucose metabolism extends to zebrafish. Indeed, BefA proteins generated by the *Aeromonas* and *Shewanella* has the ability to stimulate the proliferation of pancreatic β -cells, consequently augmenting insulin production and fine-tuning blood glucose

homeostasis³¹³. Taken together, these findings highlight the striking similarities in the role of the microbiota in gut development, immune responses, and metabolic processes between zebrafish and mammals.

To understand the complex interactions between intestinal microbes and their hosts, researchers often use gnotobiotic animal models, which allow for controlled exploration of these interactions. Zebrafish embryos are an attractive organism for studying gut microbiota and host-microbiome crosstalk because of their *ex-utero* fertilization allowing them to be easily sterilized and raised without microorganisms. This ability to rear zebrafish under GF or gnotobiotic conditions gives researchers the ability to precisely control the microbial environment in these organisms^{314,315}. The use of GF zebrafish as a convenient animal model marks the beginning of a new era in intestinal microbiome research, opening up unprecedented opportunities to unravel gut-microbiome interactions.

5.4 Immune system in zebrafish

The immune system of zebrafish is remarkably similar to that of humans, with both innate and adaptive branches. However, zebrafish do not have lymph nodes, and the hematopoietic function of the bone marrow of adult mammals is instead performed by the kidney in zebrafish. At 4 dpf, components of the adaptive immune system can be detected, such as precursor T-cells expressing *recombinant activating gene 1* (*rag1*) and *ikaros*. However, the full function of adaptive immunity does not develop until two to four weeks after fertilization. Thus, during the first week of embryonic development, the zebrafish larva relies on the innate immune system.

5.4.1 Innate immunity

Zebrafish larvae possess highly conserved innate immunity, which comprises different cell types found in mammals, such as neutrophils, eosinophils, macrophages, and dendritic cells. In the early stages of embryonic development, zebrafish blood cells such as erythrocytes and macrophages are produced by a primitive wave of hematopoiesis. Definitive hematopoiesis is initiated at about 30 hpf by the emergence of hematopoietic stem cells from the ventral wall of the aorta¹. These stem cells migrate to the caudal hematopoietic tissue before colonizing the definitive hematopoietic

tissue, the kidney³¹⁷. This definitive hematopoiesis will give rise to all blood cells, including erythrocytes, lymphocytes, and all myeloid cells (macrophages, neutrophils, dendritic cells, eosinophils, mast cells).

The PRRs of zebrafish phagocytes are diversely present on both cellular and phagosomal membranes, including TLRs, Nod-like receptors (NLRs), and other soluble receptors like that of mammalian counterparts. Surprisingly, some other non-mammalian TLRs have also been discovered in fish, such as the soluble forms of TLR4 and TLR5 (sTLR4, sTLR5), TLR19, TLR20, TLR21, TLR22, and TLR23^{318,319}. Although most TLRs and their signaling are well-studied and conserved in zebrafish, the role of these novel receptors as well as the extra copies of the mammalian orthologs is still unclear. In zebrafish, TLRs are expressed after body axis determination, while the adaptor proteins, like MyD88, are expressed even later. This is in contrast to *Drosophila*, where TLRs and adaptor proteins are maternally expressed and are involved in body patterning³²⁰. The earlier expression of TLRs in zebrafish suggests that they may fulfill functions alternative to anti-microbial recognition such as participating to tissue development or homeostasis for example.

Interestingly, novel immune-type receptors (NITRs) were recently discovered in bony fish and are characterized by immunoglobulin (Ig)-variable ectodomains³²¹. They are putative orthologs of mammalian natural cytotoxicity receptors and killer cell immunoglobulin-like receptors. In brief, they share structural features with mammalian natural killer receptors. The ligands and signal transduction pathways engaged by NITRs are still under investigation.

Upon infection, epithelial cells as well as leukocytes such as macrophages and neutrophils abundantly express cytokines, chemokines, and reactive oxygen species to mount inflammatory responses. This is a crucial process that facilitates the bacterial clearance and tissue homeostasis.

5.4.2 Adaptive immunity and evolution of the immune system

The origin of the adaptive immune system can be traced back to about 500 million years ago in jawed fish. The thymus, a critical organ for adaptive immunity, first appeared in the jawless lamprey, and later became present in all jawed fish along with other components of the adaptive immune system, such as T cells, B cells, and highly polymorphic major histocompatibility (MHC) antigens. Zebrafish, being a model organism, offers valuable insights into studying adaptive immunity, as the development of the thymus and lymphoid organs is highly conserved between zebrafish and mammals. Several studies have demonstrated that zebrafish possess a functional adaptive immune system similar to mammals, where T cells can be detected in the thymus at 72 hours. T cell progenitors are present in the thymus around 3 days post-fertilization, coinciding with the hatching from the protective chorion. However, detecting T cells outside of the thymus during the first 3 weeks of development is challenging³²². Interestingly, recent single cell RNA-sequencing data showed expression of few *nitr9* and *rorc* expressing cells in 7 dpf larvae, suggesting that some lymphoid cells or precursors could already be circulating or residing in tissues³²³.

In addition to T cells, zebrafish also have B cells. However, the class switching process, which contributes to the immune repertoire diversification in mammals and is mediated by AID (activation-induced cytidine deaminase), does not occur in teleost fishes. Indeed, they have only IgM and an IgD isotype equivalent. Considering the absence of efficient affinity maturation and class switching, it is reasonable to hypothesize that AID emerged in evolution after teleosts separated from other vertebrates.

Prokaryotic cells have evolved mechanisms to protect themselves from foreign invaders. These mechanisms include restriction enzymes and CRISPR-Cas systems for example. Since the appearance of the first eukaryotic cells, a series of additional defense mechanisms have evolved, including the innate immune system and the adaptive immune system. Invertebrates such as *Drosophila*, the sea urchin, as well as numerous plant species rely only on innate immunity, thus they had a significant proliferation of PRR families. It was only with the appearance of vertebrates that a higher degree of immune sophistication evolved. It is intriguing to note that elements of the innate immune system, such as TLRs and NLRs, are widespread across different

species in the animal kingdom. Lower vertebrates seem to heavily rely on innate immunity, as their adaptive immune responses is maturing later, and their humoral responses are lacking class switching and efficient affinity maturation³²⁴. In contrast to terrestrial mammals, which are protected during early development by maternal immunity, fish are constantly exposed to many different microbes immediately after hatching³²⁵. The most efficient way for them to deal with this external environment so early during development seem to be by rapidly distinguishing self from non-self through the recognition of bacterial motifs in innate immunity. In line with this hypothesis, they have more TLRs, as well as NITRs, NK cells³²⁶, and complement-dependent phagocytosis³²⁷. With such efficient and active innate immunity in fishes, there might not have been a significant selective pressure on the adaptive immunity, leading to the absence of lymph nodes and antibody diversification. However, as vertebrates evolved and began colonizing the terrestrial environment, conditions changed as well as the type of microbes and pathogens they were facing. In brief, the selective pressures changed. We can also take into account that the temperatures in water are different than the terrestrial ones, consequently, T cells metabolism also potentially evolved. We could hypothesize that all these environmental changes as well as their co-evolution with microbes led to the development of a more complex adaptive immune system, more elaborate structures and optimized B cells antigen recognition by improving somatic mechanisms.

5.4.3 Zebrafish IL-22 and its negative regulator

Teleost fish possess a class II cytokine system (comprising IFNs and cytokines from the IL-10 family) surprisingly similar to that of humans. The zebrafish *il22* gene contains 5 exons and is located on chromosome 4. IL-22 ortholog in zebrafish was first described in 2006³²⁸ due to its highly conserved synteny. Indeed, *il22* is flanked by the *mdm1* gene on one side and *il26* and *ifng1* on the other side (Figure 12A). Siupka et al, resolved the IL-22 three-dimensional structure by X-ray crystallography in zebrafish. Interestingly, despite its low sequence similarity, its structure is highly similar to that of human IL-22³²⁹ (Figure 12B). In addition, they produced a recombinant zIL-22 protein and showed that it signals to gut epithelial cells as its mammalian counterpart. However, the genes encoding for the IL-22 receptor chains in zebrafish are still unknown.

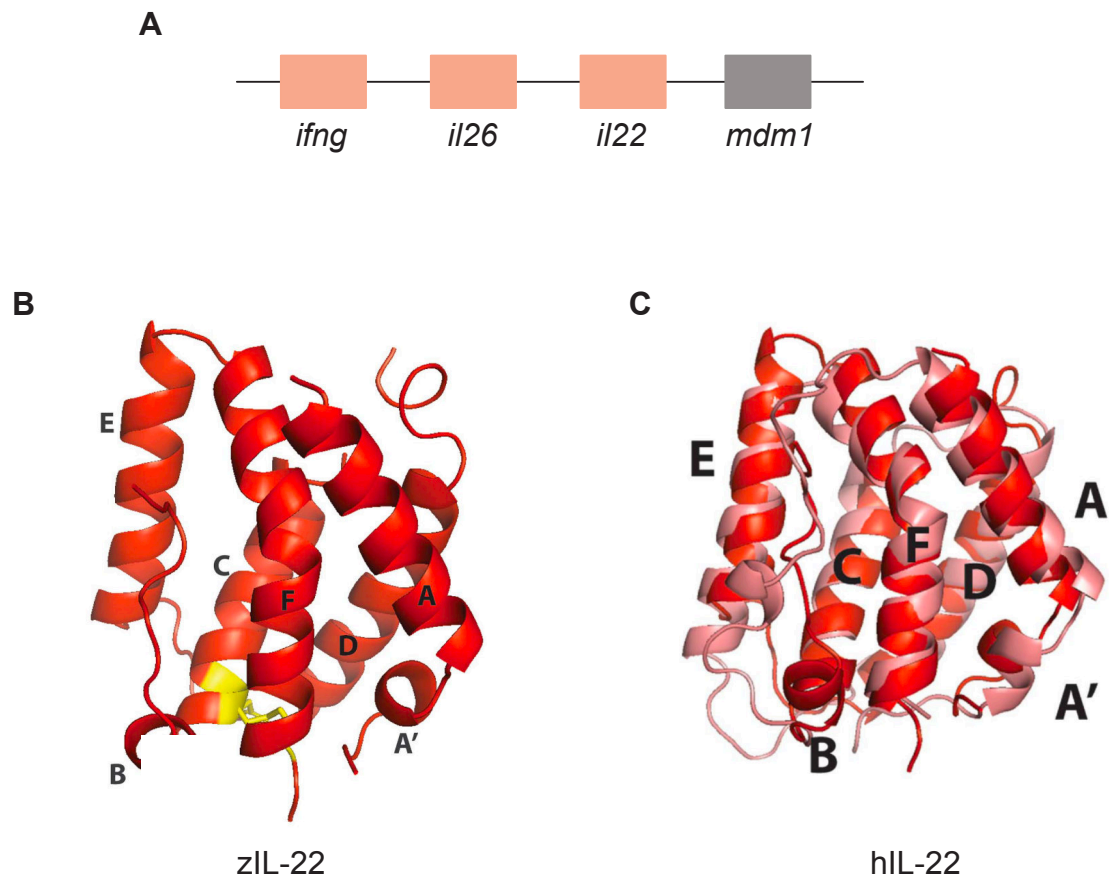


Figure 12. IL-22 gene organization and protein structure

A. Scheme showing the gene organization of the zebrafish IL-22 gene and its neighbors. **B.** Cartoon representation of the structure of zIL-22. Structural elements are labeled A' through F. Two disulfide bridges are shown in yellow and comparison in **C.** with the human IL-22 (hIL-22). Adapted from Siupka et al, 2014.

Regarding its cell sources, Hernandez et al, have reported by single-cell RNA-sequencing in *rag1*-deficient adult zebrafish (lacking B and T lymphocytes), the presence of ILC-like cells expressing *il22*³³⁰. However, these cells differ from their mammalian counterparts. Zebrafish ILC-like cells do not constitutively express cytokine receptors like human and mouse ILCs, nor AhR and PRRs. Instead, they express NITRs²⁹⁶. Despite these differences, zebrafish ILC-like cells resemble human and mice NCR+ ILC3 cells in their IL-22 expression during bacterial infections. However, the differences in receptor expression could have implications for their regulation and development.

IL-22 negative regulator also has an ortholog in zebrafish. Indeed, IL-22BP has been cloned in zebrafish³³¹, and its functional characterization in mandarin fish suggests conservation of IL-22 and IL-22BP interactions³³². In zebrafish, IL-22BP has been described to be expressed by metaphocytes (tissue-resident macrophage/dendritic cell-like cells). Interestingly, removing metaphocytes led to a dysregulation of IL-22 expression, upregulation of pro-inflammatory genes and dysbiosis suggesting that like its mammalian ortholog, the zebrafish IL-22BP acts as a negative regulator to suppress IL-22 signaling³³³. However, further investigation is required to determine whether IL-22 has similar microbiota-modulating effects in zebrafish as observed in mammals during homeostasis.

VI. Aim of the thesis: Link between IL-22 and gut physiology during post-embryonic development

I completed an internship at the laboratory of Dr. Philippe Herbomel at the Pasteur Institute in Paris, where pioneering work was conducted on the use of zebrafish to investigate the emergence of immune cells. This also encompassed the study of the development and behavior of the innate immune system in response to pathogens. During this internship, I was under the guidance of Dr. Pedro Hernandez, a postdoctoral researcher who later obtained a position as a principal investigator in the Department of Developmental Biology at the Curie Institute in Paris. As our mutual trust grew during this period, I was grateful when I got the opportunity to be his first doctoral student. Together, we embarked on the ambitious journey of establishing a new laboratory and project.

At the core of my multidisciplinary project, which aims to unravel the importance of the cytokine IL-22 in the development and physiology of the vertebrate gut, lies the foundation of extensive knowledge previously acquired and outlined about IL-22. The multifaceted roles that this cytokine plays in immunity, its early expression during development, its involvement in intestinal homeostasis, regeneration, and its connection with lipid metabolism collectively formed the basis for hypothesizing that IL-22 could potentially contribute to the physiology and intestinal development during its maturation phase. Consequently, during my PhD project, I used the zebrafish model to investigate the function of IL-22 in gut development and maturation. Previous research conducted in mice underscored IL-22 protective effects on gut epithelial cells, safeguarding them against pathogens, damage, and colon cancer. Nonetheless, the extent of IL-22 impact on gut development and maturation remained unknown. Through the use of gene-editing tools, live microscopy, transcriptomics, immune challenges, and more, I have made surprising discoveries concerning the cellular origins of this cytokine within the developing zebrafish gut. Furthermore, I have unveiled a novel role of IL-22 in modulating gut motility through microbiota and hormones regulation in the developing gut.

This project led to several collaborations worldwide, including one with the laboratory of Dr. Villablanca at the Karolinska Institute. Intriguingly, our interests aligned closely; they too sought to comprehend the potential roles of cytokines in the context of gut development and maturation. Specifically, our joint efforts were directed toward investigating interleukin-10 (IL-10), a member of the same cytokine family as IL-22. IL-10 plays a crucial role in establishing intestinal homeostasis, as evidenced by the emergence of spontaneous colitis due to mutations in components of the IL-10 signaling pathway. However, the broader scope of its functions beyond immunomodulation remained poorly understood. As a side project, I participated to the identification of IL-10 novel role in regulating goblet cell numbers through Notch signaling in the zebrafish developing gut. This collaboration resulted in a publication as second author in *Mucosal Immunology* in 2022.

In the results section, we will first explore my primary PhD project, unraveling the complex functions of IL-22 during gut development. Then, I will provide a brief introduction to the aforementioned collaborative publication. We will investigate the pathways of physiological evolution, and uncover hidden mechanisms that drive intestinal maturation. Together, we will hopefully provide a better understanding of the enigmatic interplay between immune response and gut physiology.

RESULTS

1. Interleukin-22 modulates microbiota-mediated control of gut motility during early life

In the first and main part of my PhD, I investigated the role of IL-22 in gut development and maturation using the zebrafish model. Firstly, I identified a new cell source of IL-22 in the zebrafish larval gut, the EEC. I also showed that *il22* expression is regulated by the microbiota and more specifically by the *Trpa1* receptor, which recognises bacterially derived tryptophan metabolites. I then showed that despite the difference in cell sources between mammals and the developing zebrafish gut, the protective role of IL-22 in anti-bacterial immunity is conserved between these animals. Then, surprisingly, I found a novel role for this cytokine in the regulation of intestinal motility during early life. Following these observations, I aimed to uncover the mechanism by which IL-22 regulates this key physiological process. So I asked myself: Are neurons impaired? Are the muscles affected? Is the microbiota responsible for this defect? How does the lack of IL-22 impact EECs? Is this impairment intestine-specific or does IL-22 communicate with other organs to regulate intestinal motility? Many questions have been raised and are addressed in the study presented below.

The data and analyses presented were compiled mostly by myself with the help of my team members and the expertise of collaborators. Mutant lines were generated by collaborators and I generated a gut tissue-specific rescue transgenic line. In addition, the RNA sequencing analyses were performed by myself and I also directed all the bioinformatic analyses performed by Sylvia Brugman for the 16S RNA sequencing. Aya Mikdache also contributed with the *Trpa1* activator injections, while Ignacio Medina Yanez helped me with the DSS and Fabian Guendel was in charge of the mouse experiments.

This study is close to completion and will be compiled in a manuscript presented below :

IL-22 modulates microbiota-mediated control of gut motility during early life

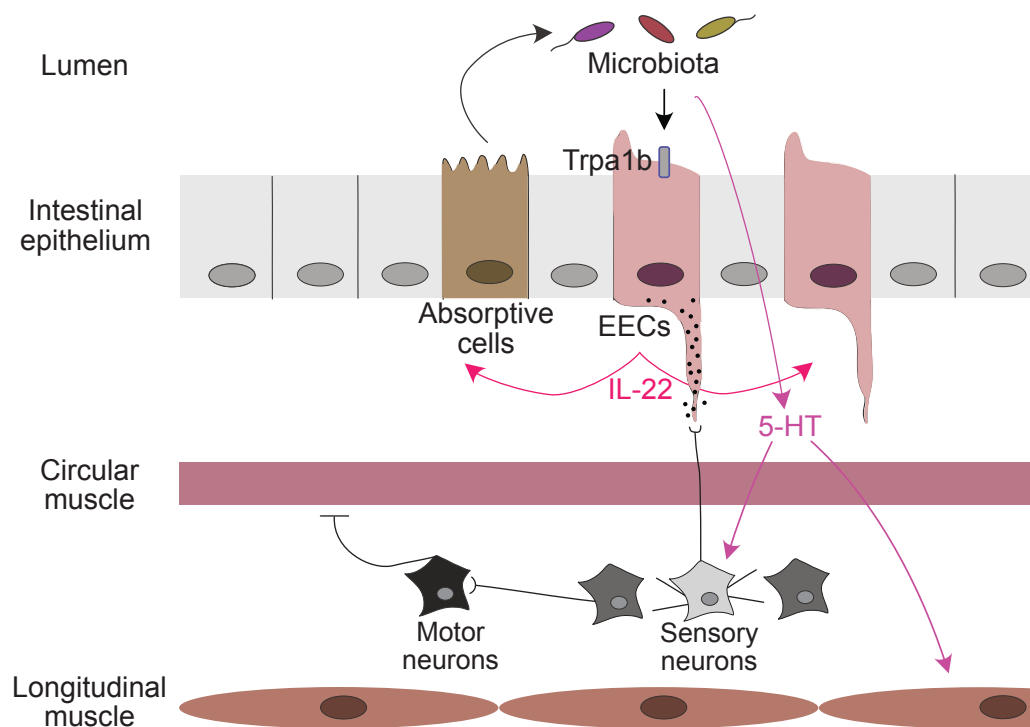
Soraya Rabahi¹, Aya Mikdache¹, Ignacio Medina-Yanez¹, Gwendoline Gros¹, Fabian Guendel⁶, Emiliano Marachlian⁵, Jos Boekhorst⁴, Camila Baudino-Garcia¹, Patricia Diabangouaya¹, Yazan Salloum¹, Jean-Pierre Levraud², Carmen Feijoo³, Sylvia Brugman⁴, German Sumbre⁵, Gerard Eberl⁶, Pedro Hernandez¹

1. Institut Curie, PSL Research University, CNRS UMR3215, INSERM U934, Paris, France.
2. Université Paris-Cité, Institut des Neurosciences Paris-Saclay, Saclay, France.
3. Facultad de Ciencias de la Vida, Universidad Andres Bello, Santiago, Chile.
4. Host-Microbe Interactomics, Wageningen University and Research, De Elst 1, 6708 WD, Wageningen, The Netherlands.
5. Institut de Biologie de l'ENS (IBENS), Département de Biologie, École Normale Supérieure, CNRS, INSERM, Université PSL, 75005 Paris, France.
6. Institut Pasteur, Microenvironnement & Immunity Unit, 75724 Paris, France; INSERM U1224, 75724 Paris, France.

Abstract

Cytokines are small secreted proteins that promote tissue defense and homeostasis. Among the key cytokines in barrier organs such as the gut, interleukin-22 (IL-22) primarily targets epithelial cells to protect this organ from pathogens and promote tissue repair, but whether this cytokine plays a role in the developing gut remains unclear. Using IL-22-deficient zebrafish, we discovered a novel role for this cytokine in modulating intestinal motility during early life. We found that enteroendocrine cells are the major source of IL-22 in the larval gut and that its expression is induced by the sensing of tryptophan-derived metabolites by Trpa1. IL-22 deficiency results in neuronal and smooth muscle defects, impaired hormone expression, dysbiosis and potential impairment in the expression of bacterial-derived metabolites. Co-housing and live bacteria transfer from WT larvae and serotonin (5-HT) treatment could rescue the intestinal motility defect. We also found conservation of IL-22 role in the regulation of intestinal motility in suckling mice. These results establish a pathway by which IL-22 expression by EECs regulates microbiota composition, which in turn influences hormone expression, particularly 5-HT, and ENS/smooth muscle function to modulate gut motility.

Graphical abstract



Highlights

- Enteroendocrine cells are the main source of *il22* in larvae
- Conservation of IL-22 signaling and protective function in zebrafish
- Lack of IL-22 results in intestinal motility impairment with dysbiosis as well as neuronal and smooth muscle defects
- Co-housing and transferring water from wild-type rescue the gut motility phenotype in *il22*^{-/-}
- Hormone expression is impaired and administration of 5-HT rescues the intestinal motility phenotype in *il22*^{-/-}

Introduction

In all organisms, the colonization of the gut lumen by its microbiota begins when the digestive tube opens to the external environment and feeding begins. During this developmental period and continuing into adulthood, the microbiota influences the development of intestinal epithelial cells, neurons, muscle and immune cells, and subsequently maintain the physiological function of the gut¹⁻⁴. Microbial stimuli such as microbial-associated molecular patterns (MAMPs) and microbial-derived metabolites often mediate effects on host physiology, particularly on intestinal epithelial cells^{5,6}. Numerous host-microbe interactions, including those regulating processes such as intestinal motility are conserved across vertebrates⁷. However, the mechanisms by which host cells integrate microbial cues to regulate early life gut maturation and physiology are not well understood.

Gut epithelial cells tightly interact with microbial communities^{8,9}. Enteroendocrine cells (EECs), a key epithelial subtype controlling gut physiology and motility, are evolutionarily conserved across insects, fish, and mammals, with preserved sensory functions^{7,10-12}. Upon activation by various luminal signals from the microbiota, EECs release hormones and neurotransmitters such as serotonin¹². Furthermore, recent findings revealed synaptic contacts between EECs and sensory neurons, providing a direct pathway for the transmission of nutrient sensory information to the brain through the intestinal epithelium¹³⁻¹⁵. Nevertheless, the precise mechanisms by which EECs regulate local and systemic host physiology upon sensing of gut luminal molecules remain unclear.

The gut immune system plays a critical role in maintaining the composition of the microbiome while protecting against harmful pathogens. Gut lymphocytes such as innate lymphoid cells (ILCs) and T cells produce cytokines in response to cues from the microbiota including metabolites. For instance, in mice, tryptophan-derived metabolites, when detected by the aryl-hydrocarbon receptor (AhR) in ILC3 and Th17 cells, lead to the production of high levels of interleukin-22 (IL-22) by these cells¹⁶⁻¹⁸. IL-22 has multiple functions, such as safeguarding the gut against bacterial and viral infections¹⁹⁻²², promoting tissue regeneration^{23,24}, controlling cancer development²⁵⁻²⁷ and regulating lipid metabolism^{28,29}. Notably, its receptor is exclusively expressed in

non-hematopoietic cells, primarily epithelial cells rather than other immune cells^{30,31}. Interestingly, ILCs express high levels of constitutive IL-22 in mice during the suckling period^{32,33}. However, the role of IL-22 during early life in modulating the microbiota and gut physiology remains unknown.

The zebrafish is a powerful model to study organ development and physiological processes during early life. In organs such as the gut, it shares with mammals a high degree of similarity in cellular composition and interactions between gut epithelial, immune, and neuronal cells^{7,10,33–37}. Recently, ILCs and T cells with the capacity to produce IL-22 have been identified in the adult zebrafish gut³⁷. Therefore, the zebrafish stands out as a valuable model to investigate the role of IL-22 during intestinal maturation and the onset of its physiological function.

In this work, we identified enteroendocrine cells as the major source of IL-22 in the zebrafish larval gut. We found that tryptophan-derived metabolites induce its expression in a specific EEC subtype through the activation of the transient receptor potential cation channel *Trpa1b*. Furthermore, zebrafish larvae deficient in IL-22 showed dysbiosis and increased susceptibility to gut bacterial infection as well as to inflammatory chemical treatment, demonstrating conservation of gut protective function with mammalian IL-22. In addition, we unveiled a novel role of IL-22 in gut physiology: *il22^{-/-}* larval guts show reduced production of serotonin, impaired gut neuronal activity and smooth muscle structure, resulting in reduced gut motility as well as slower food transit. Notably, gut motility impairment was successfully rescued through co-housing and live bacteria transfer from WT larvae, as well as by serotonin treatment. Finally, analysis of IL-22-deficient mice revealed impaired food transit primarily during early life, suggesting an evolutionarily conserved role for IL-22 in maintaining early vertebrate gut physiology.

Results

Indoles induce steady state *il22* expression in enteroendocrine cells

To determine the role of IL-22 during early life, we first aimed to identify which cell types express this cytokine in the developing zebrafish. In mammals, IL-22 expression is known to be restricted to immune cells such as T cells and ILC3³⁸. In zebrafish, single-cell RNA-sequencing (scRNA-seq) of the adult gut revealed *il22* expression in ILC-like cells, similarly to mammals³⁷. However, the identity of *il22*-expressing cells in the embryo and early larval stages, which precede the emergence of lymphocytes in the intestine, remains unknown. To address this question, we generated an *il22*:mCherry reporter line utilizing Tol2-mediated transgenesis. Interestingly, we observed strong *il22*:mCherry expression in neuromasts, a bony fish sensory organ akin to the mammalian inner ear (**Figure S1A**). Furthermore, we noted *il22*:mCherry expression in intestinal epithelial cells (IECs) when coupled with the *cldn15la*:GFP line (**Figure S1A**). To validate these results, we performed qPCR analysis on sorted *cldn15la*:GFP-positive and -negative cells (**Figure 1A**) and found *il22* expression enriched in sorted IECs (**Figure 1B**). The main mammalian IEC subtypes are conserved from mammals to zebrafish, including mucus-producing goblet cells, absorptive enterocytes, and enteroendocrine cells (EECs). The latter sense various environmental stimuli and secrete neurotransmitters and neuropeptides that regulate diverse physiological processes. By reanalyzing scRNA-seq datasets of larval sorted IECs or dissected intestines, we found *il22* expression mainly in a subtype of EECs³⁵ (**Figure 1C and S1B**). In addition, when we combined *il22*:mCherry with the *neurod1*:GFP transgenic line, which labels EECs, we observed co-localization (**Figure 1D**), further supporting that in the gut *il22* is expressed mostly in an EEC subtype. Altogether, our results show that epithelial cells and more precisely enteroendocrine cells are the main cell source of *il22* in the gut during zebrafish early life.

We next sought to investigate what regulates *il22* expression in the zebrafish larval gut. Studies in mammals have shown a central role of the microbiota and its derivatives —pathogen-associated molecular patterns and short-chain fatty acids (SCFAs)— in orchestrating IL-22 production. To determine whether zebrafish *il22* expression is dependent on the microbiota in larvae, we generated germ-free (GF) fish

(**Figure 1E**). qPCR on guts of conventionally-reared (CV) larvae and GF larvae revealed lower *il22* expression in the latter, indicating that the microbiota maintains *il22* mRNA levels at steady state (**Figure 1F**). The innate immune Toll-like receptor (TLR) signaling pathway is key for sensing microbes in all animals. In mice, TLR5 agonist flagellin induces *il22* expression in immune cells³⁹. In zebrafish, there are two TLR5 orthologs, *tlr5a* and *tlr5b*, and both are necessary to respond to flagellin⁴⁰. Interestingly, the zebrafish EEC subtype expressing *il22* exhibits high levels of *tlr5b*³⁵ (**Figure 1C**). Thus, we investigated the role of TLR5 in *il22* expression in the zebrafish larval gut. We found increased *il22* expression upon flagellin injection in the gut by qPCR (**Figure S1C,D**). However, no differences in *il22* expression were observed between WT (wild-type) and *tlr5b*^{-/-} at steady state and upon flagellin injection (**Figure S1E**), suggesting an alternative mechanism for flagellin-induced *il22* expression in zebrafish.

Dietary and gut bacteria-derived metabolites such as those of tryptophan are reported to induce IL-22 production in mammalian lymphocytes¹⁶. Interestingly, the EEC subtype expressing *il22* is the only one expressing the nutrient sensory channel *trpa1b* (**Figure 1C**). It has been recently shown that tryptophan metabolites can activate zebrafish and mammalian EECs through Trpa1⁷. Thus, we aimed to determine whether Trpa1 activation induces *il22* expression in EECs. To do so, we injected indole, a tryptophan metabolite into the gut of double transgenic *il22:mCherry neurod1:GFP* larvae and quantified the number of double-positive cells (**Figure 1G**). We found an increase of EECs expressing *il22* cells upon indole injection (**Figure 1H-J**) suggesting that tryptophan metabolites can induce *il22* expression in EECs through Trpa1 activation.

Altogether, we found *il22* expression in larval EECs, conservation of its regulation by the microbiota, and we identified Trpa1 as a novel receptor likely mediating *il22* induction upon indole sensing.

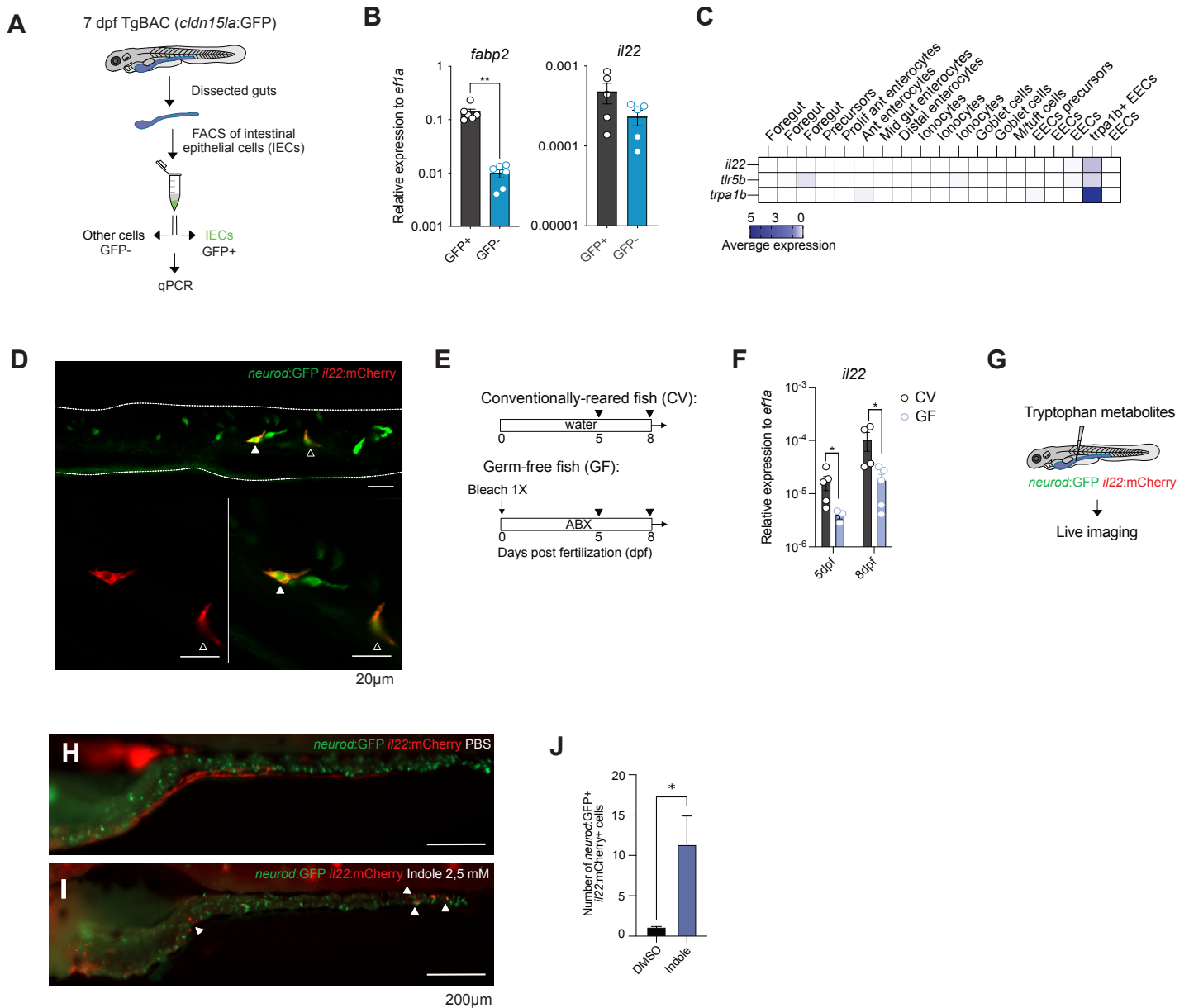


Figure 1. *il22* is expressed in enteroendocrine cells in zebrafish larvae

A. Schematic representation of FACS sorting of *cldn15la*:GFP-positive and -negative cells from dissected larval guts, followed by RT-qPCR analysis. **B.** RT-qPCR analysis measuring the expression of *fabp2* (epithelial marker) and *il22* in sorted *cldn15la*:GFP-positive and -negative cells. **C.** Heatmap showing a re-analysis of a scRNA-sequencing dataset of IECs from zebrafish larval gut from Wen et al, 2021. **D.** Confocal images of Tg(*neurod*:GFP, *il22*:mCherry) at steady state. Arrows are showing colocalization between the two transgenic lines. Scale bar: 20µm. **E.** Diagram showing bleach and antibiotic (Abx) treatment in zebrafish embryos/larvae to generate germ-free (GF) animals. **F.** RT-qPCR analysis measuring the expression of *il22* in dissected guts from 5 and 8 dpf conventionally reared (CV) or GF larvae. **G.** Schematic representation of tryptophan metabolites (indole) injection in the gut of 7 dpf Tg(*neurod*:GFP, *il22*:mCherry) larvae, followed by live imaging. **H.** Fluorescent image of Tg(*neurod*:GFP, *il22*:mCherry) injected with PBS or **I.** indole (2,5mM). Arrows are

showing the double positive cells. Scale bar: 200um. **J.** Quantification of the number of *neurod*:GFP+ *il22*:mCherry+ cells in the gut of 7 dpf larvae injected with PBS or indole. Statistical analysis were performed with Mann-Whitney * $P < 0,05$, ** $P < 0,01$. These data are representative of at least two independent experiments.

Zebrafish IL-22 promotes gut immunity and defense

We then wondered whether, despite the differences in cell sources observed in zebrafish larvae compared to mammals, IL-22 still plays a similar role in both types of animals. In mammals, IL-22 is well-known for its protective role in the gut by promoting anti-bacterial gene expression in the epithelium^{41–43}. To investigate the function of *il22* in the zebrafish gut, we generated an *il22* mutant line through CRISPR/Cas9 genome editing. We created a 5bp deletion in the exon 1 of the zebrafish *il22* gene (**Figure 2A**), resulting in a predicted premature stop codon (**Figure 2B**). To verify our *il22* knock-out line, we first sought to identify an IL-22-specific target gene to test our mutant. A previous study showed that the *mpx* gene is induced in gut epithelial cells following zebrafish recombinant IL-22 (zrIL-22) injection⁴⁴, a finding we validated using a *mpx*:GFP transgenic line (**Figure S2A**). We observed *mpx*:GFP induction in gut epithelial cells also upon bacterial extract injection, which was absent in *il22*^{-/-}*mpx*:GFP (**Figure S2B**), confirming the loss-of-function of the *il22* gene. *il22*^{-/-} fish did not show morphological or developmental defects, reached adulthood and were fertile (**Figure S2F-H**). Also, we did not observe differences in gut size, secretory and goblet cells numbers in the gut (**Figure S2G, I, J**). In order to reveal differences at the molecular level, we conducted bulk RNA-sequencing of dissected guts from 7 dpf WT and *il22*^{-/-} larvae (**Figure 2C and S2K**). 917 transcripts were significantly down-regulated in *il22*^{-/-} larval guts (**Figure 2D**). Unbiased gene ontology (GO) analysis revealed that most down-regulated genes in *il22*^{-/-} were associated with anti-bacterial responses (**Figure 2E**). IL-22 receptor signaling in mammalian IECs activates the STAT-3 signaling pathway and anti-bacterial peptide production^{45,46}. *il22*^{-/-} guts showed decreased expression of *stat3*, *socs3a/b* and *mpx*, in addition to reduced *il22* expression (**Figure S2M**). Further, zrIL-22 injection resulted in induction of these genes, as measured by qPCR of dissected guts (**Figure S2N**). Interestingly, *mpx* is a potential anti-bacterial gene⁴⁷, suggesting that IL-22 induces anti-bacterial gene expression in zebrafish gut epithelial cells through STAT-3 activation, similarly to mammals.

Next, we functionally tested the predicted gut protective role of zebrafish IL-22. To this end, we performed bath infection with live *Edwardsiella tarda*, a well-known fish gut pathogen (**Figure 2F**). *il22*^{-/-} showed increased susceptibility to bacterial infection (**Figure 2G**). In addition, we treated larvae with dextran sulfate sodium (DSS) (**Figure**

2H), which induces gut inflammation in mice and zebrafish^{41,48}. Similarly to IL-22-deficient mice⁴⁹, *il22*^{-/-} zebrafish larvae were highly susceptible to DSS treatment (**Figure 2I**).

In sum, we demonstrated the conservation of zebrafish IL-22 signaling pathways as well as protective functions against bacterial and chemical-induced inflammation during zebrafish early life.

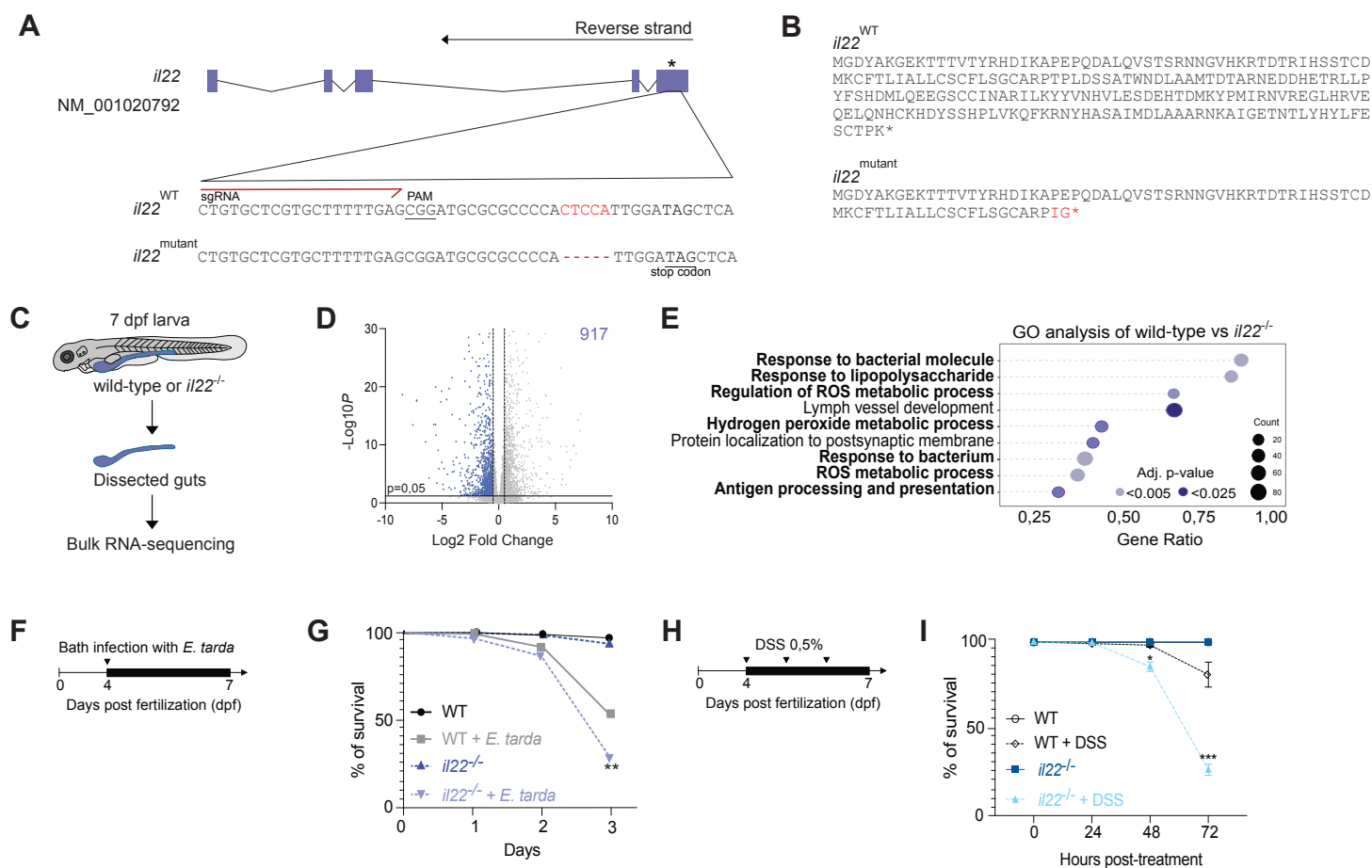


Figure 2. Conservation of IL-22 protective function in the zebrafish larvae gut

A. Schematic for the mutation generated in the zebrafish *il22* gene (-5bp) by CRISPR/Cas9. **B.** Predicted protein sequence for IL22 in wild-type (WT) and *il22* mutant individuals, according to the DNA sequences obtained. **C.** Diagram showing intestine extractions of WT or *il22*^{-/-} larvae at 7 days post fertilization (dpf) for bulk RNA-sequencing. **D.** Volcano plot showing the dysregulated genes in the *il22*^{-/-} with a $P < 0,05$ and a fold change > 1.5 . The 917 downregulated genes are labeled in blue. **E.** Gene ontology analysis comparing WT and *il22*^{-/-}. Most terms are associated with anti-bacterial immunity. **F.** Experimental strategy for a bath infection with live *Edwardsiella tarda* of WT and *il22*^{-/-} larvae. Larvae were infected from 4 to 7 dpf and survival was measured every day. **G.** Survival curve of WT, *il22*^{-/-} with or without *E. tarda* infection showing a significant increase of mortality in the mutant infected. **H.** Schematics of a chemical treatment with 0,5% DSS applied every day from 4 to 7 dpf WT and *il22*^{-/-} larvae, survival was measured every day. **I.** Survival curve of WT, *il22*^{-/-} with or without DSS-induced inflammation treatment showing a significant survival impairment in DSS-treated *il22*^{-/-}. Statistical analysis were performed with multiple comparisons 2-way ANOVA ** $P < 0,01$, *** $P < 0,001$. These data are representative of at least two independent experiments (except RNA-sequencing for which we had 4 replicates of each conditions).

Zebrafish IL-22 modulates the muscle-ENS unit development and function

We identified 1050 genes to be up-regulated in *il22*^{-/-} compared to WT guts (**Figure 3A**). Unexpectedly, GO analysis of those genes showed enrichment in processes associated with neuronal and axon development (**Figure 3B**). Of note, our RNA-seq on full dissected intestines contained gut epithelial cells and other cell types within this organ. We then validated selected dysregulated genes by qPCR (**Figure 3C, D**). To determine whether enteric neuron numbers were impaired in *il22*^{-/-}, we quantified them by immunostaining of HuC, a pan-neuronal marker, but found no significant difference (**Figure 3E, F**). However, our analysis may have overlooked impairments in neuronal subtypes not highly represented in the gut. To determine if enteric neurons present any functional defect, we used a reporter assessing neuronal activity based on the [Ca²⁺]_i-sensitive fluorescent protein Gcamp6f (*HuC:Gcamp6f*) and performed live imaging using light sheet microscopy (**Figure 3G**). Interestingly, we observed that *il22*^{-/-} neurons remain functional, but WT neurons seem to exhibit faster frequencies during each peak period compared to the mutant (**Figure 3H**). This finding suggests a role of IL-22 in regulating gut neuronal activity, although further analyses are needed to fully determine the nature of the impairment.

We next aimed to assess a physiological readout of a potential ENS impairment. One key function of enteric neurons is to regulate gut motility, thus we evaluated it by live imaging in 7 dpf larvae (**Figure 3I**). We found a strong impairment of gut motility in *il22*^{-/-}, with significantly slower movement speed (**Figure 3J, K**). Moreover, we complemented this observation with food transit analysis, measuring the distance between the intestinal bulb and the fluorescent food at different time points (**Figure 3L**). We observed significantly slower food transit in *il22*^{-/-} (**Figure 3M**), confirming IL-22 role in regulating gut motility in zebrafish larvae.

In addition to the enteric nervous system, muscles also play a crucial role in communicating with enteric neurons to drive gut motility. Interestingly, we found that several genes involved in muscle contraction tended to be down-regulated in *il22*^{-/-} (**Figure 3N**). To determine a potential impairment in muscle structure, we performed immunostaining of Desmin, a gut muscle marker, on dissected guts and quantified the number of circular smooth muscle cells (**Figure 3O**). We observed a significant

increase in the number of circular smooth muscles per area, suggesting a developmental muscular defect in *il22*^{-/-} (**Figure 3O,P**).

Altogether, our results point towards a novel role of IL-22 in regulating gut motility through the modulation of the ENS-muscle axis.

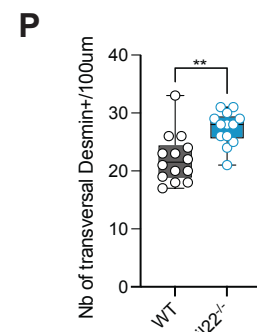
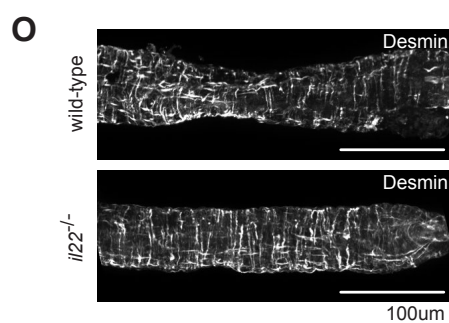
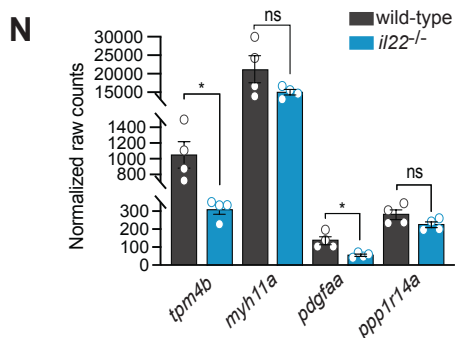
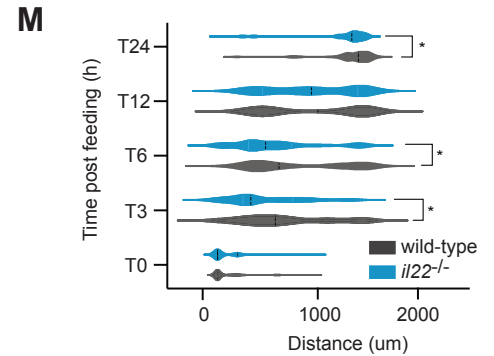
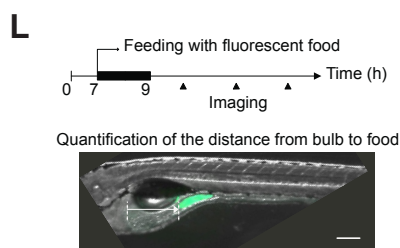
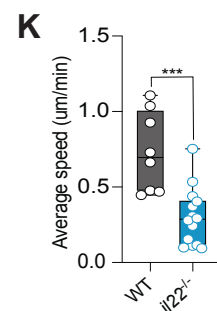
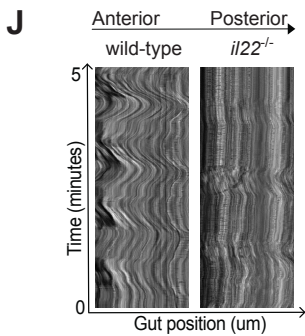
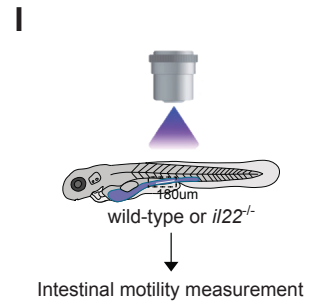
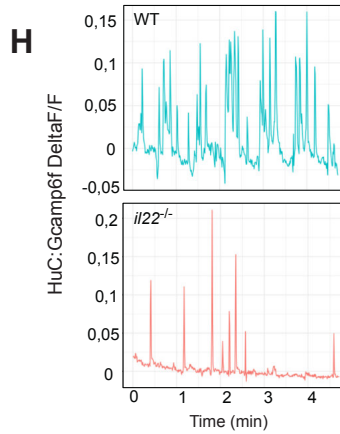
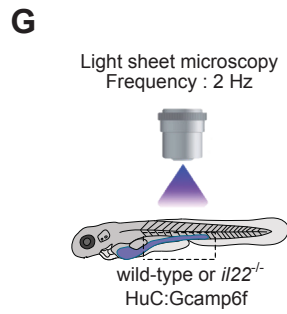
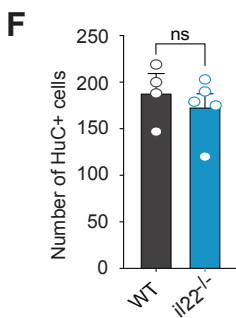
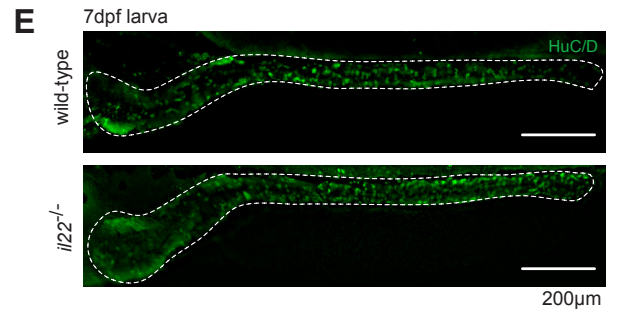
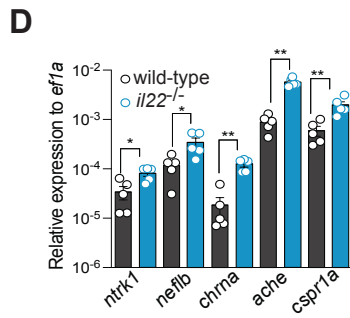
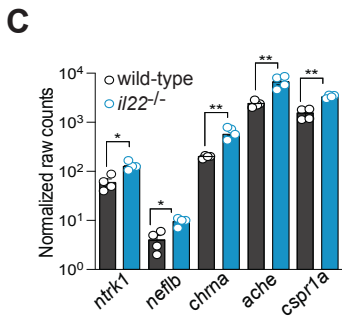
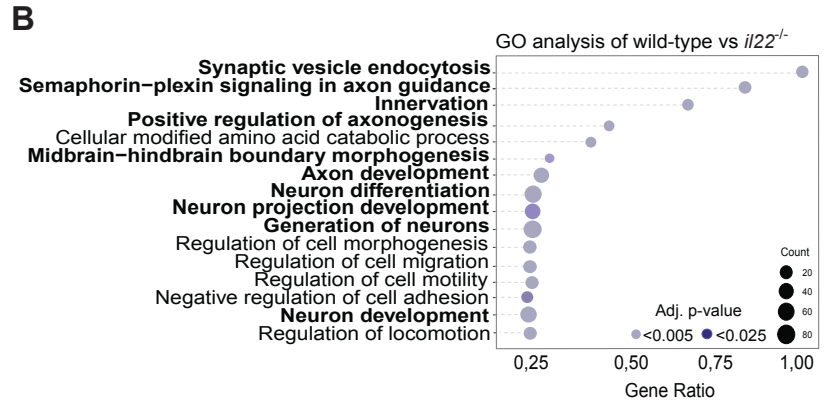
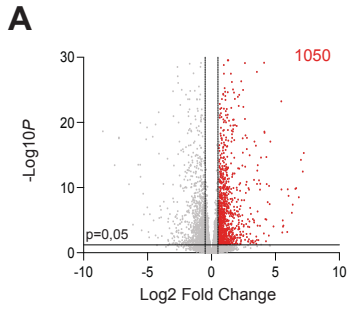


Figure 3. IL-22 plays a role in gut motility regulation

A. Volcano plot showing the dysregulated genes in the *il22*^{-/-} with a $P < 0,05$ and a fold change > 1.5 . The 1050 upregulated genes are labeled in red. **B.** Gene ontology analysis comparing WT and *il22*^{-/-} upregulated genes. GO terms in bold are associated with neuronal development. **C.** Normalized raw counts of neuronal dysregulated genes in WT and *il22*^{-/-}. **D.** RT-qPCR measurement of neuronal dysregulated genes in dissected guts from 7 dpf WT and *il22*^{-/-} larvae. **E.** Fluorescence image of WT and *il22*^{-/-} stained with an anti-HuC/D (a pan neuronal marker) antibody. Dot lines surround the zebrafish larval gut. Scale bar = 200um. **F.** Quantification of the number of HuC/D positive cells in the gut of 7 dpf WT and *il22*^{-/-}. **G.** Schematics of enteric neurons activity measurements combining the Tg(*Huc:Gcamp6f*) transgenic line with light sheet microscopy. This experiment has been performed on transgenic 7 dpf WT and *il22*^{-/-} larvae. **H.** Light-sheet imaging of calcium spikes in enteric neurons of 7 dpf WT and *il22*^{-/-} Tg(HuC:Gcamp6f) larvae. **I.** Experimental design for gut motility measurement. The analysis was performed on a segment of 180um in the midgut of 7 dpf WT and *il22*^{-/-} larvae. **J.** Kymograph analysis using ImageJ of 180um in the midgut of 7 dpf WT and *il22*^{-/-} larvae showing impairment in gut motility. **K.** Velocity measurement of the gut motility from 7 dpf WT and *il22*^{-/-} larvae. **L.** Schematic of the food transit experiment. In brief, during 2h larvae were fed with dry food coupled with fluorescent beads and the distance from the anterior bulb to the fluorescent food was measured at different time points. **M.** Location of the fluorescent food on the intestine of WT and *il22*^{-/-} larvae at different time points. **N.** Normalized raw counts of muscles associated genes from the WT and *il22*^{-/-} 7 dpf RNA-sequencing dataset. **O.** Confocal image of the dissected gut of WT and *il22*^{-/-} stained with an anti-Desmin antibody. Scale bar = 100um. **P.** Quantification of the transversal muscle fibers labeled with anti-Desmin antibody per 100um of intestine. Statistical analysis were performed with unpaired T-test : ns: not significant, * $P < 0,05$, ** $P < 0,01$, *** $P < 0,001$. These data are representative of at least two independent experiments (except RNA-sequencing for which we had 4 replicates of each conditions and the HuC:Gcamp6f measurements which are preliminary).

IL-22 control of peristalsis is gut-specific

Gut motility can be regulated by the central nervous system through the gut-brain axis and the vagal nerve, and it can also be influenced by other organs. Since IL-22 and its receptor are expressed in various organs, we sought to determine whether the action of IL-22 in controlling gut motility was specific to the gut. To this end, we aimed to establish a system allowing us to study the consequences of IL-22 signaling exclusively in the gut. To achieve this, our first step was to identify the gene encoding the IL-22-specific receptor chain in zebrafish.

In mammals, the IL-22 receptor comprises two chains: IL-10R2 and IL-22R1. IL-10R2 is a chain shared in other cytokine receptors, while IL-22R1, encoded by the *Il22ra1* gene, is specific to IL-22 and is expressed only in non-hematopoietic cells, such as epithelial cells^{38,50}. Despite this knowledge, the zebrafish ortholog of the *Il22ra1* gene was unknown. Using synteny analysis between mammals and fish, we identified a promising gene candidate previously annotated as *ifnlr1* (interferon lambda receptor 1), also known as *crfb14* (cytokine receptor family member B14). Interestingly, *crfb14* exhibited higher expression in sorted gut epithelial cells within the gut, akin to the mammalian IL-22R1 receptor (**Figure S3A**). To determine if this gene encodes the IL-22-specific receptor chain, we used CRISPR/Cas9 to generate a *crfb14* mutant line with a 5 bp insertion and a 43 bp deletion in exon 2 effectively removing an essential splicing site (**Figure S3B**). qPCR analysis confirmed significantly reduced *crfb14* gene expression in our mutant (**Figure S3C**). zrIL-22 injection into *crfb14*^{-/-} *mpx*:GFP fish failed to induce the expression of this IL-22-dependent gene, confirming that *crfb14* encodes the IL-22-specific receptor chain (**Figure S3D, E**). Importantly, we observed that gut motility was impaired in *crfb14*^{-/-}, similarly to *il22*^{-/-} (**Figure 4A, B**) further supporting that IL-22 signaling is required for proper gut peristalsis.

Since both *crfb14*^{-/-} and *il22*^{-/-} showed impaired gut motility, a process controlled by gut neurons and smooth muscle cells, we wondered if IL-22 could directly target these cell types. Upon re-analyzing various scRNA-seq datasets of zebrafish larval intestines⁵¹, we detected high *crfb14* expression only in gut epithelial cells and not in enteric neurons or smooth muscles (**Figure 4C**). Nevertheless, we aimed to determine whether the observed phenotype indeed resulted from gut epithelial cell-specific IL-22 signaling to rule out any indirect effects of IL-22 in other organs. To achieve this, we

sought to generate a transgenic line expressing *crfb14* exclusively in gut epithelial cells. We accomplished this by driving the expression of the *crfb14* gene with the gut epithelial-specific *cldn15la* promoter in *crfb14*^{-/-} fish (*crfb14*^{-/-};*cldn15la:crfb14*, from now on IEC-*crfb14* **Figure 4D**). Quantitative RT-PCR on dissected larval guts and trunks confirmed the expression of *crfb14* only in the gut and not in other body parts (**Figure 4E, F**). We then performed live imaging to examine gut motility in the IEC-*crfb14* line. Remarkably, we observed a recovery of the previously observed gut motility defect (**Figure 4G**). These data strongly indicate that IL-22 signaling specifically in IECs is sufficient to restore normal intestinal motility.

Taken all together, we have identified the gene encoding the IL-22-specific receptor chain in zebrafish and demonstrated that IL-22 controls intestinal motility through the specific activation of its receptor in gut epithelial cells.

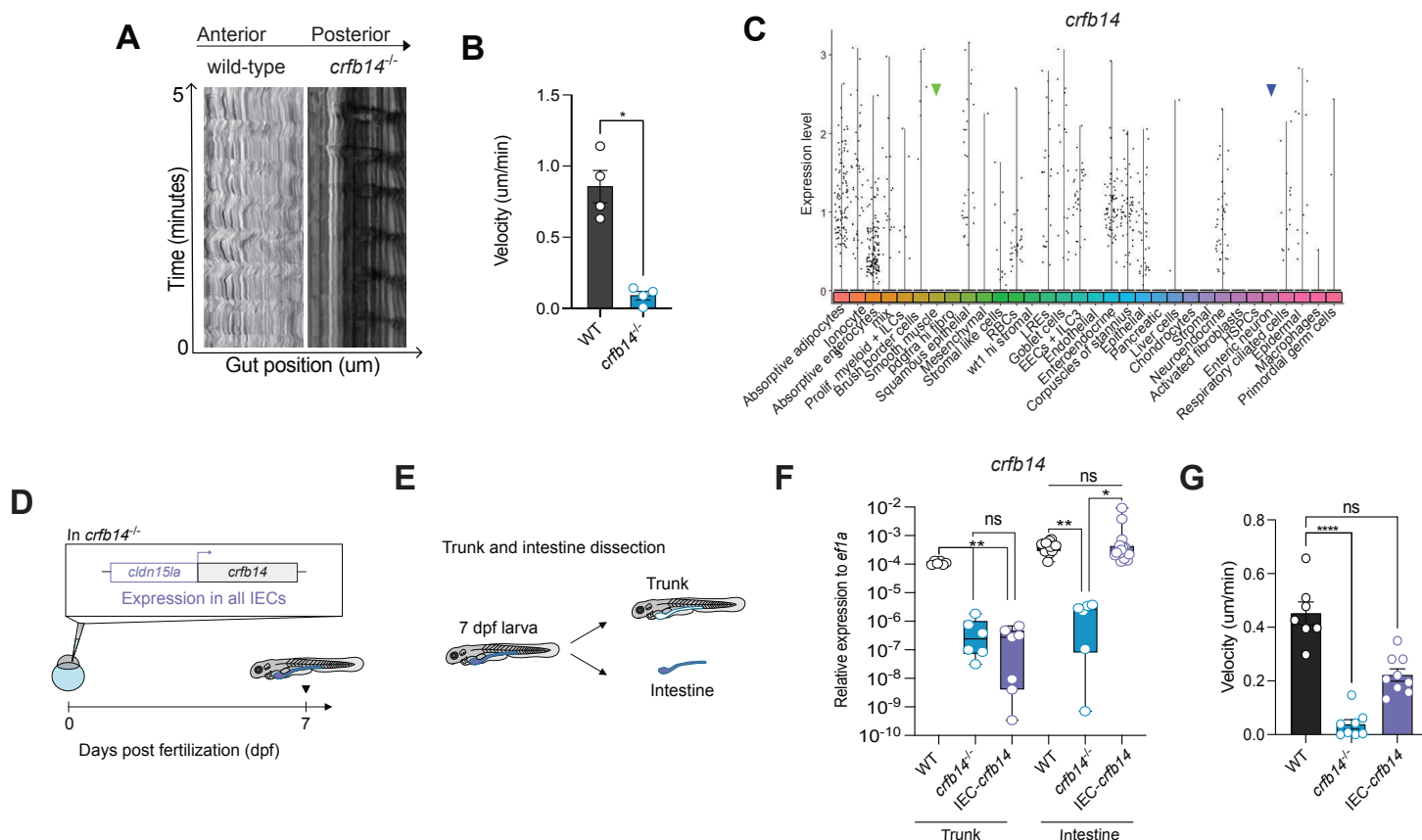


Figure 4. IL-22 regulates gut motility in an IEC-specific manner

A. Kymograph analysis using ImageJ of 180um in the midgut of 7 dpf WT and *crfb14*^{-/-} larvae showing impairment in gut motility. **B.** Velocity measurement of the gut motility from 7 dpf WT and *crfb14*^{-/-} larvae. **C.** Re-analysis of a single-cell RNA-sequencing dataset of zebrafish larvae gut published by Nayar et al, 2020 showing *crfb14* expression in IECs mostly and not in enteric neurons (blue arrow) nor smooth muscle cells (green arrow). **D.** Experimental design for generating a tissue-specific line re-expressing *crfb14* driven by an IECs specific promoter (*cldn15la*) in an *crfb14*^{-/-} background (IEC-*crfb14*). The construct was injected at 1-cell stage and a stable line was obtained after several crosses. Following experiments were performed at 7 dpf and the newly generated line will be named IEC-*crfb14*. **E.** Schematic of the trunk and intestine tissue collection performed on 7 dpf WT and *crfb14*^{-/-} larvae for RNA extraction. **F.** RT-qPCR measuring *crfb14* gene expression in the dissected trunk or gut of 7 dpf WT, *crfb14*^{-/-} and IEC-*crfb14* larvae. **G.** Velocity measurement of the gut motility from 7 dpf WT, *crfb14*^{-/-} and IEC-*crfb14* larvae. Statistical analysis were performed with Mann-Whitney : ns: not significant, * $P < 0,05$, ** $P < 0,01$. These data are representative of at least three independent experiments.

***il22*^{-/-} larvae have altered gut microbiota composition**

Our findings indicated that in the zebrafish larva IL-22 is both produced and signals in gut epithelial cells. In addition, we determined that expressing the IL-22 receptor exclusively in gut epithelial cells, and not in other cell types of the body, is sufficient to maintain normal gut motility. In mice, IL-22 shapes the composition of the gut microbiota by inducing anti-microbial peptide expression in gut epithelial cells^{52,53}. Furthermore, the gut microbiota plays a critical role in the development and function of enteric neurons⁵⁴ and muscles⁴. Since we found that the zebrafish IL-22 also induces anti-microbial gene expression in the zebrafish gut, and that this cytokine is key for proper gut motility, as well as both neuronal and smooth muscle function, we hypothesized that the phenotypes of *il22*^{-/-} could be attributed to an impaired microbiota composition.

To first analyze microbiota composition, we performed 16S RNA sequencing on dissected guts from 7 dpf WT and *il22*^{-/-} larvae. First, biological replicates from each of the sample groups consistently clustered together, suggesting that the composition of these bacterial communities are reproducible and different (**Figure S4A**). Second, the Shannon diversity index indicated a comparable level of microbial diversity between the two conditions (**Figure 5A**). The distinct separation of WT and *il22*^{-/-} samples observed in the PCA analysis was accompanied by significant differences in the relative abundances of several bacterial taxa. All samples were dominated by Proteobacteria phylum whereas Firmicutes were slightly enriched in *il22*^{-/-} (**Figure S4B**). More striking differences were observed at the family level, with an enrichment of *Enterobacteriaceae* and a strong reduction of *Rhizobiaceae* in the KO (**Figure 5B**). Interestingly, IL-22 expression has been shown to repress commensal *Enterobacteriaceae* through the induction of anti-microbial peptides in mice⁵². Altogether, our data show that IL-22 deficiency results in gut microbiota dysbiosis and suggest that this cytokine may play a conserved role in suppressing *Enterobacteriaceae* bacteria in zebrafish.

Gut microbes can modulate host production of molecules that interact with the nervous system and gut epithelial cells, and recent studies have shown that they can also produce these molecules themselves⁵⁵⁻⁵⁷. To determine if impaired interactions might take place between the gut microbiota and the host in *il22*^{-/-} larvae, we used a

published module-based analytical framework designed for the targeted analysis of metagenomic data in the context of microbiota-gut-brain communication⁵⁸. This allowed us to infer the neuroactive potential of the gut microbiota of zebrafish WT and *il22*^{-/-} larval guts. More specifically, we could know whether the bacteria identified contained the genes encoding for microbial pathways that metabolize molecules with the potential to interact with host cells neurons and epithelial cells. Each annotated module corresponds to a single production or degradation process of an active compound. We applied this framework analysis on the 20 most discriminative bacterial genus or families found by 16S RNA-seq in *il22*^{-/-} (**Figure 5C**). As expected, we noticed that the Clpb (ATP-dependent chaperone protein) module is indeed present in all the species studied as it has been described to be ubiquitous (present in >90% of gut microbial genomes)⁵⁸. Then, we observed an enrichment of species carrying the potential to synthesize kynurenine (involved in tryptophan metabolism), GABA (neurotransmitter), propionate (short-chain fatty acids (SCFAs)), quinolinic acid (immune system regulator), tryptophan (essential amino acids involved in serotonin synthesis), serotonin (neurotransmitter) and dopamine (neurotransmitter). Interestingly, tryptophan, serotonin, and GABA are all important regulators of gut motility^{7,59}. This suggests that the gut microbiota dysbiosis of *il22*^{-/-} might lead to a dysregulated production of SCFAs, hormones, and neurotransmitters that are important for proper gut motility. This impairment could hypothetically be responsible for the phenotype observed in the mutant.

Altogether, we have shown impaired gut microbiota composition in *il22*^{-/-} larvae, with dysregulated abundance of some species in a manner similar to mammals. Additionally, we have identified a potential impairment in the production of bacteria-derived molecules critical for maintaining gut motility and overall homeostasis.

Intestinal motility impairment in *il22*^{-/-} larvae is restored by co-housing and live microbiota transfer from WT larvae

Next, we aimed to determine whether the altered microbiota of IL-22-deficient larvae was responsible of the gut motility defect. To do so, we first generated GF WT and *il22*^{-/-} and compared their gut motility to their CV siblings. Surprisingly, we did not observe any difference between WT CV and GF larvae (**Figure 5E**) despite previous indications suggesting a faster gut motility in GF larvae⁶⁰. However, the difference in

the methodologies used could potentially explain this inconsistency. In mammals, the absence of microbiota has been associated with slower food transit⁶¹, thus the gut motility phenotype in GF animals seem to be highly variable. Intriguingly, *il22*^{-/-} GF showed a similar gut motility defect as their CV counterparts. Then, we co-housed WT GF or *il22*^{-/-} GF with conventional WT or *il22*^{-/-} thereby facilitating the transfer of gut microbiota and molecules (**Figure 5D, E**). We found that the gut motility impairment was not transferred to WT larvae when they were co-housed with *il22*^{-/-}. In contrast, co-housing with WT larvae was able to rescue the *il22*^{-/-} defect. These results suggested that the WT microbiota can provide crucial bacteria or factors that are necessary for normal gut motility and thus compensate for the lack of IL-22. We then sought to determine whether the bacteria and/or molecules present in the medium of WT larvae were sufficient to rescue the gut motility impairment of mutant larvae. To achieve this, we transferred water from WT CV fish to *il22*^{-/-} GF larvae during three days prior to analysis (**Figure 5F**). Interestingly, we observed that water transfer alone restored the gut motility impairment (**Figure 5G**). In parallel, we treated the water from WT larvae with antibiotics before transferring it to *il22*^{-/-} GF larvae (**Figure 5F, G**). We observed a significant reduction in the recovery of gut motility, indicating a critical role of live bacteria for the full rescue of the impairment of mutant larvae.

Collectively, we found that the transfer of live bacteria present in the water of WT larvae can rescue the gut motility defect in *il22*-deficient larvae. Our findings imply that IL-22 is crucial in maintaining a healthy gut microbiota, which in turn is necessary for proper gut movement.

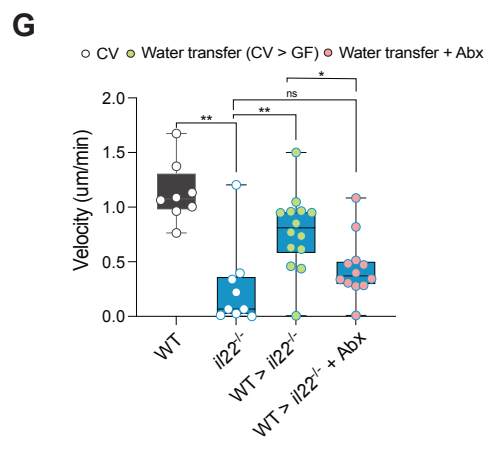
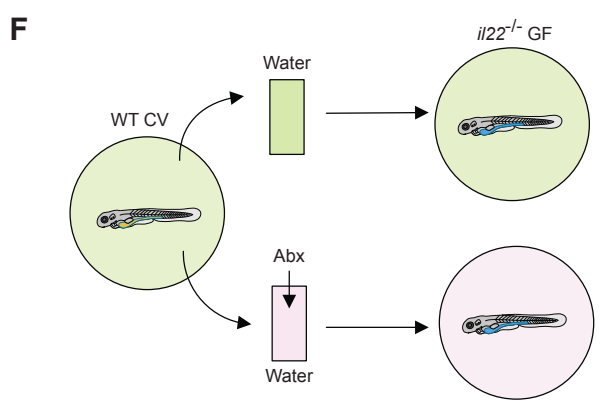
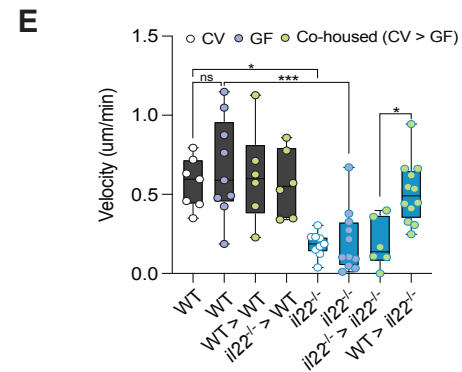
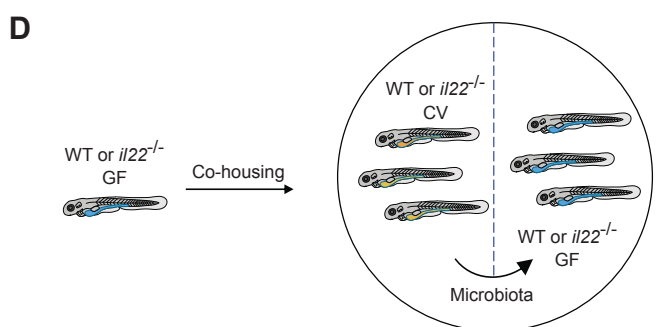
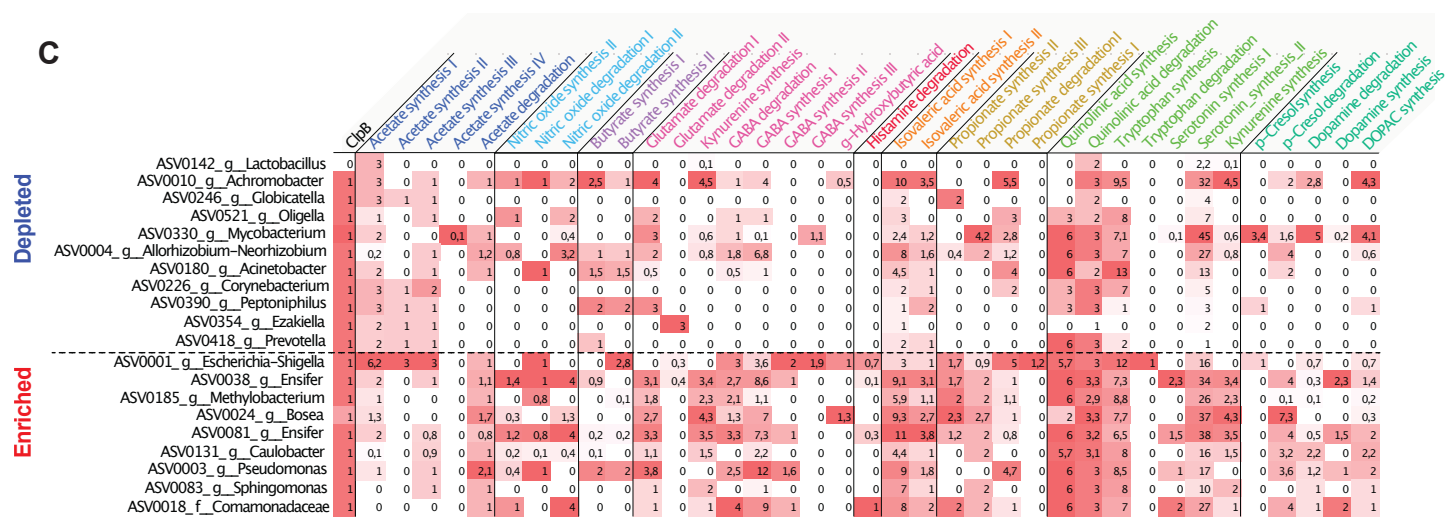
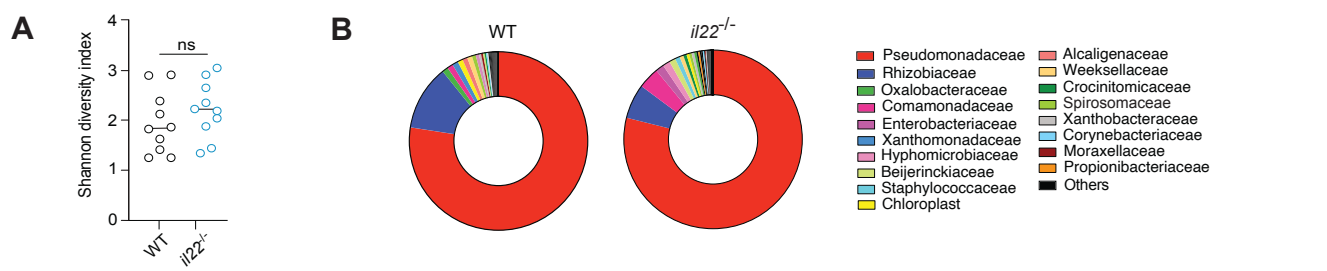


Figure 5. Lack of IL-22 leads to dysbiosis and gut motility impairment

A. 16S sequencing of WT and *il22*^{-/-} dissected intestines shows no difference in Shannon-Wiener diversity between the two groups. **B.** Taxon-based analysis at the family level between the two groups. **C.** Module-based analysis (established by Valles-Colomer and al, 2019), which analyze the synthesis or degradation of metabolites by bacteria from the microbiota. This methodology focuses on metabolites capable of communicating with neurons but also with EECs. The analysis has been performed on the 20 most discriminative species found by 16S RNA-seq in *il22*^{-/-} (enriched and depleted). **D.** Experimental drawing of the co-housing experiment. Briefly, conventionally-reared (CV) larvae were co-housed in the same petri dish with their GF counterparts for three consecutive days before being analysis allowing for transfer of the microbiota and metabolites. **E.** Velocity measurements of the gut motility from WT, *il22*^{-/-} CV alone, co-housed with their GF siblings, WT (CV) co-housed with *il22*^{-/-} (GF) and *il22*^{-/-} (CV) co-housed with WT (GF). **F.** Schematic representation of a water transfer experiment. Fresh water from WT CV was transferred to *il22*^{-/-} GF larvae every day during 3 consecutive days without or with antibiotic (Abx) treatment. **G.** Velocity measurements of the intestinal motility from WT, *il22*^{-/-} conventionally-reared (CV), from WT (CV) water transferred to *il22*^{-/-} (GF) and from WT (CV) water treated with antibiotics transferred to *il22*^{-/-} (GF). Statistical analysis were performed with Mann-Whitney : ns: not significant, * $P < 0,05$, ** $P < 0,01$, *** $P < 0,001$. These data are representative of at least three independent experiments (except the 16S RNA-sequencing for which we had 15 replicates per condition).

IL-22 deficiency impairs EECs function, but 5-HT restores gut motility

To gain insight into mechanisms by which WT microbiota might rescue the gut motility impairment of *il22*^{-/-}, we focused on critical players that coordinate microbiota with muscle and neuron function to enable proper gut motility. Interestingly, EECs are a central gut epithelial cell type that responds to dietary or bacterial cues, particularly tryptophan metabolites. They play a key role in regulating digestion, nutrient absorption and gut motility. The activity of EECs is characterized by the production of hormones and neuropeptides that target neurons and muscles. Therefore, we hypothesized that EEC dysfunction links microbiota dysbiosis and gut motility impairment in *il22*^{-/-}.

RNA sequencing comparing WT and *il22*^{-/-} guts (**Figure 2**), revealed a trend towards dysregulation in the expression levels of multiple hormones in the mutant, including *pyy* (which encodes for the neurotransmitter PYY) and *gcga* (which encodes for the hormone glucagon) (**Figure 6A**). Furthermore, we observed their significant dysregulation by RT-qPCR (**Figure S5A**). Immunostaining also revealed fewer PYY-positive cells in the gut of *il22*^{-/-} larvae (**Figure S5B, C**). Interestingly, we also observed a reduction in the expression of the *tph1b* gene, which encodes a rate-limiting enzyme in the synthesis of serotonin, commonly known as 5-HT (**Figure 6A**). The latter has been shown to play a critical role in the regulation of gut motility in both mammals and zebrafish^{7,56,62}. In addition, 5-HT has been shown to be induced upon *Trpa1b* activation by tryptophan-derived metabolites and to induce gut motility through activation of enteric neurons⁷. Immunostaining in larval guts showed a strong reduction in the number of EEC positive for 5-HT cells in *il22*^{-/-} compared to WT (**Figure 6B, C**). We then wondered whether 5-HT alone could restore the gut motility impairment in *il22*^{-/-}. To this end, we exposed larvae to 250uM 5-HT during 3 days and measured gut motility at 7 dpf (**Figure 6D**). As expected, we noticed increased gut motility in WT upon 5-HT exposure (**Figure 6E**). Intriguingly, we observed a complete recovery of the gut motility impairment in *il22*^{-/-}, indicating that 5-HT alone is sufficient to restore gut motility (**Figure 6E**).

In summary, we found an impairment of EEC function with dysregulated hormone expression, particularly 5-HT. Interestingly, 5-HT administration was sufficient to restore proper gut motility. Work is underway to further understand the mechanisms by which IL-22 maintains proper gut motility.

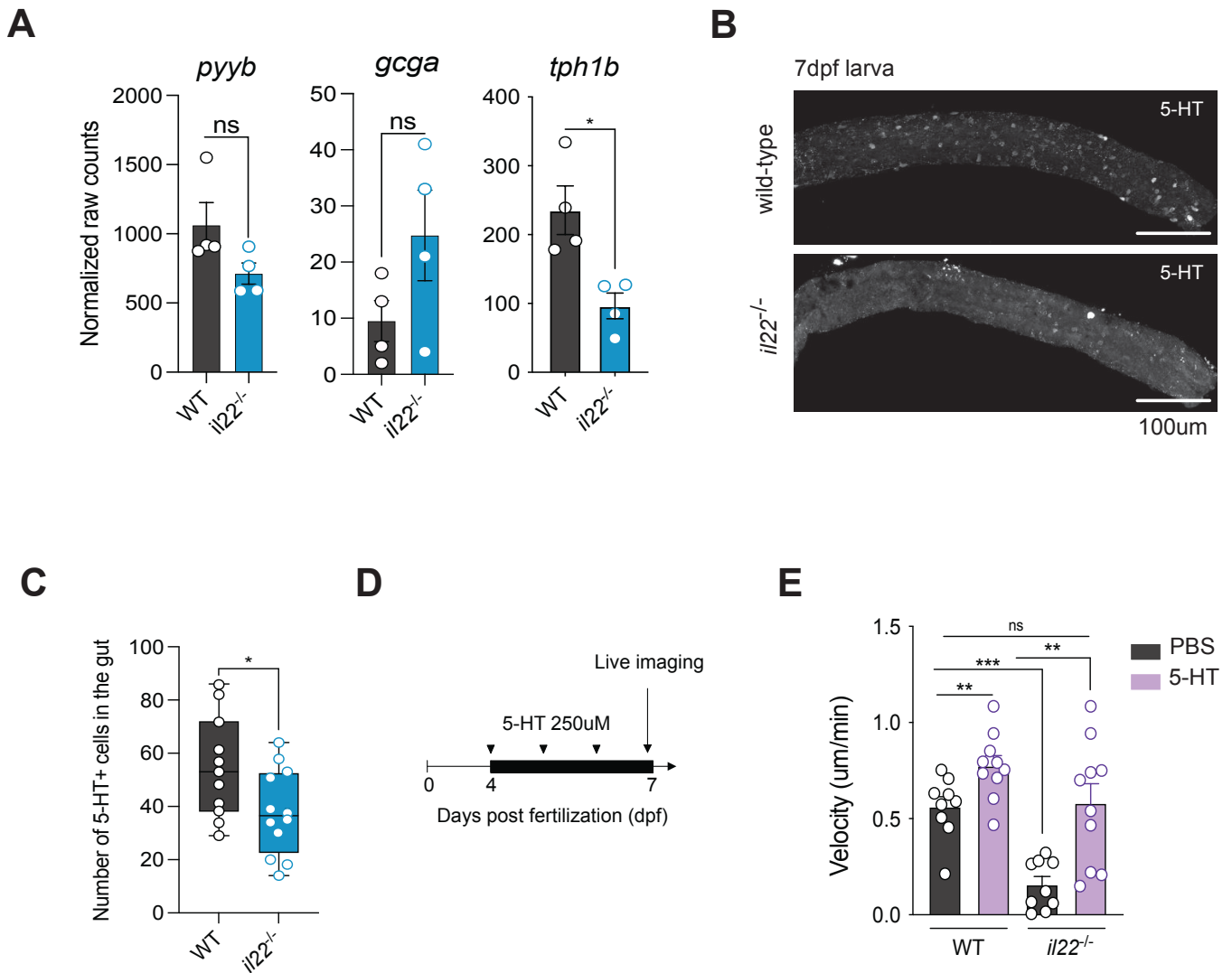


Figure 6. EEC function is impaired in *il22*^{-/-} and 5-HT administration is sufficient to restore gut motility.

A. Normalized raw counts of genes encoding for hormones (*ppyb*, *gcga*) or a rate-limiting enzyme crucial to in 5-HT synthesis (*tph1b*) in WT and *il22*^{-/-}. **B.** Confocal image of a 7 dpf WT or *il22*^{-/-} dissected intestine stained with an anti-5-HT antibody. Scale bar = 100um. **C.** Quantification of the number of 5-HT positive cells in the gut of 7 dpf WT or *il22*^{-/-}. **D.** Experimental procedure describing the administration of 5-HT in the water. Freshly prepared 5-HT (250uM) was added every day from 4 to 7 dpf to the water of WT or *il22*^{-/-} larvae. Gut motility was analyzed by live imaging at 7 dpf. **E.** Velocity measurements of the intestinal motility from WT, *il22*^{-/-} treated or not with 5-HT. Statistical analysis were performed with Mann-Whitney : ns: not significant, * $P < 0,05$, ** $P < 0,01$, *** $P < 0,001$. These data are representative of at least two independent experiments (except the RNA-sequencing data for which we had four replicates per condition)

IL-22 is necessary to maintain proper gut motility during early life of mice

To address whether our findings in zebrafish larvae also take place in mammals, we used WT or *Il22*^{-/-} mice at different stages (3-4, or 5 or 8-10 weeks old) and measured food transit speed. Interestingly, we observed a slower food transit time in *Il22*^{-/-} at 3-4 weeks (**Figure 7**). However, this phenotype did not take place at older ages, indicating that IL-22 likely influence gut motility in mice during a specific time window. Whether the regulation of intestinal motility by IL-22 occurs through similar mechanisms in mice as in zebrafish remains to be investigated.

In conclusion, our data reveal an evolutionarily conserved role for IL-22 in modulating gut motility in the early vertebrate gut.

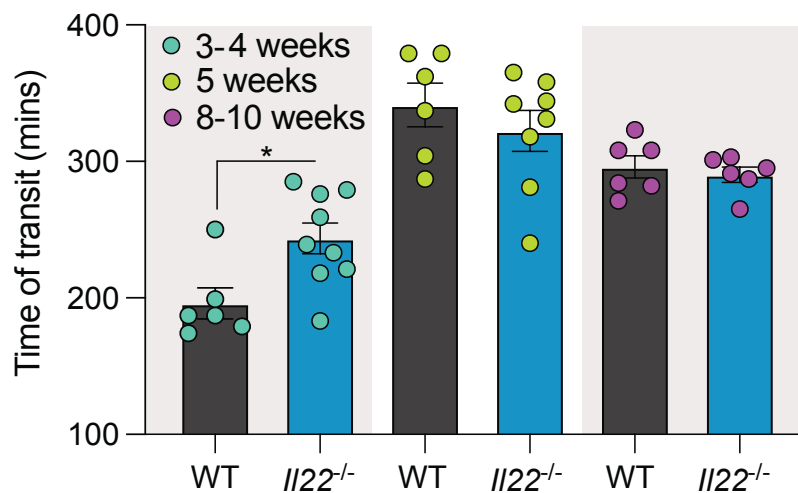


Figure 7. The lack of IL-22 impairs gut motility in mice

Food transit experiment performed on WT or *Il22*^{-/-} 3-4, 5 or 8-10 weeks old-mice. The time of transit is significantly impaired in *Il22*^{-/-} 3-4 weeks old only. Statistical analysis were performed with Mann-Whitney : * $P < 0,05$. These data are representative of two independent experiments.

Discussion

In mice, the initial studies of IL-22 primarily focused on its role in defending the gut epithelium against damage induced by bacterial infection¹⁹, chemical agents²⁶, and viral infections²². Subsequently, it was recognized for promoting DNA damage response in gut epithelial stem cells⁶³. Furthermore, the role of IL-22 in negatively regulating lipid absorption and metabolism was described²⁸. In mice, microbial and dietary signals, including tryptophan-derived metabolites *via* the aryl hydrocarbon receptor (AhR), have been identified as important inducers of IL-22 expression¹⁶. Furthermore, the significance of tightly regulating IL-22 activity is underscored by the existence of a negative regulator, the soluble receptor IL-22 binding protein (IL-22BP)⁶⁴. Notably, IL-22BP-deficient mice exhibit a higher incidence of intestinal tumors and metabolic imbalances²⁶. The zebrafish emerges as a powerful model for studying IL-22 functions, given the conservation of cell type diversity in the gut, including immune and epithelial cells, and the functional regionalization along the gut, which mirrors that of mammals⁶⁵.

Our results reveal a surprising function of IL-22 in regulating gut motility during early life, highlighting an unexpected, central role of this cytokine in regulating the onset of gut physiology. First, our study revealed the expression of IL-22 in EECs, prior to lymphocyte colonization of the zebrafish gut. Despite the difference in cell sources during early development between zebrafish and mammals, we discovered comparable regulatory mechanisms. Our findings indicate that microbiota induces *il22* expression, similar to mammals. Notably, the subtype expressing *il22* also showed elevated levels of *trpa1b*, which encodes for the TRPA1 ortholog. Trpa1 is a primary nociceptor involved in pain sensation and neuroinflammation. Trpa1 can be activated by environmental chemical irritants and inflammatory mediators⁶⁶. Recent evidence shows that it is also activated by microbes, specifically tryptophan metabolites⁷. Tryptophan, an essential amino acid, is released in the intestinal lumen through dietary protein digestion or microbial synthesis. It is widely accepted that gut microbes are capable of breaking down tryptophan to generate a diverse range of metabolites, including the first identified and frequently most prevalent indole⁶⁷. Tryptophan metabolites observed in mammals have the ability to induce IL-22 expression through the binding of an AhR ligand such as indole in ILCs¹⁸. Here, we demonstrate the

preservation of *il22* induction by tryptophan-derived indole. However, additional investigations are required to fully validate the involvement of the Trpa1 receptor.

Interestingly, we also found conservation of IL-22 anti-bacterial properties in zebrafish larvae. Notably, we found strong induction of *mpx* (*myeloperoxidase*) gene expression in IECs upon zrIL-22 injection. *Mpx* is highly expressed by neutrophils and has microbicidal and pro-inflammatory effects⁴⁷. This led us hypothesize that IL-22 induces IECs to express genes with similar functions in teleost fish and mammals despite the lack of zebrafish orthologs for key mammalian IL-22 target genes such as the anti-microbials Reg3g, Reg3b and Defb2. The precise mechanisms and potential anti-bacterial genes induced by IL-22 to protect the gut against bacterial infection in zebrafish larvae remains still unclear.

IL-22 has been demonstrated to modulate microbiota composition due to its ability to induce various anti-microbial peptides and mucins, which aid in safeguarding the epithelial barrier^{28,53,68}. Our study revealed numerous differences between the altered microbiota resulting from the absence of IL-22 compared to the microbiota in wild-type zebrafish. The absence of IL-22 resulted in a decrease of a variety of bacteria, including the Gram-positive family *Lactobacillaceae* that produces lactic acid and is generally considered a part of the healthy flora in mammals⁵³. Furthermore, we noted the enrichment of *Enterobacteriaceae*, a phenomenon previously observed in *il22*^{-/-} mice^{52,53} suggesting overall conserved mechanisms through which this cytokine regulates microbiota composition. Interestingly, we found that the alteration in microbial composition potentially results in changes in bacterial-derived metabolite production. Potential alterations were observed in bacteria capable of synthesizing tryptophan, which has already been associated with gut motility regulation through the activation of the Trpa1 receptor in zebrafish and mice⁷. Notably, other metabolites such as GABA, previously linked to gut motility⁵⁹, may also be dysregulated. Further investigations are needed to better understand whether the tryptophan metabolites/Trpa1 axis is responsible for the observed gut motility defect in *il22*^{-/-} or if other metabolites might also be involved.

Studies in mice have shown that transferring altered gut microbiota from mice that lack IL-22 to wild-type mice can increase their susceptibility to DSS-induced colitis⁵³. However, it should be noted that the composition of the microbiota in these animals, raised conventionally, was not examined at earlier ages when no inflammation was present, preventing analysis of the concurrent development of the microbiota and

inflammatory responses during early life stages. Studying microbiota changes that precede spontaneous colitis could provide valuable insights, whereas the differences in microbial composition detected between wildtype and *Il22^{-/-}* mice in this study might result from the inflammatory environment that favors bacteria exhibiting increased resistance to such conditions. In our study, we found that transferring *Il22^{-/-}* microbiota to WT did not result in gut motility impairment transfer. On the other hand, we noticed an improvement in this phenotype upon transferring WT microbiota to *Il22^{-/-}* in early life. Therefore, it is worth considering whether early life transfer of WT microbiota to *Il22^{-/-}* animals could improve colitis or other phenotypes triggered by IL-22 deficiency.

The microbiota actively communicates with the brain to regulate numerous physiological processes. Additionally, the ENS communicates bidirectionally with the central nervous system (CNS), giving rise to the microbiota-gut-brain axis. Our study points to a defect in ENS development or function due to the lack of IL-22, leading to dysregulated genes expressed in neurons. Despite the absence of differences in the total number of neurons, we discovered a noteworthy change in neuronal activity that requires further characterization. We speculate that impaired ENS-CNS crosstalk in animals lacking IL-22 might result in potential deficiencies in brain function and other organs. Furthermore, we also noticed a smooth muscle impairment. We observed that neither enteric neurons nor smooth muscle cells express detectable levels of the IL-22 specific receptor chain. Therefore, we hypothesize that dysregulation of the enteric nervous system and muscles is caused by dysbiosis resulting from impaired expression of anti-bacterial genes in epithelial cells in the absence of IL-22. The precise molecular link between gut motility impairment and dysfunction in neurons and muscles remains to be fully characterized.

90% of 5-HT in the intestine originates from enterochromaffin (EC) cells, an EECs subtype. Therefore, it has been hypothesized that 5-HT secretion by EC cells plays a crucial role in the control of intestinal motility⁶⁹. However, recent studies have contradicted this idea. Despite the lack of 5-HT production in EC cells in *Tph1^{-/-}* mice (a gene coding for a rate-limiting enzyme for 5-HT biosynthesis), only minor effects on gastric emptying, intestinal transit and colonic motility were observed⁷⁰. Thus, the precise role of 5-HT expressed in EC cells is not definitively established. Recent research indicates that 5-HT production in EC cells may facilitate gut motility changes in response to environmental or microbial stimuli, but perhaps not under normal physiological conditions⁷. Recently, it has been demonstrated that gut bacteria can

express 5-HT⁷¹. However, it is not clear if bacteria expressing 5-HT exhibit any impairment in *Il22*^{-/-} larvae. Our microbiota composition analysis utilizing a module-based analytical framework⁵⁸ revealed that there might be a dysregulation in bacterial species capable of synthesizing tryptophan, which is crucial for 5-HT production. The precise enzymes responsible for the biosynthesis of bacterial-derived 5-HT as well as its role remain a novel exciting area of research. Furthermore, although gut motility was improved in *Il22*^{-/-} larvae following administration of 5-HT, it is possible that other dysregulated hormones or factors such as PYY may also contribute. Nonetheless, studies have indicated that 5-HT targets both the neurons and smooth muscle cells to regulate their activity and consequently, gut motility. Notably, we observed impairment in both compartments, which could be due to a deficiency in 5-HT signaling. However, more research is needed to determine whether the reduction in 5-HT production by EC cells is the cause of these changes. Overall, our findings propose a model where IL-22 preserves a healthy gut microbiota composition, in which specific microbial communities or species express and/or induce 5-HT secretion from Trpa1+ EECs to modulate intestinal motility. This might represent a pathway by which IL-22 modulates 5-HT signaling and, ultimately, intestinal motility.

Our results from *Il22*^{-/-} mice revealed that the gut motility phenotype takes place only in mice aged 3-4 weeks, which coincide with the weaning process (transition from milk to solid food). Following weaning, the microbiota and immune system undergo significant transformations⁷² that possibly explain the declining impact of IL-22 deficiency on gut motility at older ages. This implies that IL-22 plays an important role in gut motility before weaning. However, further research is necessary to establish whether the mechanisms discovered in zebrafish are also taking place in mice. Nevertheless, we can conclude that the IL-22/gut motility axis is a process that has been conserved evolutionarily among vertebrates.

IL-22 has been extensively studied in IBD^{73,74} but to a lesser extent in IBS (irritable bowel syndrome), the most common disorder of gut-brain interactions diagnosed in gastroenterology⁷⁵. IBS is known to have a multifactorial etiology and its pathophysiology involves epithelial dysfunction, changes in gut microbiota composition as well as gut motility impairment⁷⁶. Following gastrointestinal infections, individuals may experience chronic abdominal pain coupled with anxiety and depression which can persist even after pathogen clearance, causing Post-Infectious IBS (PI-IBS). A recent study suggests that the Ahr/IL-22 signaling pathway is altered in

PI-IBS⁷⁷. Our work demonstrates that a lack of IL-22 results in a gut motility defect, which is also a major characteristic of IBS and a feature of more than one third of IBD patients⁷⁸. Therefore, a better understanding of this pathway could be lead to potential treatment for IL-22-associated gut motility and neurological disorders.

Material and methods

Zebrafish lines and husbandry

The maintenance of zebrafish wild-type line (AB), transgenic lines Tg(*mpx:EGFP*)¹¹⁴⁷⁹, Tg(*neurod1:GFP*)⁸⁰, TgBAC(*cldn15la:GFP*)*pd1034*⁸¹, *tlr5b*^{sa16424} (KIT, #17089) and Tg(*neurod1:GcaMP6F*)*icm05*⁸² was performed in accordance with European Union regulations on laboratory animals.

Mice

Wild-type (C57BL/6) and *Il22*^{-/-} mice were maintained in the Institut Pasteur animal facilities. Animal care and experiments were performed according to the committee on animal experimentation of the Institut Pasteur and authorized by the French Ministry of Research.

Construction of mutant zebrafish lines and genotyping

The zebrafish coding sequence for the ortholog of the human *IL22* gene (Gene name: *il22*, ENSEMBL ID: ENSDARG00000045673) was targeted by CRISPR/Cas9 with specific sgRNA: CTGTGCTCGTGCTTTTTGAG. The same method was used for the *crfb14* gene in zebrafish that we identified as the IL22RA1 ortholog (Gene name: *ifnlr1*, ENSEMBL ID: ENSDARG00000087131) with this specific sgRNA: TCAAACGGCTCTTT. For the maintenance of WT, *il22*^{-/-} and *crfb14*^{-/-} mutant stocks, zebrafish embryos derived from the incrossing of heterozygous *il22* and *crfb14* individuals were reared and genotyped when they reached adulthood by cutting off part of the caudal fin (Fin Clip). Briefly, Adult and larvae zebrafish were anesthetized with tricaine (100 ug/ml, Sigma Cat#A5040), their tails were cut and incubated during 1h at 56°C with FinClip buffer (Tris pH 8 10mM, EDTA 10mM, NaCl 200mM, SDS 0,5%) containing Proteinase K (0,2mg/mL, Invitrogen #25530-049). The mutation was genotyped using the KASP system (LGC genomics). The KASP assay was performed according to the manufacturer's instructions⁸³. The reaction mix per reaction consists of 5.1 µl KASP Master Mix containing the two allele-specific primers and one reverse primer (see Table 1) and 0.138 µl Assay Mix containing universal fluorescent probes,

Taq polymerase and dNTPs in an optimized buffer solution. The only exception was for *crfb14*^{-/-} that we genotyped by RT-qPCR (see primers Table 1).

Construction of the *il22:mCherry* transgenic zebrafish line

The 6.5 kb *SpeI*-*PstI* fragment from PAC clone BUSMP706A0151Q01 (IMAGE) covering the *il22* promoter was cloned ahead of the ORF for a farnesylated version of mCherry in a Tol2 derivative vector to yield vector pTol2-*piil22*mC-F. The fragment includes exon 1 including the first codons of the zebrafish *il22* ORF. This construct was co-injected with Tol2 mRNA into 1-cell stage eggs of AB origin. Screening for mCherry positive fish was performed by PCR.

Construction of the *cldn15la:GFP-p2a-crfb14* zebrafish line

The original plasmid was the pDestTol2pA2_349cldn15la-GFP-KRASV12 kindly provided by Filippo Del Bene. The *crfb14* coding sequence (Gene name: *ifnlr1*, ENSEMBL ID: ENSDARG00000087131) was synthesized by Genescript and replaced the *kras*^{V12} gene expression in the aforementioned plasmid. The generated plasmid construct (25 ng/μl) was then co-injected with mRNA transposase (50 ng/μl) into 1-cell stage *crfb14*^{-/-} embryos⁸⁴ and the resulting embryos were grown to adulthood for stable line screening. The rescue line name has been shortened to *crfb14*^{-/-} F1.

Length and area measurements

Larvae were anesthetized with tricaine and mounted in 3% methylcellulose for live imaging. Body and intestinal length measurements were performed from the intestinal bulb until the end of the intestine at the anal pore of the larva, and body length or area measurements in adults and larvae were done from the head to the tail. The measurements were quantified using ImageJ software (NIH).

Alcian blue staining

Fixed larvae in Paraformaldehyde 4% (Polysciences inc., #04018-1) were rinsed with acidic ethanol (70% ethanol (VWR, #20821.310) with 1% concentrated hydrochloric acid (AnalaR NORMARK, #20252.290)) before being incubated with 0.1% alcian blue (Sigma, #33864-99-2) diluted in 80% ethanol, 20% glacial acetic acid (SAFC, #ARK2183) during 3h at RT. Then, larvae were washed with acidic ethanol and imaged with an Upright Epifluorescence Microscope (Leica DM4 B) equipped with a color camera (DFC4500 Leica).

Immunofluorescence staining and imaging

Immunostaining was performed on whole larvae at 5 days post fertilization. Paraformaldehyde at 4% was used to fix zebrafish larvae overnight at 4°C. The sample were then washed with distilled water. Fixed larvae were then permeabilized with cold 100% acetone (Honeywell, #32213) during 20 min at 4°C before being washed three times with PBST (PBS 1X + 0,5% Triton-X100 (Invitrogen, #10717503)). Samples were then permeabilized with 1mg/mL Collagenase from *Clostridium histolyticum* (Sigma, #C2139) during 2h at room temperature. Samples were then washed with PBST and blocked with 10% of FBS (fetal bovine serum)/PBST at room temperature for more than 2h. The primary antibodies (see Table 1) were diluted in blocking solution solution and incubated at 4°C for more than 24h. Following primary antibody incubation, the samples were washed with PBST solution and incubated during at least 2h with secondary antibodies (see Table 1) at room temperature in the dark. Imaging was performed with THUNDER Imager Model Organism (Leica) with lens and analyzed with ImageJ software.

Neuronal activity measurements

To characterize the neuronal activity of Tg(HuC:*Gcamp6f*), larvae were first embedded laterally in a thin layer of 4% Low melting point agarose (Promega, #V2111). Then, we used selective-plane illumination microscopy (SPIM) to record the neuronal activity at cellular resolution across the gut. Optical sectioning was achieved by the generation of a micrometer-thick light sheet to excite GCaMP from the side and the front of the

larva. The GCaMP emission was collected by a camera whose optical axis was orthogonal to the excitation plane (a 488 nm laser, Phoxx 480-200, Omicron). In both arms the laser beam was first filtered by a 488 cleanup filter (F488 Omicron) and coupled to a single-mode fiber optic. The beam was expanded using a telescope ($f = 50$ mm, LA1131-A, and $f = 150$ mm, LA1433-A, Thorlabs) and projected onto two orthogonal galvanometric mirrors (HP 6215H Cambridge technology) to scan the laser beam, whose angular displacement were converted into position displacement by a scan lens ($f = 75$ mm AC508-075-A-ML, Thorlabs). The laser beams were then refocused by a tube lens ($f = 180$ mm, U-TLUIR, Olympus) and focused on the pupil of a low-NA (0.16) 5x objective lens (UPlan SAPO 4x, NA = 0.16, Olympus) facing the specimen chamber. The arrangement yielded a 1mm-wide illumination sheet and a beam waist of 3.2mm ($1/e^2$). The emitted fluorescence light was collected by a high-NA water-dipping objective (N16XLWD-PF, 16x, NA = 0.8, Nikon) mounted vertically on a piezo translation stage (PI PZ222E). A tube lens ($f = 180$ mm U-TR30IR, Olympus), a notch filter (NF03-488, to filter the laser's excitation light), a band-pass filter (FF01 525/50 Semrock) and a low-pass filter (FF01 680 SP25 Semrock, to filter the IR light) were used to create an image of the GCaMP emitted fluorescence on a sCMOS sensor (Orca Flash 4.0, Hamamatsu). The volumetric gut recordings were obtained by sequentially recording the fluorescence in 40 coronal sections spaced by 3 μ m. For this purpose, the light sheet was scanned vertically in the dorso-ventral direction in synchrony with the objective of the emission path. The camera was triggered to acquire an image every Texposure = 10 ms. Once the 40 coronal sections were recorded, the position of the light sheet and the objective of the emission path was reset to their initial dorsal position (Treset = 100 ms). This resulted in a volumetric acquisition time of 0.5 s or a rate of 2 Hz. The cell tracking and intensity measurement were performed using Imaris.

Body-intestine dissection in zebrafish larva

Larvae at 7dpf were euthanized by overdosing them with tricaine. Intestines were extracted mechanically by using tweezers (WPI, #142400). 5 intestines and their respective bodies were used for each replicates and further analyzed by RT-qPCR.

Quantitative real-time PCR

Intestines or body carcasses were pooled and RNA was extracted using the Single cell RNA purification kit (Norgen Biotek Corp, Cat. 51800) following manufacturer's instructions. Synthesis of cDNA was performed using the M-MLV Reverse Transcriptase Kit (Invitrogen). Real-time PCR was performed using the Rox SYBR Green MasterMix dTTP Blue Kit (Takyon) and run on a Thermo ABI ViiA 7 Real-Time PCR System (Thermo Applied Biosystems).

Samples were analyzed using Δ Ct method. The mean Ct value of housekeeping gene (*ef1a*) was used for normalization. Primers used for qRT-PCR are found in Table 1.

Fluorescence-activated cell sorting (FACS)

To acquire the intestinal epithelial population, approximately 100 7 days post-fertilization TgBAC(*cldn15la*:GFP) zebrafish larvae were collected, then intestines were dissected and placed into PBS on ice with a dissection time of maximum 2 hours. Intestinal cell dissociation was performed using TrypLE Express (Gibco, #12605028) for 1h at 37°C, pipetting up and down every 10 minutes to support digestion. Digested samples were spun at 1500g for 5 min at 4°C and wash twice with PBS 1X before being resuspended together with PBS 1X and 10% FBS (fetal bovine serum). Filtered cells were immediately subjected to FACS at the Institut Curie Flow Cytometry Platform with a Sony SH800 Cell Sorter. Dead cells were excluded from analysis using a combination of Calcein Blue (Invitrogen, #65-0855-39) and Propidium Iodide viability stains (Sigma, #P4864). Non-transgenic and single transgenic controls (pools of 10 dissected guts) were prepared as above and used for gating and compensation. RNA isolation was done using on average 30000 GFP+ or GFP- sorted cells with the Single Cell RNA Purification Kit from Norgen Biotek Corp and reverse transcribed using Superscript IV Reverse Transcriptase (Life Technologies, #18090050) following manufacturer's instructions. Quantitative PCR was performed with gene-specific primers (see Table 1). qPCR was performed using Low ROX SYBR Master Mix dTTP Blue (Takyon, #UF-LSMT_B0701) on an Applied Biosystems StepOnePlus Real-Time PCR System. Data were analyzed with the Δ Ct method.

Bulk RNA-sequencing and analysis

10 guts of 7 days post-fertilization WT and *il22^{-/-}* larvae were dissected per replicates (4 replicates per genotype, 8 in total). Total RNA was extracted with the Single-Cell RNA Purification kit (Norgen Biotek, #51800) following manufacturer's instructions. The RNA integrity and concentration were analyzed on Agilent 4200 TapeStation system using the high sensitivity RNA ScreenTape Analysis kit (Agilent, #5067-5579) and apparatus. RNA sequencing libraries were prepared from 500 ng of total RNA using the Illumina TruSeq Stranded mRNA Library preparation kit. cDNA quality was checked on Agilent 2100 Bioanalyzer using Agilent High Sensitivity DNA kit (Agilent #5067-4626). After quality control, libraries were sequenced with 100-bp paired-end (PE100) reads on the NovaSeq 6000 (Illumina) sequencer. Raw data were checked for quality using FastQC (v0.11.8) and aligned to the reference genome for *Danio rerio* danRer11 from Genome Reference Consortium. Analysis strategy includes unsupervised analyses such as PCAs and differential expression analyses (done with DEBrowser bioconductor package or on R with edgeR package).

Microbial DNA extraction and 16S rRNA sequencing

Dissection of 15 intestines from WT and *il22^{-/-}* 7 dpf larvae per replicates followed by bacterial DNA isolation using the DNeasy PowerSoil kit (Qiagen, #47014) following manufacturer's instructions. They were directly stored at -20°C until sequencing. Two primers were used to amplify the 16S rRNA genes covering the hypervariable regions V3 to V4: 338F: ACTCCTACGGGAGGCAGCAG and 806R: GGACTACH-VGGGTWTCTAAT. Amplified regions were sequenced by BGI technologies using the DNBSEQ™ sequencing technology platform.

From the resulting raw data, redundancy analysis was done with Canoco 5.15⁸⁵ with ASV relative abundance as response variables, after transforming with the formula $\log(1000 \times \text{relative_abundance} + 1)$. RDA p-values were determined through permutation testing (500 permutations). Bacterial genome sequences were downloaded with the NCBI tool "datasets" (<https://www.ncbi.nlm.nih.gov/datasets/docs/v2/reference-docs/command-line/datasets/>), 16S sequences were extracted with BioPython (<https://biopython.org/>).

ASVs were compared to 16S sequences with blastn 2.9⁸⁶. HMM screening was done with hmmsearch from HMMER3.3 (<http://hmmer.org>).

Redundance analysis shows a significant link between relative abundance of ASVs and genotype (explained variation 13.3%, $p \leq 0.002$). For the ASVs contributing most to the separation, the genomic potential for functionality, as reported by Valles-Colomer⁵⁸, was determined. In brief, for the top 25 ASVs associated with the KO and the top 25 ASVs associated with the WT, the NCBI Genbank bacterial genome collection was screened for all genomes encoding a 16S gene with exactly the ASV sequence (full-length ASV sequencing, 100% sequence identity). This subset of genomes was screened with Hidden Markov models of the KEGG orthologous groups as reported⁵⁸, and the average number of hits for each function was used as the predicted genomics potential score, as shown in **Figure 5C**.

Generation of germ-free (GF) larva and co-housing experiments

Fertilized zebrafish eggs were treated with bleach (0,05%) for maximum 2 min at 3–4 hpf and then washed twice with sterile E3 medium for 5 min. Embryos were incubated in chlorine hypochlorite (0.003%) for 20 minutes. After washing, embryos were left in sterile E3 medium containing Ampicillin (200 µg/mL), Kanamycin (5 µg/mL), Ceftazidime (200ug/mL) and Chloramphenicol (20ug/mL) and placed at 28 °C in isolated containers. Media was renewed every day in sterile conditions until the day of sample collection. Sterility of larvae and E3 water was monitored every 2 days by incubating fish water in TBS media for 24h at 37°C. GF zebrafish were co-housed from 4 dpf to 7 dpf with conventionally-raised larvae to allow microbiota transfer. At 7 dpf, samples were processed for RT-qPCR or further analyzed by live imaging.

Recording *in vivo* intestinal motility

7 dpf larvae were anesthetized using 100 ug/ml of Tricaine for several minutes at 28°C. Larvae were then embedded in a liquified 0,5% low melting point agarose (Promega, #V2111) and covered with E3 water containing tricaine (100 ug/ml). Imaging was performed using the THUNDER Imager Model Organism (Leica). Movies were taken with 100ms exposure time at 10 frames per second. They were later analyzed by

making kymographs on 180um length region of the larval intestine using the macro “velocity” in ImageJ software (NIH).

Food transit experiment

Larvae were trained from 4 dpf to 7 dpf with usual food (SDS scientific fish food, # 824867). On test day, larvae were fed during 2h with food coupled with non-digestive fluoresphere carboxylate 2um (Invitrogen, #F8827). Only fish having the intestinal bulb filled with fluorescent food were used for the experiment. Pictures were taken using the THUNDER Imager Model Organism (Leica) 3, 6, 12 and 24h after feeding. The distance between the anterior end of the intestinal bulb and the fluorescent food in the intestine was measured using ImageJ software (NIH).

Zebrafish *Edwardsiella tarda* infection

The day before challenging *Edwardsiella tarda* FL60 (kindly provided by Dr. Phillip Klesius (USDA, Agricultural Research Service, Aquatic Animal Health Research Unit), the bacteria grew in TSB medium + tetracycline (15ug/mL) at 28°C overnight. On the day of the challenge, a 1:100 dilution was performed, and the bacteria grew to OD600 = 0.250 (approximately 10⁸ CFU/mL). The bacteria were then centrifuged twice at 4500 rpm for 5-10 minutes and resuspended in E3 1X water to OD600 = 0.250. Six larvae per 6 ml of liquid were incubated in E3 water containing bacteria for 5 hours at 28°C. The infected larvae undergo three washes with E3 1X water. Survival was monitored every 12 hours for 3 days post infection.

Chemical treatments in larval zebrafish

Gut injection: 6 to 7 dpf larvae were microinjected in the gut with ultrapure Flagellin (InvivoGen, #tlrl-epstfla) at a concentration of 100ng/uL or Indole (Sigma, #I3408). 4h post-injection, larval intestines were dissected and stored at -20°C or directly processed for RT-qPCR.

Water incubation: A groups of 25 larvae were kept in E3 1X medium (controls) or DSS 0,5% (MPbio, #02160110-CF) or 5-HT 250uM (Sigma, #H9523) from 4 dpf to 7 dpf. The medium was changed daily. The experiment was done at least 3 times with

different egg batches. Survival analysis or live imaging were performed on treated samples.

Food transit experiment in mice

WT and *I122^{-/-}* mice male or female aged of 3-4, 5 or 8-10 weeks were used. Carmine red (Sigma, #C6152) was given by gavage to 3h-fasted mice (10 mg/ml of water, 10 μ l/g body). The total intestinal transit time was measured by determination of time between ingestion of carmine red and first appearance of the dye in the feces.

Statistical analysis

Statistical analyses were conducted using Rstudio or GraphPad Prism. The types of statistical tests and significance levels are described in respective figure legends. The results were considered statistically significant when P value was lower than 0.05 and were marked in the figures as ***P < 0.001, **P < 0.01, and *P < 0.05.

Table 1. Key resources table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|----------------|--|
| Antibodies | | |
| Mouse anti-HuC/D monoclonal antibody | Invitrogen | Cat# A21271 |
| Rabbit anti-chicken Desmin Polyclonal Antibody | Sigma | Cat# D8281 |
| Rabbit anti-serotonin whole Polyclonal antibody | Sigma | Cat# 5545 |
| Rabbit anti-mouse PYY antibody | PMID: 28614796 | Custom antibody generated in Liddle Laboratory, aa4-21 (mouse) |

| | | |
|--|---------------------------|-------------------------------|
| Chicken anti-GFP Polyclonal antibody | Abcam | ab13970 |
| Living Colors anti DsRed Polyclonal Antibody | TAKARA | Cat# 632496, RRID:AB_10013483 |
| Goat anti-Chicken AF488 | Life technologies | Cat# A-11039 |
| Goat anti-Rabbit Cy3 | Jackson ImmunoResearch | Cat# 111-166-003 |
| Goat anti-Mouse AF647 | Life technologies | Cat# A32728 |

Oligonucleotides

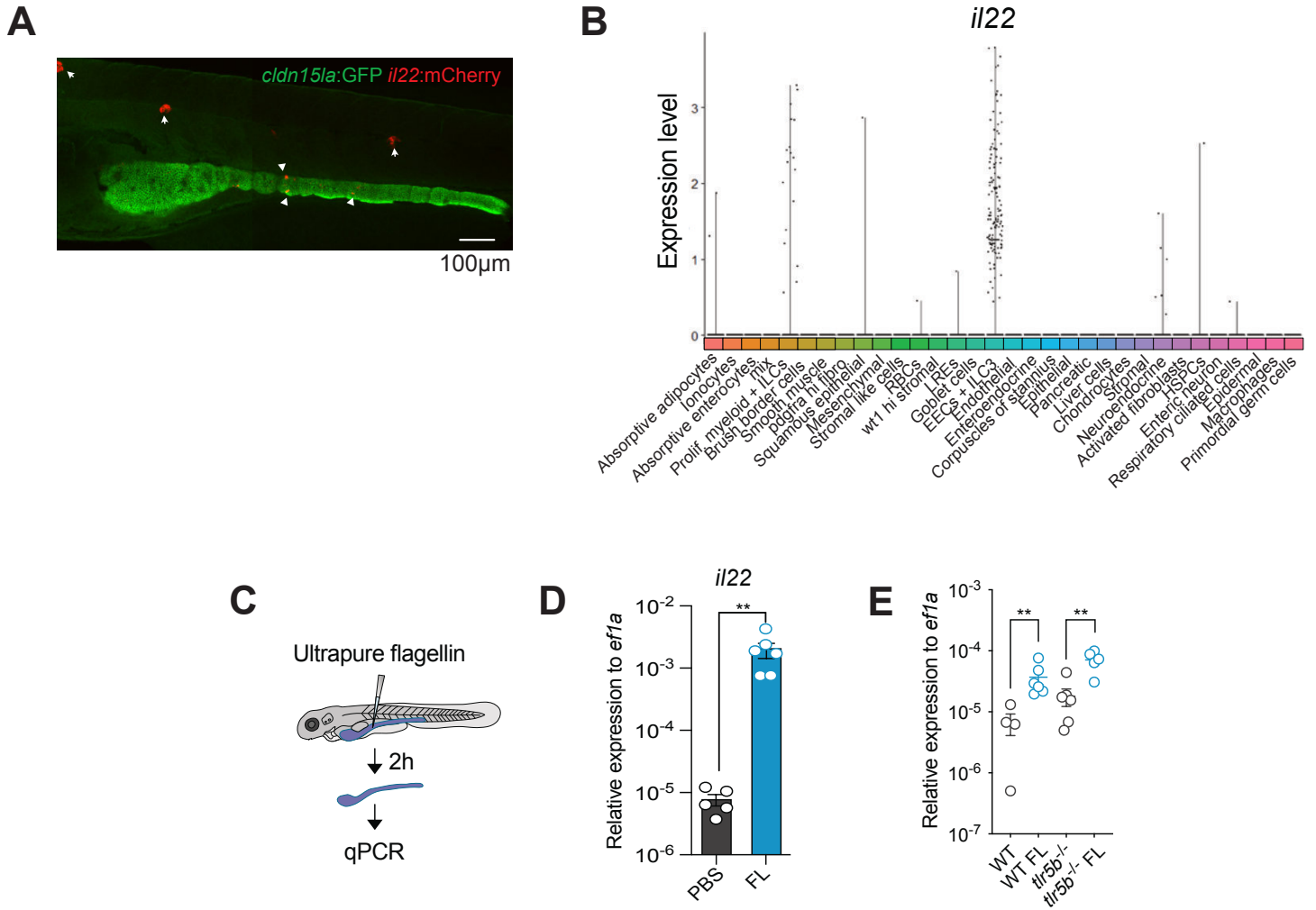
| | | |
|--|-------------------|---|
| RTqPCR for zebrafish gene: <i>il22</i> | Eurofins Genomics | TGCAGAATCACTGTAAACACGA CTCCCCGATTGCTTTGTTAC |
| RTqPCR for zebrafish gene: <i>stat3</i> | Eurofins Genomics | CTGACCGACCCCTTGTCATC CACGTCACCTGACTCCTTGT |
| RTqPCR for zebrafish gene: <i>socs3a</i> | Eurofins Genomics | GCCGAGACTCGACACTCTGTA CGATACACACCAAACCCTGA |
| RTqPCR for zebrafish gene: <i>socs3b</i> | Eurofins Genomics | TAAAACGCCCATTTTGG ACTGTACCACAGGAAGGTCATCT |
| RTqPCR for zebrafish gene: <i>cxcl8b</i> | Eurofins Genomics | GCTGGATCACACTGCAGAAA TGATGAAAGGACAATTCAGTGG |
| RTqPCR for zebrafish gene: <i>ntrk1</i> | Eurofins Genomics | GCAATATTTCTGTGTTTCAGCACATT CCTTGCCAAAAGCTCCTTCAC |
| RTqPCR for zebrafish gene: <i>neflb</i> | Eurofins Genomics | AGGTGGTTGGAGCTATCTTGA CAAACGAGGTTGAAGTCCAGT |

| | | |
|--|-------------------|---|
| RTqPCR for zebrafish gene: <i>chrna</i> | Eurofins Genomics | GTGGGGCTGCAACTCATTCA CCGTAATCGTCCGGATTCCA |
| RTqPCR for zebrafish gene: <i>ache</i> | Eurofins Genomics | AACTCGCATGGTGTGTGTA TCTCCAATGGCAGGCCAAAT |
| RTqPCR for zebrafish gene: <i>csrpr1a</i> | Eurofins Genomics | ACAGCTCAGTTGAGCCACTT CAAGAGGCATCCTGCTAGGT |
| RTqPCR for zebrafish gene: <i>crfb14</i> | Eurofins Genomics | AACGGCTCTTTACAGTGTCCA TGCATCCATCACATCAGTCAGA |
| RTqPCR for zebrafish gene: <i>pyyb</i> | Eurofins Genomics | GTGCATTGGCTTTCTTCACCC GTTTGGCTCATGCTGGTTTCT |
| RTqPCR for zebrafish gene: <i>gcga</i> | Eurofins Genomics | AATGCATTTGCGTCCCCTG CTTCATGGTCGTCAAACCCG |
| RTqPCR for zebrafish gene: <i>fabp2</i> | Eurofins Genomics | TGGGCGTCACCTTTGACTAT GCGTGTCTCCCTCTATGACC |

KASP assay

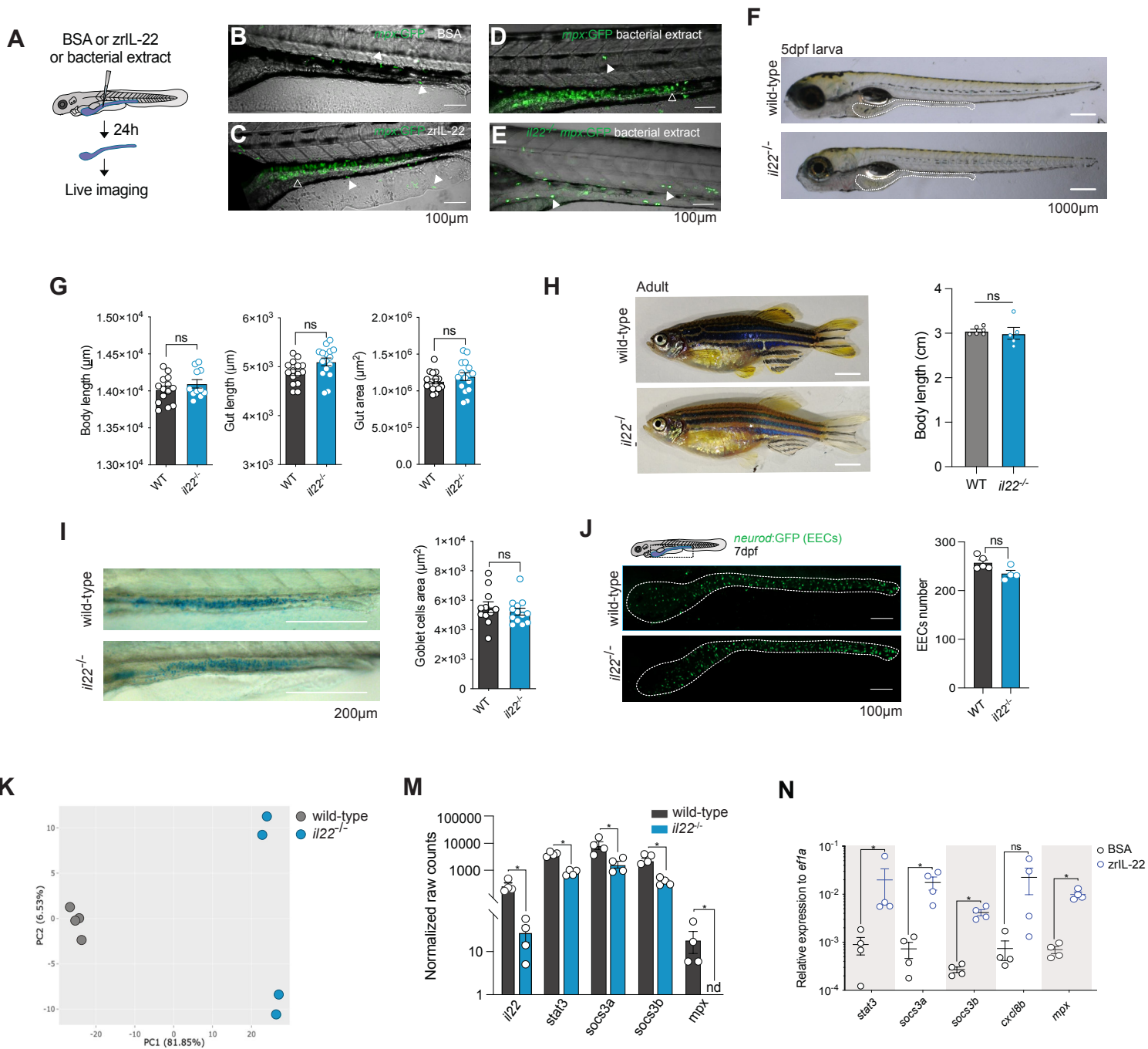
| | | |
|--|----------------------------|--|
| Primers for the zebrafish gene : <i>il22</i> | LGC Biosearch Technologies | Primer allele X GGTGGCTGAGCTATCCAATGGA Primer allele Y GTGGCTGAGCTATCCAATGGG Primer common CTTATTGCTTTGCTGTGCTCGTGCTT |
| Primers for the zebrafish gene : <i>tlr5b</i> | LGC Biosearch Technologies | Primer allele X CTGCACAAGATAAGAAAAGATATCAAGATTAATC Primer allele Y TGACAAGATAAGAAAAGATATCAAGATTAATT Primer common TTCCTCACGCTTTATTTCTTTGGTCGTTT |

EXTENDED DATA FIGURES



Supplementary Figure 1. *i/22* is expressed by enteroendocrine cells and TLR5 does not induces its expression

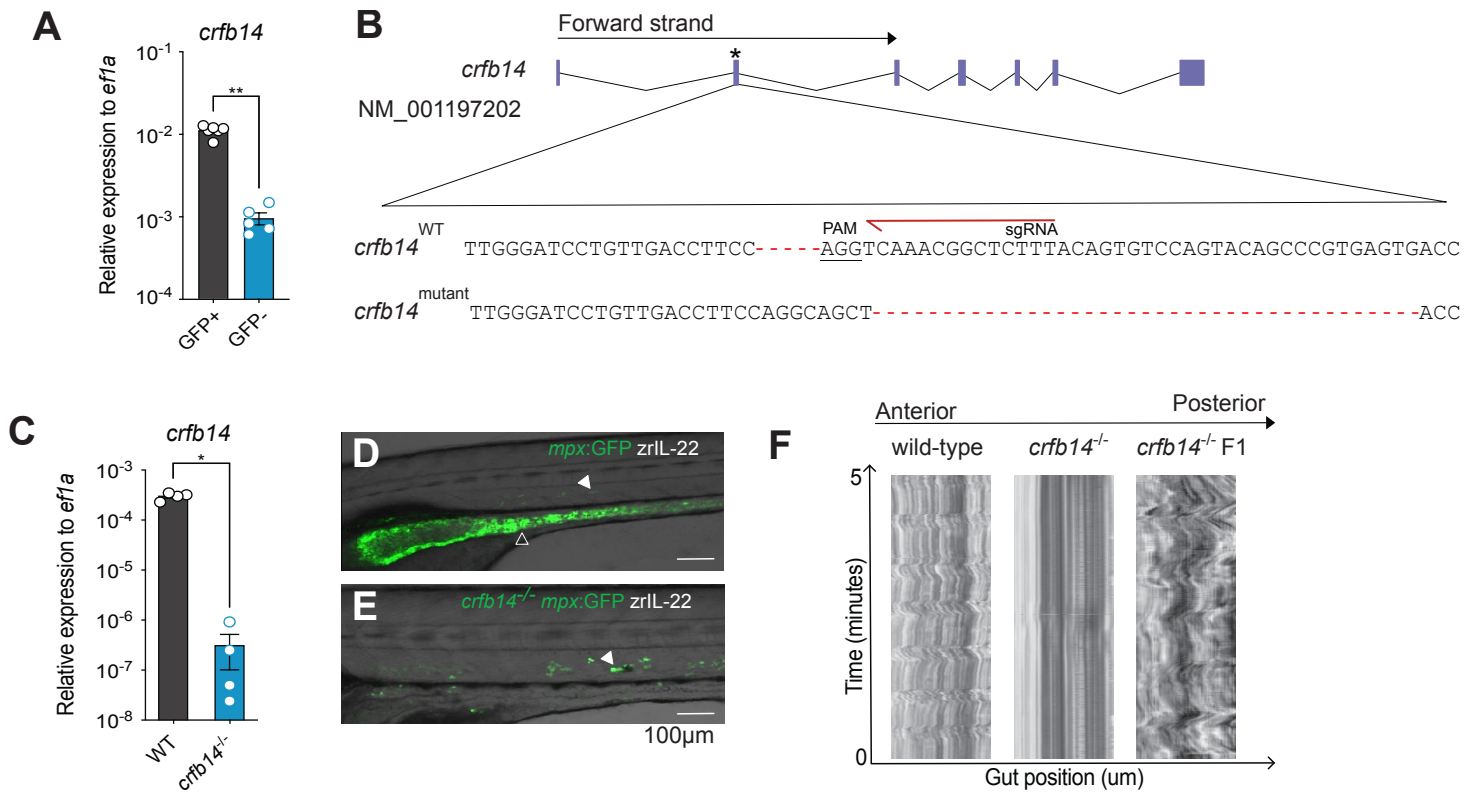
A. Confocal image of 7 dpf Tg(*cldn15la*:GFP);Tg(*i/22*:mCherry) larvae. The arrow shows the neuromasts while the triangle targets IECs expressing *i/22*. Scale bar = 100um. **B.** Re-analysis of a single-cell RNA-sequencing dataset of zebrafish larvae gut published by Nayar et al, 2020 showing *i/22* expression in EECs mostly. **C.** Schematic representation of ultrapure flagellin (FL) injection in the intestine of a 7 dpf larvae, 2h post injection the intestines were dissected and processed for RT-qPCR. **D.** RT-qPCR analysis of *i/22* expression 2h post upon FL injection in the gut. **E.** RT-qPCR analysis of *i/22* expression in WT or *tlr5b*^{-/-} 2h upon PBS or FL injection.



Supplementary Figure 2. Characterization of the *il22^{-/-}* zebrafish line

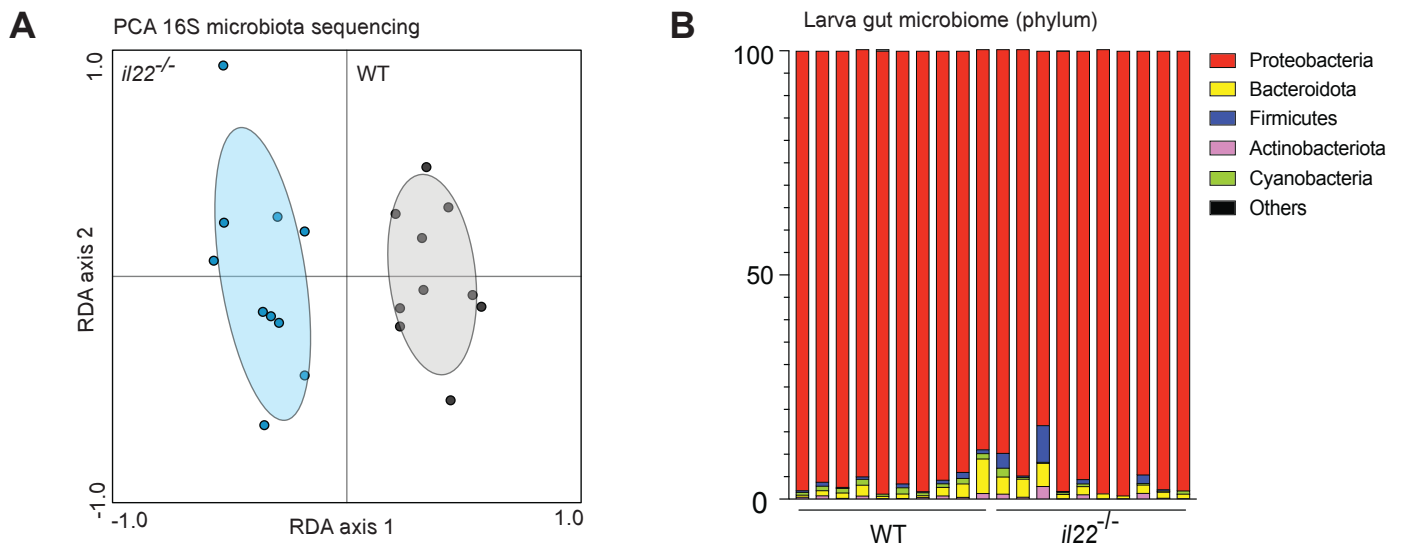
A. Schematic representation of BSA or zrIL-22 (zebrafish recombinant IL-22) protein injection in the gut of 5 dpf larvae, 24h post injection larvae were imaged. **B.** Image of a 5 dpf Tg(*mpx:GFP*) larvae injected with BSA or **C.** zrIL-22. Filled white arrow are showing neutrophils while empty arrows highlight IECs. Scale bar = 100 μm . **D.** Image of a 5 dpf Tg(*mpx:GFP*) or **E.** *il22^{-/-}* Tg(*mpx:GFP*) larvae injected with a bacterial extract. Filled white arrow are showing neutrophils while empty arrows highlight IECs. Scale bar = 100 μm . **F.** Brightfield pictures of 5 dpf WT and *il22^{-/-}* larvae. The gut is surrounded by a dotted line while. Scale bar = 1000 μm . **G.** Quantification of the body length as well as the gut length and area. No differences were found between 5 dpf

WT and *il22*^{-/-}. **H.** Brightfield pictures of adult WT and *il22*^{-/-} fish and quantification of their body length. Scale bar = 200um. **I.** Alcian blue staining of 5 dpf WT and *il22*^{-/-} and quantification of goblet cells area in the gut. **J.** Confocal microscopy of Tg(*neurod*:GFP) labelling EECs in WT or *il22*^{-/-} 7 dpf larvae and quantification of the number of GFP positive cells in the gut. The gut is surrounded by a dotted line while. Scale bar = 100um. **K.** PCA plot of the bulk RNA-sequencing comparing WT and *il22*^{-/-} 7 dpf larvae dissected intestines. **M.** Normalized raw counts of *il22* associated genes. **N.** RT-qPCR analysis on dissected guts of *il22* associated genes after BSA or zebrafish recombinant IL-22 (zrIL-22) injection in WT and *il22*^{-/-} 6 dpf larvae.



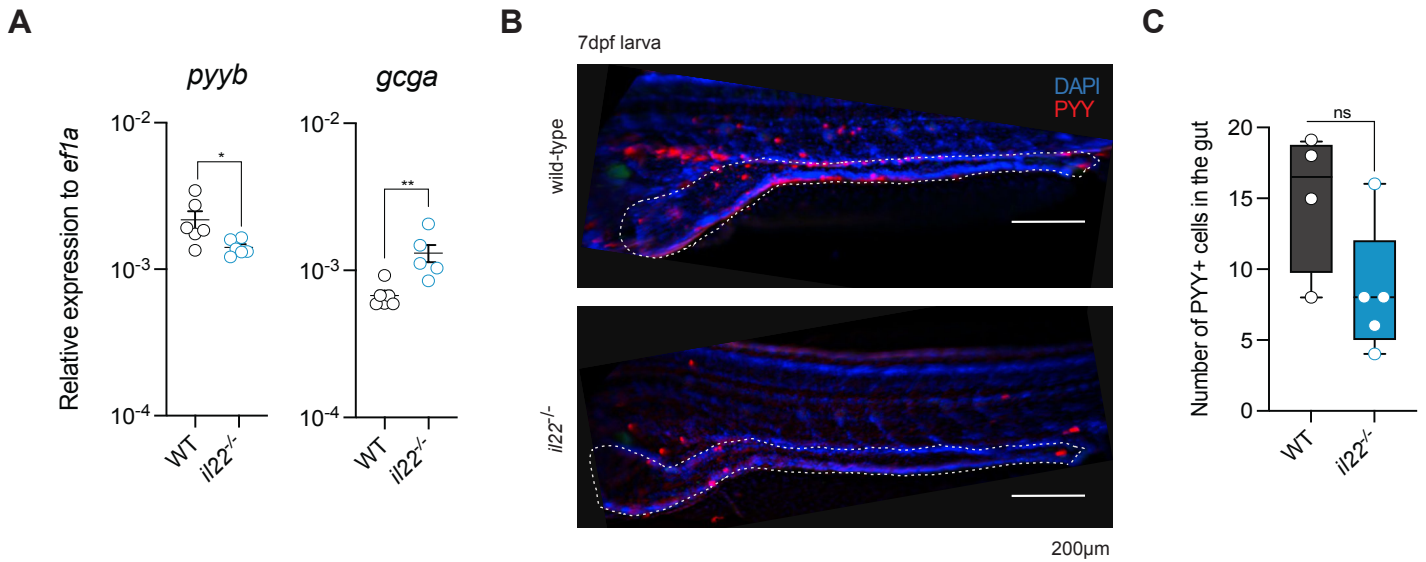
Supplementary Figure 3. Identification of the IL-22 specific receptor chain in zebrafish

A. RT-qPCR analysis of *crfb14* gene expression in *cldn15*:GFP positive and negative sorted cells. **B.** Schematic for the mutation generated in the zebrafish *crfb14* gene by CRISPR/Cas9. **C.** RT-qPCR analysis on dissected guts measuring *crfb14* gene expression in WT and the newly generated *crfb14*^{-/-} line. **D.** Image of a 5 dpf WT or **E.** *crfb14*^{-/-} Tg(*mpx*:GFP) larvae injected with zrIL-22. Filled white arrow are showing neutrophils while empty arrows highlight IECs. Scale bar = 100μm. **F.** Kymograph analysis using ImageJ of 180μm in the midgut of 7 dpf WT, *crfb14*^{-/-} and IEC-*crfb14* (*cldn15la:crfb14* line) larvae.



Supplementary Figure 4. Dysbiosis in larvae lacking IL-22

A. PCA of the microbiomes of WT and *il22*^{-/-} 7 dpf larvae. Each dot corresponds to one replicate. **B.** Taxon-based analysis at phylum level between WT and *il22*^{-/-}.



Supplementary Figure 5. Hormones dysregulation in *il22*^{-/-}

A. RT-qPCR analysis on WT or *il22*^{-/-} 7 dpf larvae dissected guts of *ppyb* and *gcga* expression. **B.** Fluorescent image of a 7 dpf WT or *il22*^{-/-} stained with DAPI and anti-PYY antibody. The gut is surrounded by a dotted line while. Scale bar = 200µm. **C.** Quantification of the number of PYY positive cells in the gut of 7 dpf WT or *il22*^{-/-}. Statistical analysis were performed with Mann-Whitney : * $P < 0,05$, ** $P < 0,01$. (B and C are still preliminary experiments).

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2. Interleukin-10 regulates goblet cell numbers through Notch signaling in the developing zebrafish intestine



In the second part of my PhD work, I investigated the role of IL-10 in zebrafish gut maturation. This collaboration was led by Rodrigo Morales, a post-doc in Eduardo Villablanca laboratory at the Karolinska Institute. IL-10 is a crucial cytokine within the immune system that plays an important role in regulating immune responses and maintaining immune balance¹⁶⁴. Often referred as an anti-inflammatory cytokine, IL-10 is responsible for suppressing excessive immune reactions and preventing the immune system from causing excessive damage to the tissues¹⁶⁴. It achieves this by inhibiting the production and activity of pro-inflammatory cytokines and immune cells. IL-10 is produced by various immune cells, including T cells, B cells, macrophages, and dendritic cells¹⁸³. IL-10 has already been linked with intestinal epithelial cell regeneration after injury³³⁴, and with proliferation of intestinal stem cells in organoids³³⁵, suggesting a potential role of this cytokine in IEC differentiation. Intriguingly, our study demonstrated that IL-10-deficient zebrafish larvae exhibited an increase in goblet cell numbers alongside with a reduced Notch signaling activity. These findings were validated using mouse intestinal organoids, uncovering an evolutionarily conserved IL-10/Notch axis controlling goblet cell differentiation.

I contributed to this study by doing immunostainings using 2F11 antibody (for secretory cells) and image quantification, which revealed a higher number of secretory cells in the intestines of *il10^{-/-}* larvae. Moreover, I performed FACS sorting of IECs and did the following qPCR to measure *il10* expression. The latter analysis indicated that non-epithelial cells mainly express this cytokine in the larval zebrafish gut. The rest of the experiments and writing of the paper were done by Rodrigo Morales and his colleagues. This study is presented as paper format and has already been published in *Mucosal Immunology* in 2022.

ARTICLE OPEN



Interleukin-10 regulates goblet cell numbers through Notch signaling in the developing zebrafish intestine

Rodrigo A. Morales^{1,2}, Soraya Rabahi³, Oscar E. Diaz^{1,2}, Yazan Salloum³, Bianca C. Kern^{1,2}, Mikaela Westling^{1,2}, Xinxin Luo^{1,2}, Sara M. Parigi^{1,2}, Gustavo Monasterio^{1,2}, Srustidhar Das^{1,2}, Pedro P. Hernández³ and Eduardo J. Villablanca^{1,2}

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Cytokines are immunomodulatory proteins that orchestrate cellular networks in health and disease. Among these, interleukin (IL)-10 is critical for the establishment of intestinal homeostasis, as mutations in components of the IL-10 signaling pathway result in spontaneous colitis. Whether IL-10 plays other than immunomodulatory roles in the intestines is poorly understood. Here, we report that *il10*, *il10ra*, and *il10rb* are expressed in the zebrafish developing intestine as early as 3 days post fertilization. CRISPR/Cas9-generated *il10*-deficient zebrafish larvae showed an increased expression of pro-inflammatory genes and an increased number of intestinal goblet cells compared to WT larvae. Mechanistically, IL10 promotes Notch signaling in zebrafish intestinal epithelial cells, which in turn restricts goblet cell expansion. Using murine organoids, we showed that IL-10 modulates goblet cell frequencies in mammals, suggesting conservation across species. This study demonstrates a previously unappreciated IL-10-Notch axis regulating goblet cell homeostasis in the developing zebrafish intestine and may help explain the disease severity of IL-10 deficiency in the intestines of mammals.

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
INTRODUCTION

The intestine is a semi-permeable and highly regenerative tissue responsible for nutrient and water absorption while keeping the host protected from environmental and microbiota challenges. The control of these functions is orchestrated by complex cellular crosstalk between intestinal epithelial cells and tissue-resident immune cells, among others. In this regard, cytokines, and particularly IL-10, have been considered key mediators in the maintenance of intestinal homeostasis and function.¹ This has been demonstrated in mice defective in IL-10 signaling, which develop spontaneous colitis.² In humans, mutations in the IL-10 signaling genes *IL10*, *IL10RA*, and *IL10RB* are strongly associated with a very early onset of inflammatory bowel diseases (VEO-IBD),^{3,4} which is characterized by clinical manifestations in children before 6 years of age, and a significant number of cases occurring in infants younger than 1 year.⁵ VEO-IBD has a strong genetic component compared to the 5–10% familial cases observed in adult inflammatory bowel diseases (IBD).⁶ In addition, VEO-IBD is phenotypically distinct from adult IBD and is characterized by extensive colitis, growth failure, and unresponsiveness to conventional therapies.⁶ Common clinical manifestations in children with VEO-IBD include bloody diarrhea and mucous stool,⁷ suggesting altered goblet cell activity.

Intestinal immune cells, including macrophages, B cells, and T cells, are the predominant IL-10 producers and responders. IL-10 signaling in intestinal macrophages is critical to maintaining intestinal homeostasis, as conditional deletion of IL-10Rα in

CX3CR1⁺ macrophages is sufficient to trigger spontaneous colitis in mice.⁸ Interestingly, intestinal epithelial cells also express IL-10 receptors and are therefore equipped to mount a cellular response upon IL-10R stimulation.^{9–11} In this regard, IL-10 deficiency in myeloid cells results in impaired regeneration of the epithelium following damage, whereas administration of recombinant IL-10 promotes intestinal epithelial cell proliferation *in vivo*.¹² Furthermore, IL-10 promotes intestinal stem cell (ISC) proliferation in organoid cultures,¹³ suggesting an intrinsic role of IL-10 signaling in intestinal epithelial cells (IEC) differentiation. In line with this, specific IL-10R deficiency on IECs results in goblet cell hyperplasia in the colon of adult mice.¹⁴ Altogether, the combined evidence not only shows that IL-10 signaling can modulate IEC differentiation but also raises the possibility that early-stage intestinal development is impaired in individuals with mutations in the IL-10 signaling pathway, which may lead to the phenotypes observed in VEO-IBD.

The intestinal tract develops at early embryonic stages from the endodermal germinal layer. It is initially formed as a flat tube of epithelial and mesenchymal cells that proliferate and stratify in a synchronic fashion, which results in the morphogenesis of the villi and crypts. At the bottom of the crypt, intestinal stem cells are constantly self-renewing and giving rise to specialized epithelial cells with either absorptive or secretory functions. The main drivers of intestinal stem cell differentiation include the orchestrated activity of signaling pathways such as Wnt/*Int-1* (Wnt), bone morphogenetic proteins (BMP), and Notch,

¹Division of Immunology and Allergy, Department of Medicine Solna (MedS), Karolinska Institutet and University Hospital, 17176 Stockholm, Sweden. ²Center for Molecular Medicine (CMM), 17176 Stockholm, Sweden. ³Institut Curie, PSL Research University, INSERM U934, CNRS UMR3215, Development and Homeostasis of Mucosal Tissues Group, 75248 Paris, France. email: rodrigo.morales@ki.se; eduardo.villablanca@ki.se

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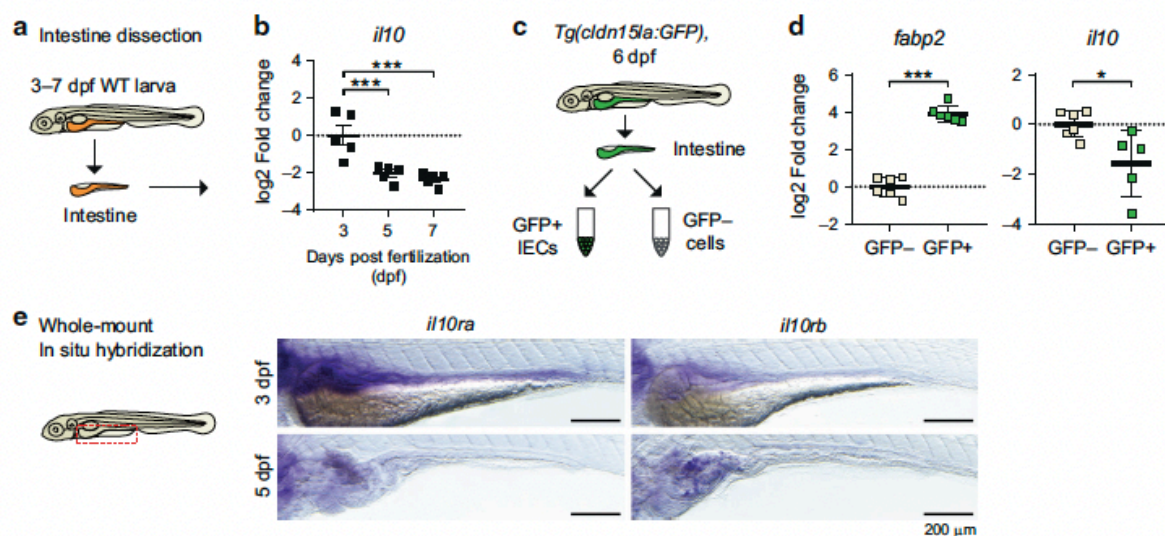


Fig. 1 Early expression of *il10* during intestinal development in zebrafish larvae. **a** Diagram showing intestine extractions of wild-type (WT) larvae, from 3 to 7 days post fertilization (dpf). **b** Expression of *il10* transcripts measured by qRT-PCR. Each dot represents independent experiments with a pool of 20 intestines used for RNA extraction ($N = 5$). **c** Sorting strategy for the isolation of intestinal epithelial cells. Intestines from *Tg(cldn15la:GFP)* larvae were extracted from the body, disaggregated to cell suspensions, and FACS sorted based on the expression of GFP. GFP⁺ and GFP⁻ cell collections were subsequently used for RNA transcriptomic analyses. **d** Transcriptomic expression analyses from sorted GFP⁺ and GFP⁻ cells by qRT-PCR. Each dot represents an individual sorting experiment with around 30,000 cells collected ($N = 6$). **e** Whole-mount in situ hybridization for the genes *il10ra* and *il10rb* in 3dpf and 5dpf zebrafish larvae. Representative pictures of the stainings at each developmental stage are shown. Scale bar = 200 μ m. One-way ANOVA with Fisher's LSD multiple comparisons test was performed in **b**, while two-tailed *t*-tests were performed in **d** (* $p < 0.05$; *** $p < 0.001$).

which antagonize each other to promote or attenuate cell replication.^{15,16} Particularly, Notch signaling plays a critical function in stem cell maintenance and regulation of secretory cell differentiation across species.^{17–19} In mice, Paneth and goblet cells are expanded after Notch inhibition,²⁰ and the lack of Notch target genes, such as *hairy/enhancer of split 1 (Hes1)* show a skewed epithelial cell differentiation towards the secretory lineage.^{21,22} By contrast, continuous Notch activation by over-expression of the Notch intracellular domain (NICD) results in the expansion of the intestinal stem cell pool and impaired secretory-IEC differentiation.²³ Expression of the Notch1 and Notch 2 receptors and their main target genes *Hes1*, *Hes5*, and *Hes6* have been detected in the dividing cells within the intestinal crypt.²⁴ Interestingly, the Notch pathway regulates T cell function,²⁵ including the production of IL-10 by Th1 cells.²⁶ However, whether IL-10 signaling regulates tissue differentiation by controlling Notch or other stem cell-related pathways remains unknown.

Zebrafish have been successfully used to model key biological processes associated with intestinal disorders, as physiological and cellular features are highly conserved between fish and humans.²⁷ Using zebrafish, we and others have addressed the impact of dietary compounds,²⁸ environmental pollutants²⁹ and IBD risk genes³⁰ in intestinal homeostasis, highlighting zebrafish as a powerful tool to investigate the function of genetics in intestinal development. The zebrafish ortholog for the human *IL10* gene³¹ shows a conserved immunomodulatory function.^{32,33} Here, we took advantage of zebrafish to investigate the effect of IL-10 signaling in the developing intestine. Using CRISPR/Cas9 we generated *il10* mutants, which combined with histological and immunological stainings, transcriptional analysis and fluorescent transgenic reporters enabled us to investigate the intestinal cell composition of IL-10-deficient larvae. We found that IL-10 deficiency resulted in an increased number of goblet cells, which was independent of the microbiota. Mechanistically, the increase in goblet cells was associated with a decreased activity of intestinal Notch signaling, and ectopic Notch signaling activation restored alcian blue⁺ goblet cell numbers in IL-10-deficient larvae. Moreover, IL-10 treatment in mouse small intestine (SI) organoids led to decreased frequencies of goblet cells

and increased Notch activity, which suggests an evolutionarily conserved IL-10-Notch axis that regulates intestinal goblet cell differentiation across vertebrates.

RESULTS

Zebrafish *il10* is expressed during early intestinal development

The development of the larval zebrafish intestine, after the formation of the tube and lumen between 30 and 52 hours post fertilization (hpf), can be divided into an early stage of high proliferation and polarization of the intestinal epithelium (52–74hpf), followed by a later stage of tissue compartmentalization and the emergence of differentiated intestinal cell types (74–120 hpf).^{34,35} To gain insights into the developmental stage in which IL10 might control zebrafish intestinal organogenesis, we analyzed *il10* transcript levels in zebrafish larvae starting at 72 hpf or 3 days post fertilization (dpf) until the stage in which a functional intestine is appreciated (5dpf and 7dpf). Using qRT-PCR to analyze *il10* transcript levels from dissected zebrafish larval intestines (Fig. 1a), we found higher transcript levels of *il10* in 3dpf intestines, compared to 5dpf and 7dpf (Fig. 1b). Dissected intestines contain intestinal epithelial cells (IECs) among other intestine-associated cells that interact with IECs, therefore we sought to determine which group of cells was responsible for *il10* expression. We then FACS-sorted epithelial cells versus the rest by using the zebrafish reporter line *Tg(cldn15la:GFP)* (Fig. 1c), in which the *cldn15la* promoter drives GFP expression exclusively in intestinal epithelial cells.³⁶ qPCR analysis from sorted cells showed that *il10* expression was not detectable in GFP⁺ cells but in GFP⁻ intestine-associated cells (Fig. 1d). To gain insights into a potential active IL-10 signaling in intestinal cells, we analyzed the expression of the orthologs for the receptors of *il10*, defined as *il10ra* and *il10rb* in the zebrafish database ZFIN. Whole-mount in situ hybridizations (WISH) showed that both *il10ra* and *il10rb* genes were expressed in the zebrafish developing intestine at 3dpf, while *il10rb* was predominantly expressed at 5dpf (Figs. 1e and S1). Altogether, these results suggest that *il10* signaling may act in zebrafish intestinal cells during early developmental stages.

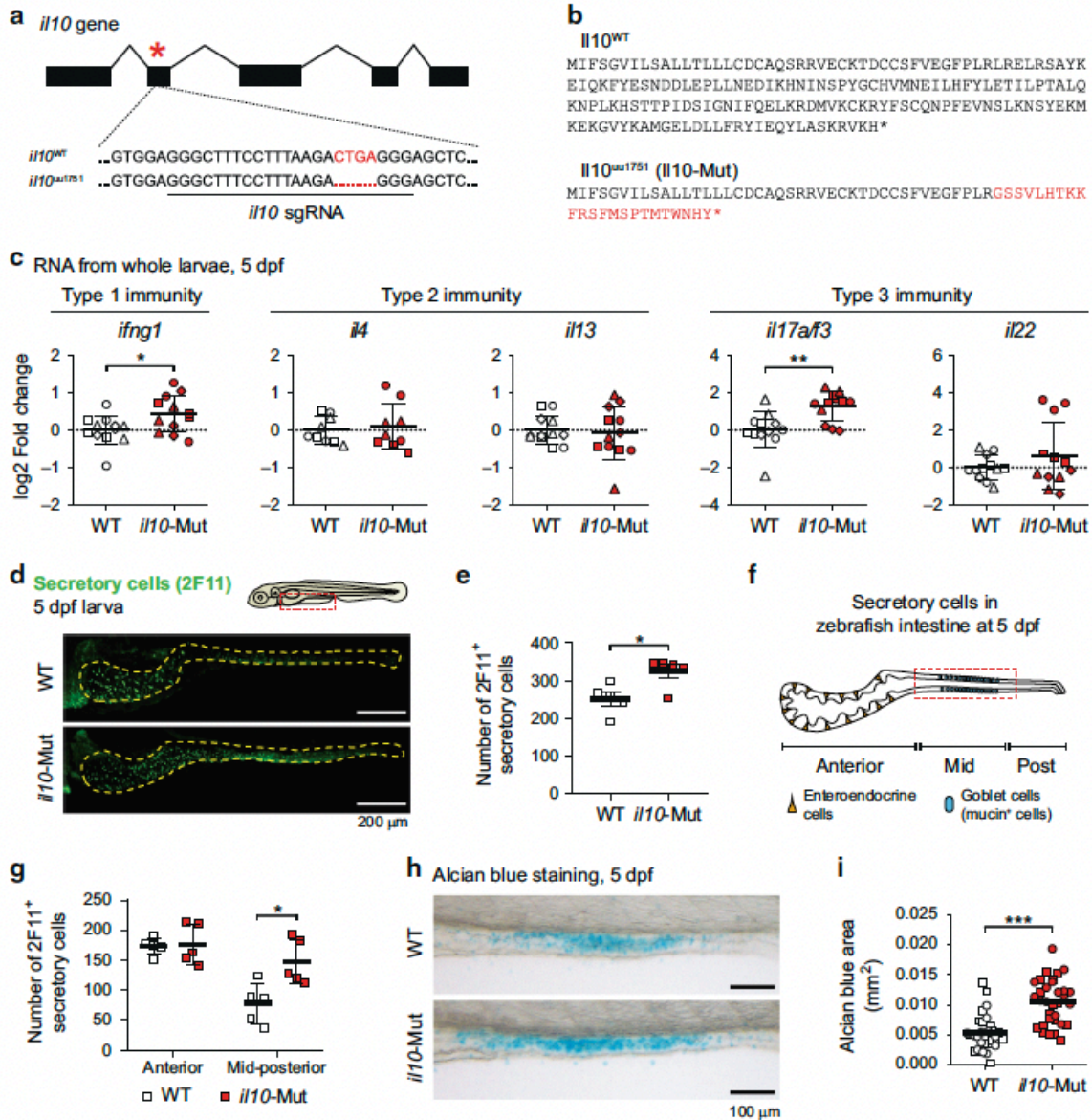


Fig. 2 Increased pro-inflammatory expression and alcian blue⁺ goblet cells in *il10* mutant zebrafish larvae. **a** Schematic for the mutation generated in the zebrafish *il10* gene (*il10*^{uu1751}, -4bp) by CRISPR/Cas9. **b** Predicted protein sequences for Il10 in WT and *il10*-Mut individuals, according to the DNA sequences obtained. **c** Whole-body expression levels of type 1, type 2, and type 3 cytokines in WT (white symbols) and *il10*-Mut larvae (red symbols) by qRT-PCR. Each dot represents a pool of 5–10 larvae collected in 3–4 independent experiments. **d** Whole-mount immunofluorescence staining in 5dpf WT and *il10*-Mut larvae using the pan-secretory marker antibody 2F11. Scale bar = 100 μ m. **e** Quantification of 2F11⁺ cells in the intestines of WT and *il10*-Mut larvae. Each dot represents individual larvae collected in 2 independent experiments. **f** Diagram for the larval zebrafish intestinal tract showing the location of Goblet cells in the mid-intestinal region. **g** Stratification of the number of 2F11⁺ cells in the anterior or mid-posterior intestines of WT and *il10*-Mut larvae ($N = 5$). **h** Alcian blue (ab) staining on 5dpf WT and *il10*-Mut larvae. **i** Automatic quantifications of the ab-stained area in the mid intestines of WT and *il10*-Mut larvae. Each dot corresponds to an individual larva collected in two independent experiments. Different symbol shapes represent independent experiments in **c** and **i**. Two-tailed student *t* tests were used for the comparisons in **c**, **e**, **g** and **i**. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Altered immune and intestine-associated profiles in *il10* mutant larvae

Next, we aimed to determine whether *il10* can modulate the development of the larval zebrafish intestine by using loss-of-function genetic approaches. Using CRISPR/Cas9,³⁷ we generated a 4 bp deletion in the exon 2 of the zebrafish *il10* gene (*il10*^{uu1751}) (Fig. 2a), predicted to generate a premature stop codon (Fig. 2b). *il10*^{uu1751/uu1751} mutant fish (hereinafter referred to as *il10*-Mut) did not show any apparent morphological or developmental defect while in homozygosity in larva (Fig. S2a), reached adulthood and were fertile (Fig. S3a, b). Analysis of selected type 1, 2, and 3 cytokines by qPCR from the whole larvae at 5dpf revealed that type 2 cytokines (*il4* and *il13*) and the type 3 cytokine *il22* were

comparable whereas *ifng1* (type 1) and *il17af3* (type 3) were significantly elevated in *il10*-Mut larvae compared to wild-type (WT, *il10*^{wt/wt}) larvae (Figs. 2c and S2b). Thus, Il10 deficiency results in altered pro-inflammatory cytokine levels in the whole larvae. We then sought to investigate whether Il10 deficiency affects the development of the intestinal tract. Absorptive and secretory cells, namely enteroendocrine and goblet cells, can be distinguished by 5dpf in zebrafish larvae.¹⁸ To analyze enteroendocrine and goblet cells, we performed whole-mount immunofluorescence using the pan-secretory marker antibody 2F11.¹⁸ By 5dpf, the *il10*-Mut larvae showed a higher number of secretory cells in the intestine compared to WT (Fig. 2d, e). Secretory cells in the zebrafish larvae are differentially distributed along the anterior-distal intestine,³⁴

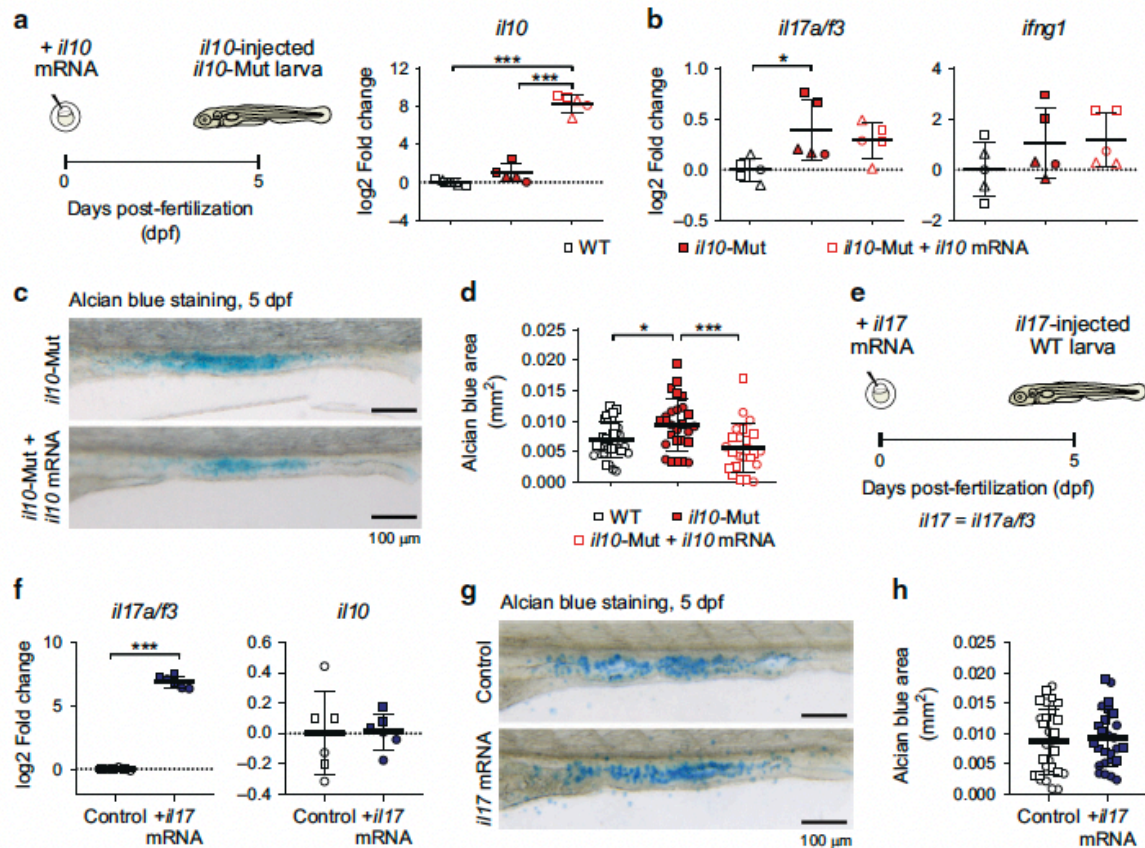


Fig. 3 Altered alcian blue⁺ goblet cell homeostasis in *il10* mutant larvae is rescued after *il10* mRNA administration. **a** Experimental strategy for *il10* rescue experiments and expression levels of *il10* mRNA at 5 dpf. **b** Whole-body expression levels of *il17a/f3* and *ifng1* by qRT-PCR. Each dot represents a pool of 5–10 larvae collected in three independent experiments. **c** Alcian blue stainings on 5 dpf WT, *il10*-Mut larvae and injected with *il10* mRNA. Scale bar = 100 µm. **d** Quantification for the ab-stained area in the *il10* mRNA-injected *il10*-KO larvae, compared to control *il10*-KO and WT larvae. *N* = 2 independent experiments. **e** Experimental strategy for the injection and analysis of *il17* (*il17a/f3*) mRNA-injected WT zebrafish embryos. **f** Whole-body expression levels of *il17a/f3* and *il10* by qPCR. Dots represent pools of 5–10 larvae collected in 2 independent experiments. **g** Alcian blue stainings on 5 dpf control and *il17*-injected WT larvae. Scale bar = 100 µm. **h** Quantification of the ab-stained area of control and *il17*-injected larvae. *N* = 2 independent experiments. Each independent experiment is shown with a different symbol shape in **a**, **b**, **d**, **f** and **h**. One-way ANOVA was used for statistical analysis in **a**, **b** and **d**, whereas two-tailed student t-test were used in **f** and **h** (**p* < 0.05; ****p* < 0.001).

with enteroendocrine cells being concentrated in the anterior intestine and mucin-producing goblet cells in the mid intestine (Fig. 2f). Therefore, we further stratified 2F11⁺ cells based on location. We observed that the number of 2F11⁺ secretory cells in the mid-posterior, but not in the anterior segment, was significantly increased in *il10*-Mut compared to WT (Fig. 2g). The increase in secretory cells in the mid-posterior segment prompted us to hypothesize that goblet cells are increased in *il10*-Mut compared to WT larvae. In line with our hypothesis, whole-mount alcian blue (ab) staining labeling mucin production as an indicator of ab⁺ goblet cell abundance in the mid intestine, displayed a higher staining area in 5 dpf *il10*-Mut larvae compared to WT (Fig. 2h–i). Area measurements were complemented with ab intensity and stained length (Fig. S2c), showing an overall increase of the ab⁺ signal detected in *il10*-Mut larvae. Likewise, whole-mount staining with fluorescently labeled wheat germ agglutinin (WGA), which binds to the N-acetylglucosamine present in the mucus produced by goblet cells³⁴ showed that the number of WGA⁺ goblet cells in the mid intestine was increased in 5 dpf *il10*-Mut larvae, compared to WT (Fig. S2d, e), further confirming that *il10*-deficiency results in goblet cell hyperplasia within the developing zebrafish embryo. To analyze if absorptive intestinal populations were affected in *il10*-Mut larvae, we used neutral red to stain a subset of mid intestine absorptive enterocytes with acidified lysosomes, namely lysosome-rich enterocytes (LREs).^{38–40} Area, intensity and length analysis from neutral red-stained

enterocytes in 5 dpf larvae did not show differences between WT and *il10*-Mut (Fig. S2f, g), suggesting no impairments in the differentiation of absorptive intestinal lineages. Further, to determine whether the increased number of goblet cells in *il10*-Mut zebrafish was age-dependent, we analyzed different intestinal regions from >1-year-old WT and *il10*-Mut zebrafish (Fig. S3c). Ab staining on paraffin-embedded intestinal sections showed a higher number of goblet cells per villus area in *il10*-Mut larvae, compared to WT (Fig. S3d, e). Altogether, our results point towards a disbalance in both pro-inflammatory cytokine expression and the abundance of goblet cells in *il10*-deficient zebrafish.

The abundance of intestinal goblet cells depends on *il10*

To determine whether the phenotypes observed in the *il10*-Mut were dependent on IL10 signaling rather than off-target effects we performed rescue experiments using an in vitro-generated version of the zebrafish *il10* mRNA. Injection of the full *il10* mRNA at 1-cell stage resulted in a significant increase of *il10* transcripts in the whole 5 dpf larvae (Fig. 3a). *il10* mRNA-injected *il10*-Mut 5 dpf larvae showed a non-significant reduction of *il17a/f3* transcript levels compared to *il10*-Mut, whereas *ifng1* levels were comparable (Fig. 3b). In addition, the average ab-stained area was reduced in the intestines of *il10* mRNA-injected *il10*-Mut larvae compared to *il10*-Mut, reaching comparable levels to those of WT larvae (Fig. 3c, d). A recent publication showed that IL-17 signaling on intestinal epithelial cells promotes goblet cell differentiation.⁴¹

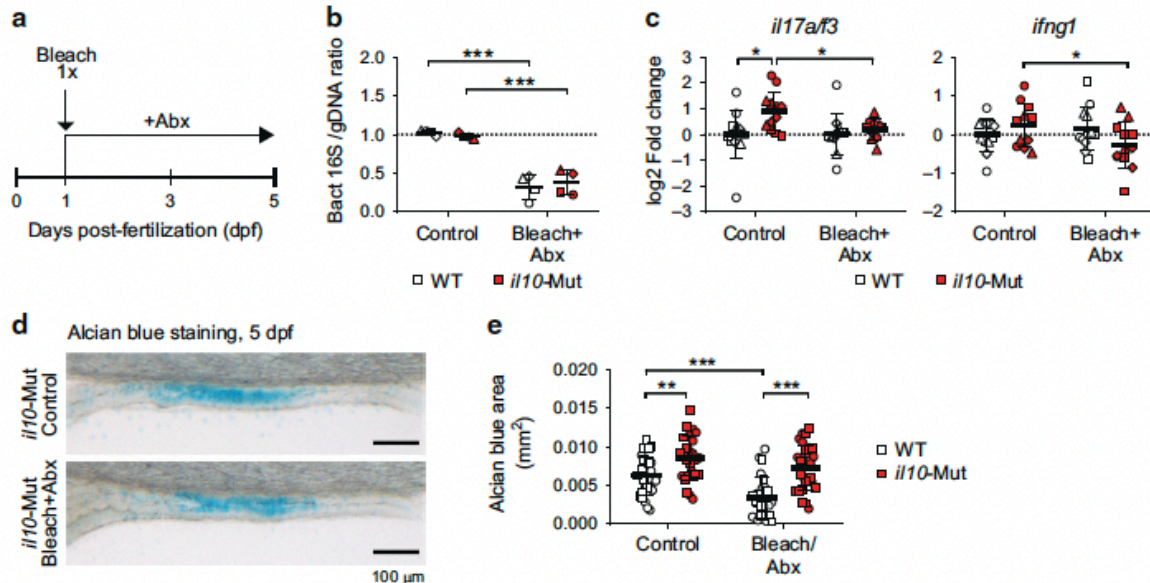


Fig. 4 Increased alcian blue⁺ goblet cells in *il10* mutant larvae are independent of the microbiota. **a** Diagram showing bleach (sodium hypochlorite 0.004%) plus antibiotic (Abx) treatment in zebrafish embryos/larvae (Abx: Ampicillin 100 µg/mL, Kanamycin 5 µg/mL). **b** Quantification of bacterial 16S DNA levels over zebrafish genomic DNA measured by PCR. Each dot corresponds to a pool of 2 larvae from 4 independent experiments. **c** Transcriptomic expression analysis of cytokines from treated larvae by qRT-PCR. Each dot represents a pool of 8–10 larvae collected from 4 independent experiments (**d, e**) Alcian blue staining and analysis on 5 dpf WT and *il10*-Mut larvae treated with Bleach+Abx and controls ($N = 2$ independent experiments). Scale bar = 100 µm. Independent experiments are shown with different symbol shapes in **b, c** and **e**. Two-way ANOVAs with Fisher's LSD multiple comparisons tests were performed in **b, c** and **e** (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

To assess the role of Il17 signaling on the increased goblet cell numbers observed in *il10*-Mut larvae, we analyzed the abundance of intestinal goblet cells in WT larvae injected with an in vitro-generated *il17* (*il17a/f3*) mRNA at 1-cell stage (Fig. 3e). While *il17a/f3* transcripts were high in 5 dpf *il17*-injected larvae (Fig. 3f), ab analysis did not show differences in the ab-stained area between *il17a/f3* mRNA-injected and control larvae (Fig. 3g, h), suggesting that the abundance of goblet cells does not depend on Il17 signaling in zebrafish larvae. To further confirm our findings, we measured the ab-stained area in a second CRISPR/Cas9-generated mutant line for the *il10* gene, this time containing a 17 bp insertion replacing 3 bp (+14 bp total insertion) in exon 3 (*il10*^{uu1762}, referred to as *il10*-Mut2), which is predicted to generate a premature stop codon (Fig. S4a, b). Similar to the *il10*-Mut, we observed increased staining for ab in 5 dpf *il10*-Mut2 larvae compared to WT (Fig. S4c, d). Altogether, these results indicate that *il10* modulates ab⁺ goblet cell differentiation, but not cytokine expression, in zebrafish larvae.

Increased goblet cells in *il10* mutant larvae are microbiota-independent

Previous findings suggest that goblet cell differentiation is dependent on the microbiota.⁴² To investigate a potential role of the microbiota in the phenotypes observed in *il10*-Mut larvae, we established a protocol to reduce bacterial loads in zebrafish embryos (Fig. 4a). Briefly, 24–28 hpf embryos were treated with E3 water containing 0.004% sodium hypochlorite (bleach) for 5 min, and then they were incubated in the presence of an antibiotic cocktail (Abx, Ampicillin and Kanamycin) from 1 dpf to 5 dpf. We confirmed a reduction of total bacterial loads after bleach+Abx treatments in larvae by comparing bacterial 16S versus targeted zebrafish genomic DNA amplification levels by PCR (Fig. 4b). Bleach + Abx-treated 5 dpf *il10*-Mut larvae showed a reduced expression of both *il17a/f3* and *ifng1*, compared to non-treated siblings (Fig. 4c), suggesting that cytokine modulation is microbiota-dependent. On the other hand, ab levels remained enhanced in *il10*-Mut compared to WT larvae, regardless of the

reduced bacterial load (Fig. 4d, e). These results indicate that the increased ab⁺ goblet cells observed in *il10*-Mut larvae are independent of the bacterial microbiota.

Impaired Notch signaling in the intestines of *il10* mutant larvae

To gain insights into the potential mechanisms by which *il10* modulates goblet cell numbers in zebrafish larvae, we analyzed the activity of signaling pathways involved in goblet cell differentiation, such as Notch,¹⁸ RAR,²⁸ and ARP/ASCL factors.^{43,44} For this purpose, we dissected intestines from 5 dpf WT and *il10*-Mut larvae (Fig. 5a) and we analyzed the expression of Goblet cell markers and key target genes for the above-mentioned signaling pathways by qRT-PCR. We did not observe differences in the expression of the RAR signaling target gene *cyp26a1*, the ARP/ASCL-associated genes *atoh1a* and *ascl1a*, nor in the expression of the goblet cell markers *agr2* and *muc2.1* (Fig. S5a). In addition, we did not detect differences in the expression of *olfm4.2*, ortholog for the mammalian stem cell marker Olfm4, nor in the expression of the teleost intestinal progenitor marker *sox9b* (Fig. S5b). However, we found a reduction in the intestinal expression levels of *her6* and *her9*, both Notch target genes described as the ortholog of human *HES1* and *HES5*, respectively (Fig. 5b). To confirm our observations, we combined the *il10*-Mut fish with the Notch activity reporter *Tg(tp1:EGFP)*,⁴⁵ which detects Notch-responding cells in the larval intestinal tract.⁴⁶ In vivo imaging of the intestines of 5 dpf *Tg(tp1:EGFP)* larvae in a WT and *il10*-Mut background proved that the number of GFP⁺ cells in the intestines of *il10*-Mut reporters was lower compared to WT (Fig. 5c, d), thus demonstrating an impaired Notch activity in *il10*-Mut larval intestines. In mammals, intracellular IL-10 downstream signaling relies predominantly on the activity of the transcription factor Stat3,⁴⁷ and the zebrafish intestine was shown to contain Stat3-responsive cells with progenitor-like features.⁴⁸ To determine whether Stat3 activity is impaired in the intestines of *il10*-Mut larvae, we crossed *il10*-Mut with the Stat3 activity reporter *Tg(7xStat3:EGFP)*.⁴⁸ Imaging and quantification of the number of

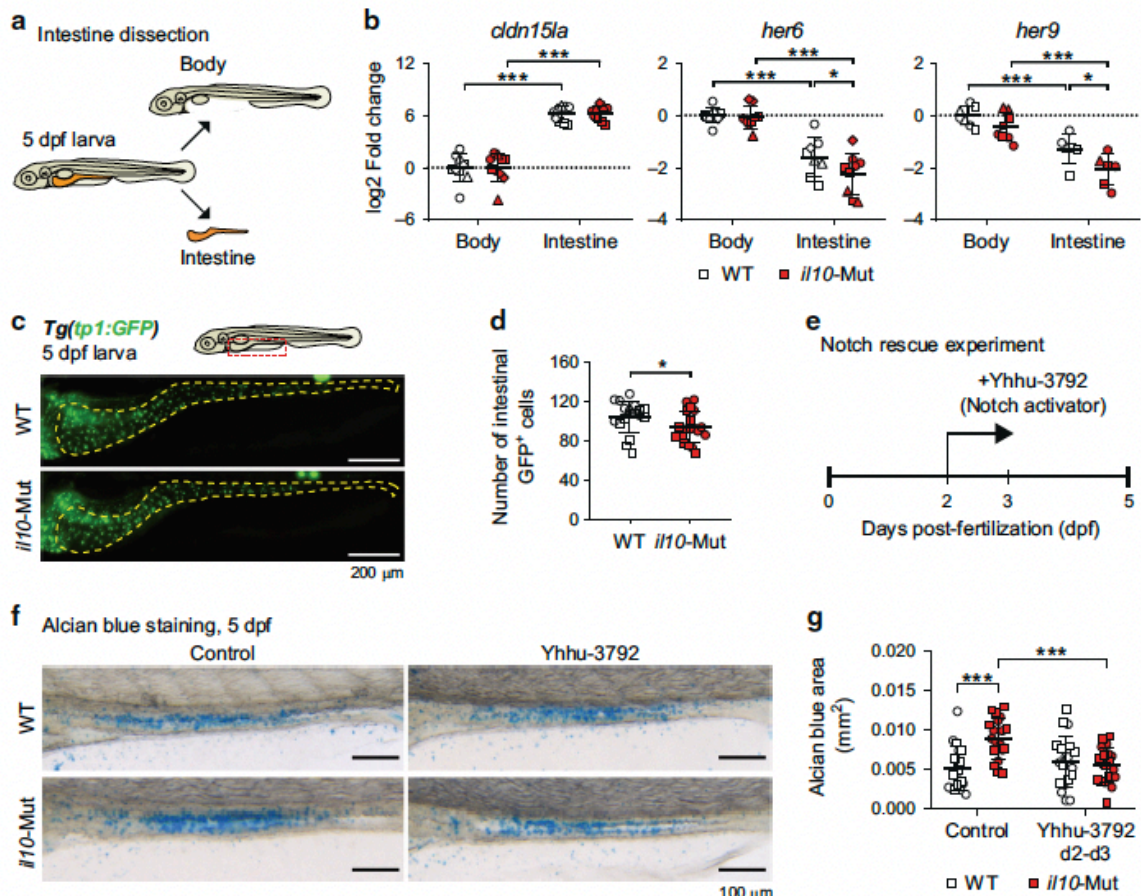


Fig. 5 Decreased Notch signaling activity in the intestines of *il10* mutant larvae. **a** Schematics showing the body-intestine tissue collection for RNA expression analyses. **b** qRT-PCR for intestinal (*ddn15la*) and Notch signaling pathway markers (*her6* and *her9*). Each dot represents a pool of 10 separated intestines and body remnants collected from 4 independent experiments. **c** Representative images of *Tg(tp1:GFP)* in WT or *il10*-Mut genetic backgrounds. Scale bar = 200 μ m. **d** Quantification of the number of *tp1:GFP*⁺ cells in the intestines of WT or *il10*-Mut individuals. Each dot corresponds to 1 larva. Two independent experiments were performed. **e** Schematics for the rescue experiments in which *il10*-Mut larvae are exposed to the Notch activator Yhhu-3792. **f** Representative pictures of alcian blue stainings from 5 dpf WT and *il10*-Mut larvae after treatment with Yhhu-3792. Scale bar = 100 μ m. **g** Quantification of the ab-stained area after Yhhu-3792. Each dot represents individual larvae collected from two independent experiments. Independent experiments are shown with different symbol shapes in **b**, **d** and **g**. Two-way ANOVAs with Fisher's LSD multiple comparisons tests were performed in **b** and **g**, while a two-tailed t-test was performed in **d** (* $p < 0.05$; *** $p < 0.001$).

intestinal GFP⁺ cells showed no differences between WT and *il10*-Mut larvae (Fig. S5c, d), suggesting that Stat3 signaling is not affected in *il10*-Mut larvae.

It has been previously shown that Notch signaling controls secretory cell differentiation in a time-specific manner, particularly during the phase of intestinal proliferation and polarization between 64–74 hpf.⁴⁹ We validated these findings by using the γ -secretase/Notch inhibitor DAPT at 2 different time points: from 48 to 72 hpf (hereinafter referred to as 2–3 dpf) and from 72 to 120 hpf (referred to as 3–5 dpf, Fig. S6a). We found that Notch inhibition during 2–3 dpf led to an increased ab-stained area in WT larvae, while 3–5 dpf inhibition did not modulate ab levels (Fig. S6b, c). Additionally, we tested goblet cell abundance after inhibiting Stat3 signaling with the Jak2/Stat3 inhibitor AG-490⁴⁸ and found a reduced ab-stained area in AG-490-treated compared to control larvae, at both 2–3 dpf and 3–5 dpf time points (Fig. S6d–f). These results are opposite to our observations in *il10*-Mut larvae and suggest that Stat3 signaling is unlikely to play a role in the goblet cell hyperplasia observed in *il10*-Mut larvae. Therefore, we sought to determine whether boosting Notch activity between 2–3 dpf restores ab-stained area to normal levels in *il10*-Mut larvae. For this purpose, we took advantage of the Notch activator Yhhu-3792⁵⁰ and tested its activity in zebrafish larvae. Incubation of WT larvae with 1 μ M of Yhhu-3792 was

sufficient to upregulate the expression of *her6* transcripts (Fig. S7a, b). We then analyzed goblet cell abundance in WT and *il10*-Mut larvae exposed or not to 1 μ M of Yhhu-3792 at 2–3 dpf (Fig. 5e). Yhhu-3792 treatment of *il10*-Mut larvae resulted in reduced ab-stained area compared to vehicle-treated *il10*-Mut and comparable to WT larvae (Fig. 5f, g). As expected, Yhhu-3792 treatments in *il10*-Mut larvae at 3–5 dpf did not result in changes in the ab-stained area (Fig. S7c–e). These results indicate that the increased abundance of goblet cells in *il10*-Mut larvae is restored by activating Notch signaling in a specific temporal window.

IL-10 enhances Notch signaling and restricts mucin production in mouse small intestine organoids

To address whether our findings in zebrafish can be translated to mammals, we used a 3D murine small intestine (SI) organoid system which models key features of intestinal differentiation and development in vitro.⁵¹ We isolated crypts from wild-type C57/Bl6 mice and we cultured them in EGF-, Noggin-, and R-Spondin-containing media (ENR medium; see methods) supplemented or not with recombinant murine IL-10 for 4 days (Fig. 6a). Crypt domains per organoid and organoid areas were comparable between treatments on day 4 (d4) of treatment (Fig. S8a–c). However, transcriptional analyses of d4 organoids showed that IL-10 treatment increased the expression of the stem cell-related

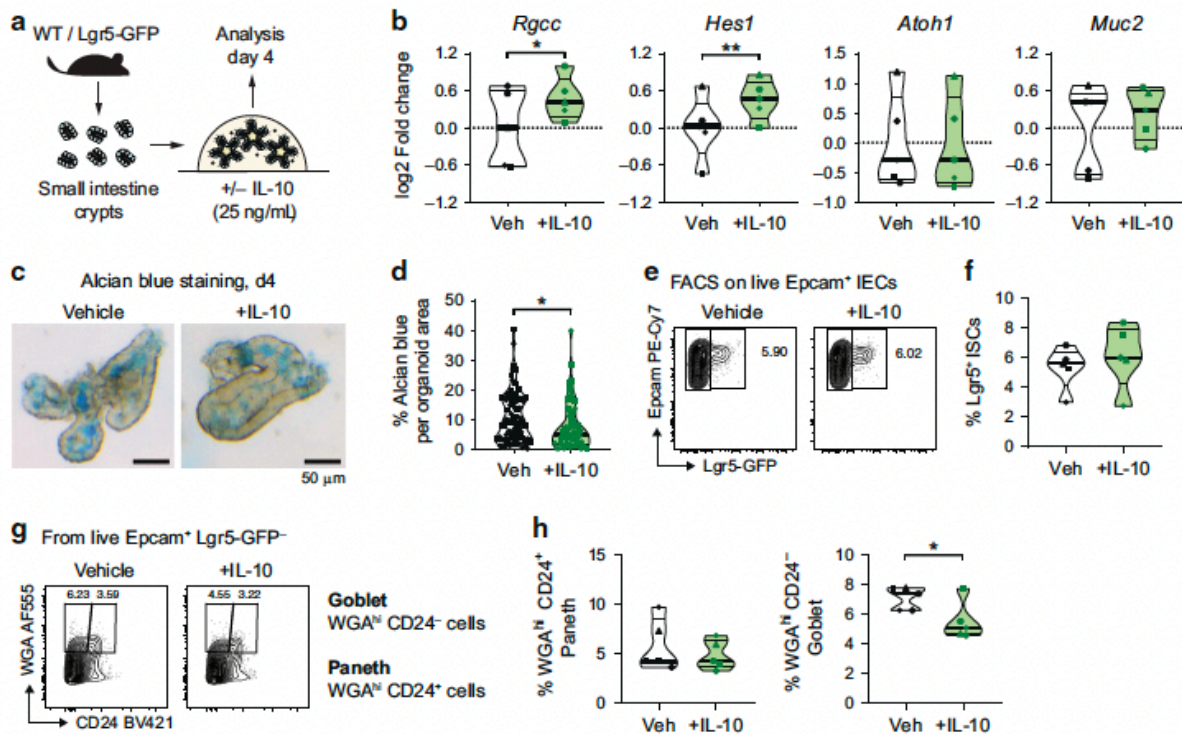


Fig. 6 Enhanced Notch signaling and decreased goblet cell frequencies in mouse small intestine organoids treated with IL-10. **a** Schematics for mouse SI organoid cultures. Organoids were grown in ENR media (containing EGF, Noggin, and R-Spondin) supplemented with recombinant murine IL-10 or BSA (Vehicle control). **b** RNA expression analysis by qRT-PCR from organoids treated with IL-10. Each dot represents SI organoids generated from an individual mouse ($N = 5$ independent mice). **c** Alcian blue staining of SI organoids in vehicle- and IL-10-treated organoids. Scale bar = 50 μm . **d** Percentage of the ab-stained area from the total organoid area. Dots represent single organoids imaged from 2 independent experiments/mice. **e** Flow cytometry analysis of organoids generated from SI crypts of Lgr5-GFP mice. Representative contour plots showing the percentage of intestinal stem cells (Epcam⁺ Lgr5-GFP⁺ cells) are shown. **f** Frequency of Lgr5⁺ intestinal stem cells (ISCs) after IL-10 treatments. Dots represent pools of organoids collected from 5 different mice. **g** Representative contour plots of WGA/CD24 stainings on disaggregated organoids treated with IL-10. **h** Frequencies of goblet cells (Epcam⁺ Lgr5-GFP⁻ WGA^{hi} CD24⁻) and paneth cells (Epcam⁺ Lgr5-GFP⁻ WGA^{hi} CD24⁺) from vehicle and IL-10-treated SI organoids. $N = 5$ independent mice. Independent experiments are represented by different symbol shapes in **b**, **d**, **f** and **h**. Conventional two-tailed t -test was performed in **d**, whereas paired two-tailed t tests were performed in **b**, **f** and **h** ($*p < 0.05$, $**p < 0.01$).

gene *Rgcc* and the Notch target gene *Hes1*, compared to vehicle-treated organoids (Fig. 6b). Similar to zebrafish, we did not observe differences in the expression of the secretory cell differentiation driver *Atoh1* and the goblet cell marker *Muc2* (Fig. 6b), nor in additional markers for intestinal stem cells (*Olfm4*) and other secretory cell types including enteroendocrine (*Chga*), tuft (*Dclk1*) and Paneth cells (*Lyz1*) (Fig. S8d). Further, we did not find differences in the expression levels of the intestinal differentiation marker *Krt20* and the Wnt member gene *Wnt3* (Fig. S8d). To analyze goblet cells abundance in control and IL-10-treated organoids, we modified the zebrafish ab staining protocol to perform whole-mount staining in matrigel-embedded SI organoids (see methods). IL-10-treated SI organoids exhibited a reduced percentage of ab staining per organoid area when compared to vehicle-treated organoids (Fig. 6c, d). To confirm our findings and expand our analysis to additional intestinal cell populations, we performed flow cytometry analysis of d4 SI organoids generated from Lgr5-GFP mice⁵² and stained with Epcam (Fig. S9a, b), together with WGA and CD24 to detect intestinal stem and secretory cells populations, as previously described.^{53,54} IL-10 treatments did not affect the frequency of organoid ISCs (defined as live Epcam⁺ Lgr5-GFP⁺ cells, Fig. 6e, f). However, IL-10 treatments reduced specifically the frequencies of goblet cells (defined as live Epcam⁺ Lgr5-GFP⁻ WGA^{hi} CD24⁻ cells), while Paneth cells (live Epcam⁺ Lgr5-GFP⁻ WGA^{hi} CD24⁺ cells) remained unaltered (Fig. 6g, h). Further, immunofluorescence stainings of d4 SI organoids with WGA and the Paneth cell marker Lysozyme (Lyz)⁵⁵ confirmed the presence of a group of WGA⁺ Lyz⁻

cells corresponding to goblet cells (white arrows on Fig. S9c), and its reduction after IL-10 treatments (Fig. S9d). Therefore, these results conclude that IL-10 signaling in intestinal epithelial cells modulates goblet cell frequencies across vertebrates through the activation of Notch signaling.

DISCUSSION

How IL-10 signaling controls intestinal immune homeostasis has been an active area of research over the past two decades. However, due to the viviparous nature of the sample sources such as humans and mice, many of these studies were performed in adult specimens, and studies understanding the role of cytokines in intestinal development remain elusive. The ex utero embryonic development and the transparency of the zebrafish larva made it an ideal model to investigate the role of cytokines in the development of intestinal mucosal immunology. Here, we combined zebrafish and mouse organoids to demonstrate that IL-10 signaling controls goblet cell homeostasis in a mechanism involving Notch signaling. Thus, we provided additional evidence showing that cytokines can act beyond leukocytes to orchestrate tissue development and that zebrafish is an excellent model organism to investigate the development and maturation of intestinal mucosal barrier functions.

The relationship between IL-10 signaling and goblet cell function has been a matter of study over the past years. An early study showed that *Muc2* was predominantly overexpressed but aberrantly sulfated in the colon of IL-10 deficient mice.⁵⁶ This was

further addressed in a later report, which showed alterations in the colonic mucus layer of IL-10 deficient mice, specifically a thicker adherent mucus layer which was however more permeable to bacteria⁵⁷. In line with a role in orchestrating epithelial homeostasis, IL-10R signaling disruption specifically in intestinal epithelial cells (IECs) skewed IEC differentiation towards goblet cells over absorptive enterocytes.¹⁴ Moreover, using mouse organoids treated with recombinant IL-10 it was proposed that goblet cell modulation could be a consequence of altered intestinal stem cell (ISC) maintenance and/or differentiation.¹³ However, how IL-10 signaling might control goblet cell homeostasis, whether IL-10 signaling acts directly on ISC, and if such cytokine-IEC crosstalk occurs early in development remained elusive. Using zebrafish, our study proposes that IL-10 signaling regulates intestinal goblet cell homeostasis early in intestinal development in a mechanism involving Notch signaling and without affecting the ISC pool. The latter was investigated in murine organoids and zebrafish, using the *Lgr5* reporter mice and zebrafish markers such as *olfm4.2*, the ortholog of the mammalian stem cell marker *OLFM4* and a potential marker for intestinal stem cells in adult zebrafish.⁵⁸ In addition, we did not observe differences in the telost intestinal progenitor marker *sox9b*,^{48,59,60} nor in the proportion of absorptive neutral red⁺ lysosome-rich enterocytes (LREs), suggesting that *il10* deficiency in zebrafish leads to a specific increase in goblet cells in the larval mid intestine.

We found a temporal correlation between intestinal *il10* expression and Notch activity in controlling secretory cell differentiation. Further, our data highlighted IL-10 signaling as a potential upstream regulator of Notch activity in the zebrafish intestine, which helped to explain the increase of goblet cells observed in *il10*-Mut larvae. Although the inhibition of Notch activity at the peak of intestinal *il10* expression (2-3dpf) recapitulated the phenotypes observed in *il10*-Mut larvae, rescue experiments are needed to directly link Notch as a downstream effector of IL-10 signaling. To rescue Notch activity, a previous study used transgenic animals that overexpressed the Notch intracellular domain (NICD), leading to a permanent activation of Notch signaling.⁴² In our approach, we used a novel synthetic Notch signaling activator Yhhu-3792⁵⁰ which allowed us to rescue Notch signaling specifically at the 2-3dpf time window. Since intestinal ab⁺ goblet cells were restored to WT levels in *il10*-Mut after Yhhu-3792-mediated Notch activation at 2-3dpf, our data strongly suggest that early expression of *il10* is key in controlling goblet cell homeostasis in zebrafish larvae through the regulation of Notch activity. Although Stat3 could serve as a potential link between IL-10 and Notch signaling, as previously reported on monocytes,⁶¹ our data suggest a Stat3-independent mechanism linking both signals. An alternative mechanism could be PI3K/Akt signaling, previously described to be activated by IL-10,^{62,63} or other members of the Stat family which can be activated by IL-10, such as Stat1 and Stat5.⁴⁷ However, further studies will be required to find the exact mechanisms which might explain the connection between *il10* and Notch signaling in the intestine of zebrafish.

Our results from mouse small intestine (SI) organoids revealed that the IL-10-Notch axis is also present in mammals and further confirms its independence from the microbiota. Although we did not find differences in the transcript levels for secretory cell markers in organoids, it is possible that these changes may be visible at a protein level, as observed in zebrafish. In addition, SI organoids do not show elevated numbers of goblet cells under normal sterile growth conditions, thus the addition of inducers of goblet cell differentiation, such as type 2 cytokines or Notch inhibitors⁶⁴ would be needed to potentially visualize its regulation by IL-10. Nevertheless, our results from SI organoids match previous findings from single-cell transcriptomic analysis of mouse intestinal organoids, which showed an increased *Rgcc* expression

after IL-10 treatments.¹³ Given that both *Rgcc* and *Hes1* are used as markers for intestinal progenitors in mice,⁶⁵ there is the possibility that IL-10 is modulating the intestinal stem cell (ISC) pool. However, we did not observe changes in Epcam⁺ Lgr5-GFP⁺ ISCs in our organoid settings after IL-10 treatments. Therefore, the decreased average in the ab-stained area from IL-10-treated organoids, as well as the decreased frequency of Epcam⁺ Lgr5-GFP⁺ WGA^{hi} CD24⁻ cells observed by FACS, indicate that goblet cell homeostasis is specifically modulated by IL-10 in mice. Deeper analyses, focusing on specific intestinal cell populations, are required to better elucidate if IL-10 signaling impacts the specification of intestinal precursors to a secretory lineage or acts directly on goblet cells to control its function.

Our study might have implications for a better understanding of pediatric IBD, as mutations in IL-10 signaling pathway-associated genes are frequent in very-early onset (VEO) IBD cases, and dysregulation of mucus biosynthesis and goblet cell numbers are classic features of IBD. Thus, it is likely that defective IL-10 signaling may contribute to the severity of VEO-IBD by affecting early intestinal epithelial cell development, which may impact subsequent processes such as microbiota colonization. The fact that IL-10 can act beyond immune responses and directly control the differentiation of IECs raises several points to be considered in clinical studies. First, it suggests that aberrant immune responses are not the only driving cause for the development of VEO-IBD in patients carrying mutations in IL-10 signaling-related genes, which explains their resistance to immunosuppressive therapies⁶⁶ and improving defective intestinal development and differentiation might also be considered for therapeutics.⁶⁷ Second, although alterations in mucus and goblet cells are observed in active VEO-IBD cases,^{7,68} there is no clear information about the intestinal cell composition before the onset of the disease and with no active inflammation, which could change the view on the causal factors and the physiological consequences of IBD pathogenesis. Third, despite the lack of clinical evidence directly linking IL-10 signaling, goblet cells, and VEO-IBD, data from experimental models support this idea, and further investigation needs to be conducted to better understand how these factors play a role in the initiation of the disease.

Thus, we propose the existence of an IL-10-Notch axis in the early development of intestinal epithelial cells which regulates mucin production, goblet cell differentiation, and is conserved across vertebrates.

MATERIAL AND METHODS

Animals

Zebrafish (*Danio rerio*, AB strain) were reared and kept in the Karolinska Institutet (KI) Zebrafish Core Facility and the Institut Curie Animal Facility, according to standard protocols. Ethical permits used for zebrafish husbandry were Nr 5756/17 (to Eduardo Villablanca), 14049/19 (to KI Zebrafish Core Facility), and APAFIS #21197-2019062521156746-v2 (To Pedro Hernandez). Zebrafish embryos were collected by natural spawning of adults and were kept at 28 °C in E3 water. The zebrafish line *TgBAC(cldn15a-GFP)^{pd1034,36}* referred to in the text as *Tg(cldn15a:GFP)*, was provided by Prof. Michael Bagnat (Duke University Medical Center, USA); the line *Tg(Tp1b:globexGFP)^{um14,45}* referred in the manuscript as *Tg(tp1:EGFP)* was kindly provided by Prof. Olov Andersson (Department of Cell and Molecular Biology CMB, Karolinska Institutet, Sweden); and the line *Tg(7xStat3-Hsv.Ul23:EGFP)⁴⁸* referred in the manuscript as *Tg(7xStat3:EGFP)*, was kindly provided by Prof. Francesco Argenton (Università degli Studi di Padova, Italy).

Wild-type mice (*Mus musculus*, C57/Bl6 strain) were purchased from Charles River Laboratories or Taconic and were kept in the Comparative Medicine facility from Karolinska University Hospital (AKM). The transgenic strain *Lgr5-EGFP-IRE5-creERT2*,⁵² referred to in the text as *Lgr5-GFP*, was maintained in a C57/Bl6 background at the Comparative Medicine facility from Karolinska Institutet (KM-B). All mice used for experiments were 6–10 weeks old. Animals were handled according to protocols approved by the Stockholm Regional Ethics Committee (Nr 3227-2017 and

6778–2020). All experiments were performed following the national and institutional guidelines and regulations.

Isolation of fluorescent intestinal epithelial cells from zebrafish larvae

To acquire the intestinal epithelial population, approximately 100 of 6dpf *TgBAC(cldn15la:GFP)* zebrafish larvae were collected for the FACS experiment, then intestines were dissected and placed into PBS on ice with a maximum dissection time of 2 h. Intestine dissociation was performed using TrypLE Express (Gibco, Cat. No. 12605028) for 1 h at 37 °C, pipetting up and down every 10 min to support digestion. Digested samples were spun at 1500 g for 5 min at 4 °C and washed twice with PBS 1× before being resuspended together with PBS 1× and 10% FBS (fetal bovine serum). Filtered cells were immediately subjected to FACS at Institut Curie Flow Cytometry Platform using the Sony SH800 Cell Sorter. Dead cells were excluded from analysis using the combination of Calcein Blue (Invitrogen, Cat. No. 65-0855-39) and Propidium iodide viability stains (Sigma-Aldrich, Cat. No. P4864). Non-transgenic and single transgenic controls (pools of 10 dissected guts) were prepared as above and used for gating and compensation. RNA isolation was done using on average 30,000 GFP⁺ or GFP⁻ sorted cells with the Single-Cell RNA Purification Kit from Norgen Biotek Corp and reverse transcribed using Superscript IV Reverse Transcriptase (Life Technologies, Cat. No. 18090050) following the manufacturer's instructions.

Whole-mount in situ hybridization in zebrafish larvae

Portions of the coding sequences for the ZFIN-annotated orthologs of the human *IL10RA* (Gene name: *il10ra*, Gene ID: 777651, ENSEMBL ID: ENSDARG00000100383) and *IL10RB* genes (Gene name: *il10rb*, Gene ID: 619391, ENSDARG00000078042) were amplified by PCR using the primers from Table S1, and subsequently cloned into a pCRII-TOPO vector (Invitrogen, Cat. No. 450640). Probe and control RNA sequences were synthesized in vitro by using T7 and SP6 RNA polymerases and subsequently purified by Lithium chloride (LiCl) precipitation. Whole-mount in situ hybridizations were performed in 3 dpf and 5 dpf zebrafish larvae as previously described²⁰ with the following modifications. Permeabilization was done by treating fixed larvae with 10 µg/mL of Proteinase K (Qiagen, Cat. No. 19131) prepared in PBS supplemented with Tween-20 0.1% and DMSO 1% for 30 minutes at room temperature. Pre-hybridization was done at 60 °C for at least 3 h in Hybridization media (HM, Formamide 50%, Sodium citrate solution 5×, citric acid 9.2 mM, Heparin 50 µg/mL, RNase-free yeast tRNA 500 µg/mL). Probe hybridization was done using 500 µL of HM containing 4 ng/mL of either probe or control RNA, and samples were incubated overnight at 60 °C. Anti-dig-AP antibody (Roche, Cat. No. 1109327493) was diluted 1:3000 in PBS supplemented with Tween-20 0.1%, sheep serum 2%, and bovine serum albumin (BSA) 2 mg/mL. Development of probe hybridizations was done by diluting NBT-BCIP stock solution (Roche, Cat. No. 11681451001) 1:50 in alkaline phosphatase buffer (Tris-HCl 100 mM pH 9.5, MgCl₂, NaCl, Tween-20 0.1%). After 3 h of developing, samples were washed extensively with PBS plus Tween-20 0.1%, fixed in PFA 4%, washed again in PBS Tween-20 0.1%, and subsequently dehydrated in Methanol and stored at -80 °C for at least one night, to allow clearing of background signal. Larvae were rehydrated, transferred to Glycerol 85%, and imaged using a Nikon SMZ25 stereoscope equipped with a DS-FI3 camera.

Generation of *il10*-Mut zebrafish lines

The zebrafish coding sequence for the ortholog of the human *IL10* gene (Gene name: *il10*, Gene ID: 553957, ENSEMBL ID: ENSDARG00000078147)³¹ was targeted by CRISPR/Cas9³⁷ with specific sgRNAs (Table S1). For the maintenance of wild-type and *il10* mutant stocks, zebrafish embryos coming from the incross of heterozygous *il10* individuals were raised, and genotyping was performed once they reached adulthood by fin-clipping a portion of the tail fin. The *il10*^{uu1751} mutation created a restriction enzyme site for EcoNI, which is absent in wild-type fish. Carriers of the *il10*^{uu1751} mutation were screened by PCR followed by enzymatic digestion with EcoNI. Zebrafish carrying the *il10*^{uu1762} mutation were screened by PCR using combinations of primers in which the forward primer was specific for either the WT or mutant sequence. Primers used for genotyping are shown in Table S1.

Cloning of the zebrafish *il10* and *il17a/f3* genes and rescue experiments

The coding sequences for the zebrafish *il10* and *il17a/f3* genes were amplified by PCR using specific primers for both genes (Table S1) and then

cloned in a pCR2.1-TOPO vector (Invitrogen, Cat. No. 450641). In vitro-transcribed mRNA was synthesized using the T7 mMessage mMachine kit (Invitrogen, Cat. No. AM1344), following the manufacturer's guidelines. For injections, 1 nL of 200 ng/µL of in vitro-transcribed mRNA was co-injected with 0.5% of Rhodamine-Dextran MW10000 (Invitrogen, Cat. No. D1824) in 1-cell stage wild-type or *il10*-Mut embryos. Embryos injected with 0.5% Rhodamine-dextran were used as control. At 2 dpf, Rhodamine-positive embryos were selected and used for subsequent analysis.

Body-intestine isolation in zebrafish larva

Larvae from 3 dpf to 5 dpf were euthanized by overdosing them with MS-222 and intestines were isolated mechanically with the use of needles to pierce the tissue and extract the intestines. Around 10 intestines and their respective bodies were collected for analysis.

RNA extraction and qRT-PCR from zebrafish samples

For zebrafish samples, up to 10 tissue samples (i.e. whole larvae, intestines, or body carcasses) were pooled and collected in Trizol reagent (Invitrogen, Cat. no. 10296010), and tissues were homogenized by pipetting the samples repeatedly through 23G and 27G needles. Total RNA was extracted following Trizol manufacturer's instructions. Synthesis of cDNA was performed using the iScript cDNA synthesis kit (BioRad, Cat. No. 1708841). Quantitative PCR was performed using iTaq Universal SYBR Green supermix (BioRad, Cat. No. 1725124), as previously reported.⁶⁹ Primers used for zebrafish qRT-PCR analysis are found in Table S2.

Bleach and antibiotic treatments in zebrafish larvae

Zebrafish embryos between 24–28 hpf were treated with chlorine hypochlorite (0.04%) for 5 minutes and then washed twice with sterile E3 medium for 5 min. After washing, embryos were left in sterile E3 medium containing Ampicillin 100 µg/mL and Kanamycin 5 µg/mL and placed at 28 °C in isolated containers. Media was renewed every day in sterile conditions until the day of sample collection. For the quantification of bacterial loads, genomic DNA from 2 larvae per condition was extracted by (heat-basic), and PCR against 16S All Bacteria³⁰ and zebrafish gDNA (for *il23r* gene) was performed. PCR products were run in a 2% agarose gel, and band intensities were measured in Fiji/ImageJ (NIH).

Alcian blue, neutral red stainings and quantifications in zebrafish larvae

Stainings for Neutral red (Sigma, Cat. No. N4638) and Alcian blue (Sigma, Cat. No. B8438) in 5 dpf zebrafish larvae were performed as previously described.⁷⁰ Alcian blue-stained larvae were additionally treated with H₂O₂ 1.5%/KOH 0.5% to remove pigments before imaging. Larvae were mounted in a lateral position using 1% low-gelling point agarose (Sigma, Cat. No. A9414), and RGB images were acquired using a Nikon SMZ25 stereoscope equipped with a DS-FB camera. Images were cropped to keep the mid-intestine section. Automatic unbiased analyses of the Alcian blue-stained area in the intestine of individual larvae were performed in Fiji/ImageJ software (NIH) using "Colour Deconvolution 1.7" and selecting the "Alcian blue & H" vector to identify the Alcian blue-stained area. For the automatic detection of the Neutral red-stained area, the function "Color Threshold" was used. The length of the alcian blue- and neutral red-stained regions were measured manually in randomized images.

Immunofluorescence staining in zebrafish larvae

Immunostaining was performed on whole larvae at 5 days post fertilization. Paraformaldehyde at 4% was used to fix zebrafish larvae overnight at 4 °C. The samples were then washed with distilled water. For whole-mount 2F11 immunostaining, fixed larvae were permeabilized with cold 100% acetone for 20 min at 4 °C before being washed three times with PBST (PBS supplemented with 0.5% Triton-X-100). Samples were further permeabilized with 1 mg/mL Collagenase from *Clostridium histolyticum* (Cat. No. C2139) for 2 h at room temperature. Samples were then washed with PBST and blocked with 10% of FBS/PBST at room temperature for more than 2 h. The primary mouse monoclonal 2F11 antibody (1:200; Abcam, Cat. No. ab71286) was diluted in blocking solution and incubated at 4 °C for more than 24 h. Following primary antibody incubation, the samples were washed with PBST solution and incubated for at least 2 h with secondary anti-mouse Alexa Fluor 488 antibody (1:500; Invitrogen, Cat. No. A11001) at room temperature in the dark. For whole-mount WGA staining, larvae were washed in distilled water for 1 h at

room temperature, permeabilized with cold 100% acetone for 20 min at -20°C , and washed with PBST. Larvae were incubated in Alexa Fluor 555-conjugated Wheat Germ Agglutinin (1:5000 from 5 mg/mL stock; Invitrogen, Cat. No. W32464) overnight at 4°C and washed extensively with PBST. Imaging was performed using the Zeiss Axio Zoom.V16 stereoscope or the Zeiss LSM800 confocal microscope and analyzed with ImageJ software.

Chemical treatments in larval zebrafish

At 2 days post fertilization, pools of 10 larvae per milliliter were exposed to the Notch/ γ -secretase inhibitor DAPT (50 μM ; Tocris, Cat. No. 2634), the Jak2/Stat3 inhibitor Typhostin AG-490 (50 μM ; Sigma-Aldrich, Cat. No. T3434), or the Notch activator Yhhu-3792 (Tocris, Cat. No. 6599), or. As a control for Notch activity induced by Yhhu-3792, RNA was extracted from treated larvae and the expression levels of the Notch downstream gene *her6* were measured by qRT-PCR.

Fluorescent reporter imaging and analysis

The fluorescent reporters for Notch activity *Tg(tp1:GFP)* and Stat3 activity *Tg(7xStat3:EGFP)*, in either wild-type or *il10^{tm1.751}* genetic background, were imaged at 5 dpf in an SMZ25 stereoscope equipped with a CoolLed laser set and a DS-FB camera (Nikon), focusing on the intestinal region. Z-stacks were merged in Fiji/ImageJ software, and the number of GFP⁺ cells in the intestinal region was counted manually in Fiji/ImageJ.

Dissection, sectioning, and staining of adult zebrafish intestines

Zebrafish older than 1 year of age were euthanized with an overdose of MS-222. Intestines were collected and fixed in neutral buffered formalin (Sigma, Cat. No. HT-501128) overnight at room temperature, and subsequently transferred to ethanol 70% for at least 24 h. Tissues were dehydrated and embedded in paraffin, and 10 μm sections were collected and stained with alcian blue. Briefly, tissue sections were deparaffinized and rehydrated to PBS, and incubated in 3% alcian blue solution (Sigma) for 5 min. After staining, tissues were counterstained with nuclear fast red (Sigma, Cat. No. N3020). Images were acquired in a Nikon SMZ25 stereoscope and quantifications of goblet cells per villus area were performed using QuPath 0.2.3 (University of Edinburgh, UK).

Mouse intestinal organoid cultures

Mouse organoids were generated from crypts derived from the entire small intestine (SI). Briefly, SIs from WT mice were collected and flushed with PBS, cut opened longitudinally, and subsequently cut into five pieces of similar size. Tissue pieces were placed in PBS and were vigorously shaken to remove mucus. Tissue pieces were then transferred to cold PBS-EDTA 10 mM and incubated for 1 h in ice. After incubation, SI villi were removed by gentle scraping of the luminal side using two glass slides. Then SI crypts were scraped from the tissue pieces by applying stronger pressure with the glass slides and collected in recipient tubes filled with cold PBS. Collected crypts were centrifuged for 5 min at $300\times g$ and 4°C , and then quantified using an upright microscope by placing 10 μL of crypt solution on a glass slide. The basic culture medium (ENR) contained advanced DMEM/F12, 1 \times penicillin/streptomycin, 1 \times Glutamax (Thermo Fisher Scientific), 10 mM HEPES (Thermo Fisher Scientific), 1 \times B27 supplement (Life Technologies, Cat. No. 17504044), 1 \times N2 supplement (Life Technologies, Cat. No. 17504048), 1 mM N-acetylcysteine (NAC, from Sigma, Cat. No. A9165) and was supplemented with 50 ng/mL of recombinant murine epidermal growth factor (EGF, from R&D, Cat. No. 2028-EG), 250 ng/mL recombinant murine R-Spondin (R&D, Cat. No. 3474-RS) and 100 ng/mL recombinant murine Noggin (Peprotech, Cat. No. 250-38). SI crypts were resuspended in 30–40% basic culture medium with 60–70% Matrigel (Corning, Cat. No. 356231) and 20 μL containing approximately 500 crypts were plated in a pre-warmed flat-bottom 48-well plate (Sarstedt, Cat. No. 83.3923). The plate was placed at 37°C and allowed to solidify for 15 min before 200 μL of ENR medium (containing the different stimuli) was overlaid. The medium was replaced every 2 days with fresh medium and cultures were maintained at 37°C in fully humidified chambers containing 5% CO_2 . During the first 2 days of culture, the ENR medium was supplemented with 10 μM of the ROCK inhibitor Y-27632 (Sigma, Cat. No. Y0503). For organoid in vitro stimulation, 25 ng/mL of recombinant murine IL-10 (Peprotech, Cat. No. 210-10) diluted in 0.1% bovine serum albumin (BSA, Sigma) was added in the ENR medium for the entire duration of the organoid cultures. Control

organoids were supplemented with a similar volume of 0.1% BSA vehicle. Each condition was plated in technical triplicates. On day 4 of culture, crypt domains per IL-10-treated or vehicle-treated organoids were quantified in 2–3 wells/condition. Each dot in the quantification plot represents one mouse and an average of 2–3 technical replicates.

Alcian blue stainings on mouse SI organoids

The Alcian blue protocol used for the staining of mouse SI organoids was adapted from the protocol used for whole-mount Alcian blue stainings in zebrafish. Organoids were collected after 4 days of culture, washed 5 times with cold PBS-BSA 0.1%, and replated in a new Matrigel stock in 24-well plates, to remove debris from the initial culture. After Matrigel gelification, organoids were fixed in PFA 4% for 45 minutes, washed twice with PBS-BSA 0.1%, and washed twice with acidic ethanol (70% Ethanol supplemented with 1% HCl). Organoids were stained with Alcian blue solution (Sigma, Cat. No. B8438) for 1 h at room temperature. After the incubation, stained organoids were washed extensively with acidic ethanol and were left overnight in acidic ethanol at 4°C , to remove excessive staining from the Matrigel. Once the Matrigel became clear, organoids were washed with PBS-BSA 0.1% and imaged using the Nikon SMZ25 system.

RNA extraction from mouse organoids and qRT-PCR

After 4 days of culture, treated organoids were harvested and Matrigel was removed by consecutive washes with cold PBS-BSA 0.1%. Cleaned organoids were resuspended in RLT-plus buffer supplemented with 1% β -Mercaptoethanol, and RNA extraction was performed using the RNeasy extraction kit (Qiagen, Cat. No. 74136), following the manufacturer's instructions. Similar to zebrafish samples, synthesis of cDNA was performed using the iScript cDNA synthesis kit (BioRad), and quantitative PCR was performed using IQ SYBR Green mix (BioRad). Primers used for mouse qRT-PCR analysis are found in Table S2.

Flow cytometry from organoid cell suspensions

For flow cytometry experiments, SI organoids were generated from crypts extracted from Lgr5-GFP mice. After 4 days of treatment, organoids were harvested and washed 5 times with cold PBS-BSA 0.1%. Organoids were disaggregated to single cells by incubating with TrypLE supplemented with DNase I for 5 min at 37°C . Cells were incubated in Fc block (1:1000; Invitrogen, Cat. No. 14-0161-85) and Fixable Viability Dye eFluor 780 (1:1000; eBioscience, Cat. No. 65-0865-14) for 15 min at 4°C , and subsequently stained with anti-Epcam PE-Cy7 (1:200; BioLegend, Cat. No. 118215), anti-CD24 BV421 (1:200; BioLegend, Cat. No. 101825) and WGA Alexa Fluor 555 (1:5000; Invitrogen) for 1 h at 4°C . Flow cytometry was performed using an LSRFortessa flow cytometer (BD Biosciences, USA), and analysis was performed using FlowJo v10 (Treestar, USA).

Immunostaining of small intestine organoids

For immunofluorescence experiments, Matrigel-embedded isolated crypts extracted from small intestines of WT mice were plated in pre-warmed 8-well 15 μm glass-bottom chambers (Ibidi, Cat. No. 80826) and cultured as described above. On day 4 of culture, organoids were fixed in PBS-buffered Paraformaldehyde 4% for 45 minutes, permeabilized with Triton X-100 0.5% for 30 minutes and treated with Glycine 0.1 M for 1 h to block free aldehyde groups. Organoids were incubated in blocking solution (Normal goat serum 5%, Triton X-100 0.25%, BSA 0.1% in PBS) for 30 min before incubating with WGA Alexa Fluor 555 (1:5000, Invitrogen) and rabbit polyclonal anti-Lysozyme Ab-1 (1:200; Thermo Scientific, Cat. No. RB-372-A) overnight at 4°C . After 3 washes with PBS-BSA 0.1%, organoids were incubated with goat anti-rabbit Alexa Fluor 647 (1:1000; Invitrogen, Cat. No. A21244) overnight at 4°C . On the final day of staining, organoid nuclei were stained with DAPI (1 $\mu\text{g/mL}$, Molecular Probes, Cat. No. D1306) for 30 min at room temperature, washed, and kept in PBS until imaging within 0–3 days. Images from stained organoids were acquired in a Zeiss LSM800 confocal microscope (Zeiss, Germany) and analyzed using ImageJ.

Statistical analysis

Quantitative data were analyzed using GraphPad Prism v8.0 software (GraphPad, San Diego, CA, USA). Group comparisons were considered statistically significant when they reached a p -value below 0.05.

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AUTHOR CONTRIBUTIONS

Idea conception and experimental design: R.A.M., S.D., P.P.H., E.J.V. Experiment execution and data analysis: R.A.M., S.R., O.E.D., Y.S., M.W., B.C.K., X.L., G.M., S.M.P., S.D. Manuscript preparation: R.A.M., E.J.V. All authors have revised and accepted the final version of the manuscript.

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The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Rodrigo A. Morales or Eduardo J. Villablanca.

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Figure S1

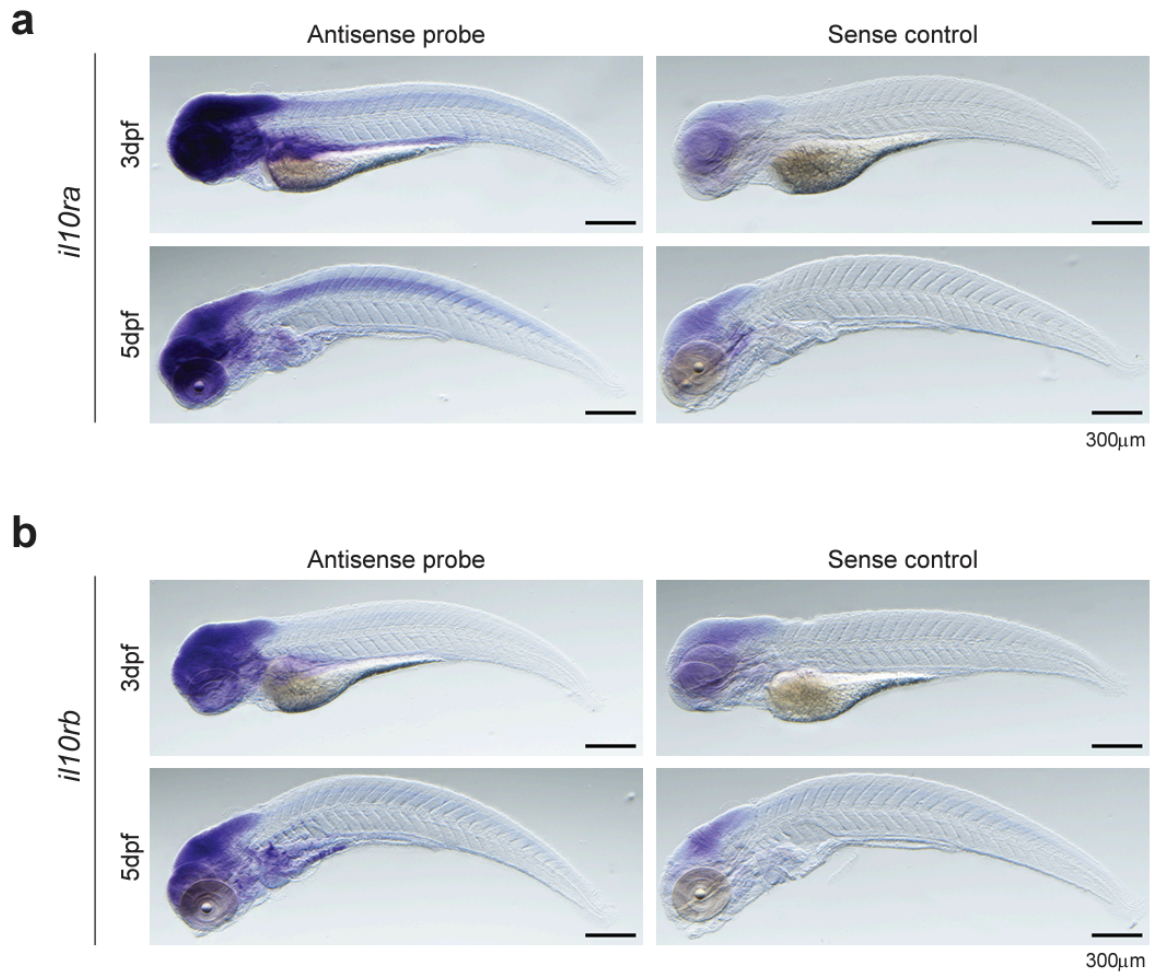


Figure S1. Specificity of the whole-mount in situ hybridizations against *il10ra* and *il10rb* in zebrafish larvae.

Representative pictures of complete 3dpf and 5dpf larvae labeled with antisense probe and sense control oligos against *il10ra* (a) and *il10rb* (b). Scale bar = 300 μ m.

Figure S2

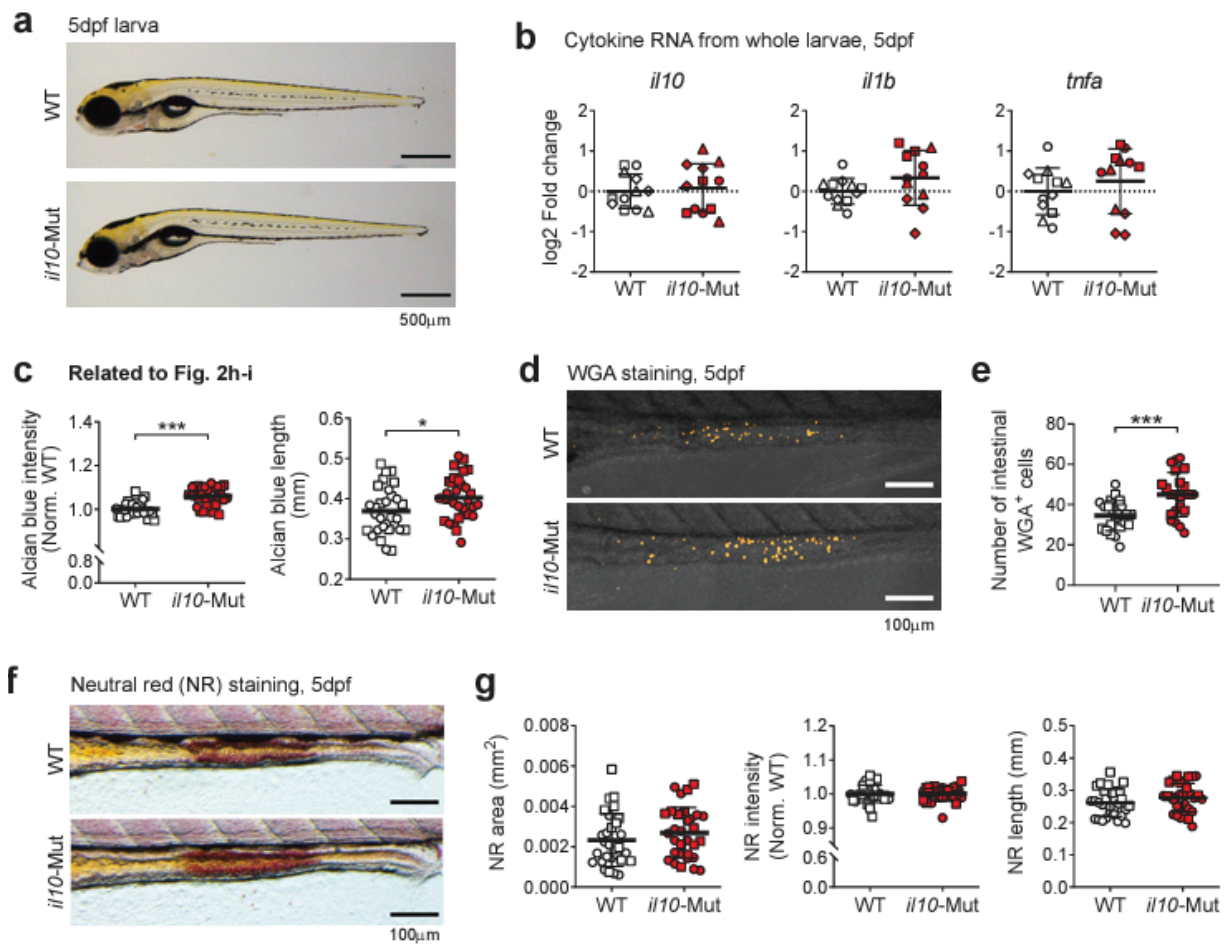


Figure S2. Characterization of *il10*-Mut zebrafish larvae.

(a) Representative images of 5dpf WT and *il10*-Mut zebrafish larvae. Scale bar = 500 μ m. (b) Whole-body expression analysis of cytokines by qRT-PCR. Each dot represents a pool of 5-10 larvae collected from 3-4 independent experiments. (c) Alcian blue signal intensity and length from analysis performed in Figure 2h-i. N = 2 independent experiments. (d) Confocal images from whole-mount WGA stainings on 5dpf WT and *il10*-Mut larvae. Scale bar = 100 μ m. (e) Quantification of the number of WGA⁺ goblet cells in the mid intestines of 5dpf WT and *il10*-Mut larvae (N = 2 independent experiments). (f) Neutral red stainings on 5dpf WT and *il10*-Mut larvae. Scale bar = 100 μ m. (g) Area, intensity, and length measurements for the neutral red-stained regions. Each dot represents an individual larva collected from 2 independent experiments. Two-tailed t-tests were performed in b, c, e and g (* p < 0.05; *** p < 0.001).

Figure S3

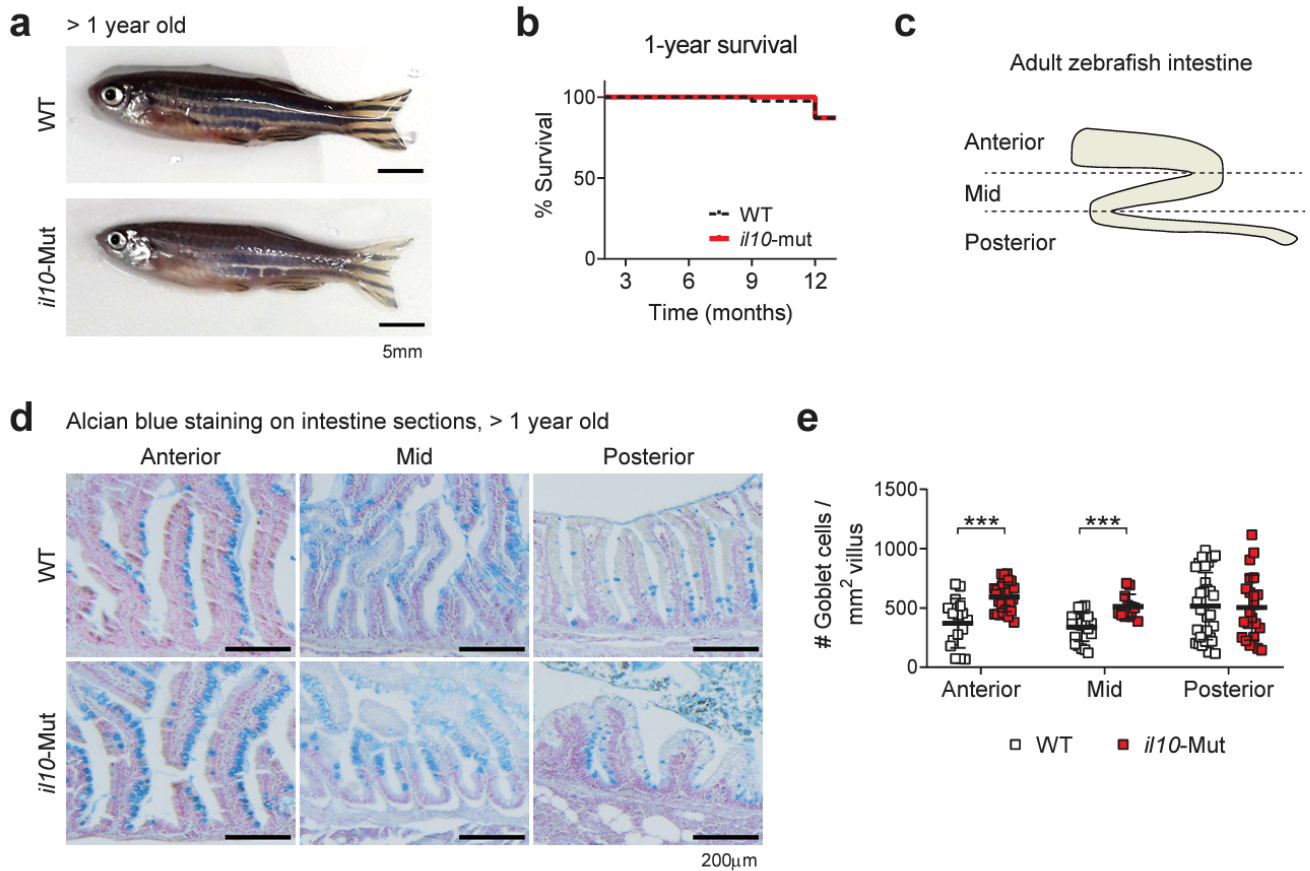


Figure S3. Characterization of *il10-Mut* adults.

(a) Images of WT and *il10-Mut* zebrafish adults. Scale bar = 5mm. (b) Survival of WT and *il10-Mut* zebrafish over a period of 1 year (N= 38 for WT and 39 for *il10-Mutant*, out of 2 independent breedings). (c) Representation of an adult zebrafish intestine and the sections used for analysis. (d) Representative alcian blue staining images from intestinal sections of WT and *il10-Mut* zebrafish. Scale bar = 200µm. (e) Quantification of goblet cells (ab+ cells) from WT and *il10-Mut* intestine sections. The number of goblet cells was normalized by the area of the villi analyzed. Unpaired two-tailed t-tests were used in e (**p < 0.01, ***p < 0.001).

Figure S4

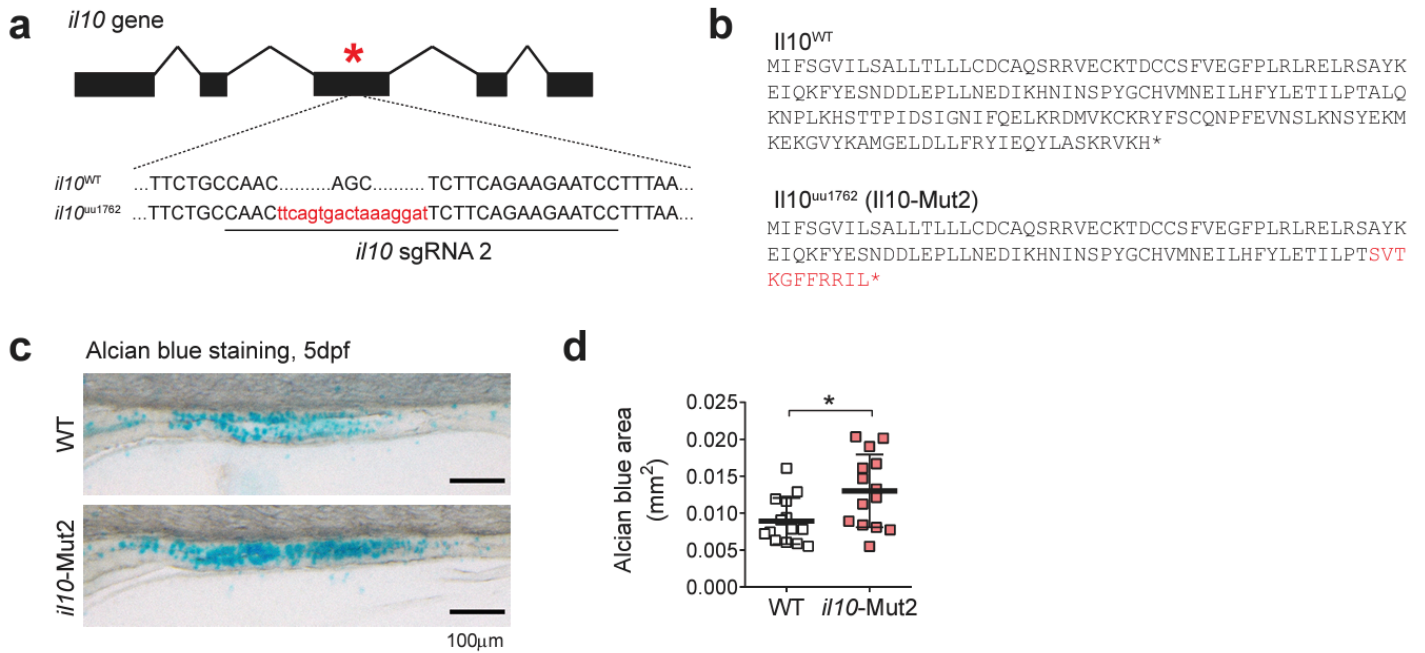


Figure S4. Increased intestinal alcian blue⁺ goblet cells in a second mutant line for *il10*.

(a) Schematics for the second mutation generated in the zebrafish *il10* gene (*il10*^{uu1762}, +14bp) by CRISPR/Cas9. (b) Predicted sequences for Il10 protein in *il10*-Mut2 individuals, compared to WT. (c) Alcian blue stainings in the mid intestines of 5dpf WT and *il10*-Mut2 larvae. (d) Automatic quantification of the ab-stained area of WT and *il10*-Mut larvae. A two-tailed t-test was performed in b (**p*<0.05).

Figure S5

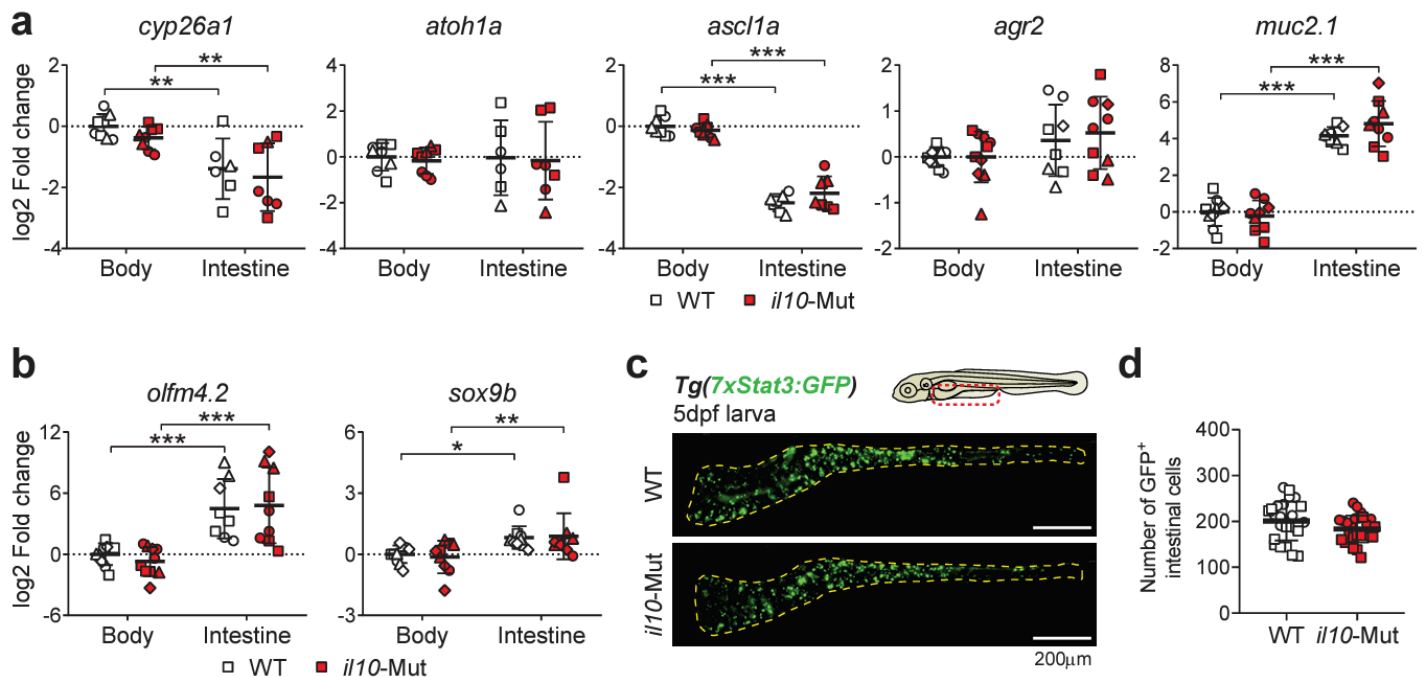


Figure S5. Body-intestine transcriptomic profile of WT and *il10*-Mut zebrafish larvae.

RNA from dissected intestines and body remnants from 5dpf WT and *il10*-Mut larvae was used to perform qRT-PCR analysis against RAR, ARP/ASCL signaling pathways, and markers of goblet cells (a) and for the candidate markers of intestinal progenitors *olfm4.2* and *sox9b* (b). Each dot corresponds to RNA from a pool of 10 intestines or body remnants, collected in 3-4 independent experiments. (c) Representative pictures of the intestines of *Tg(7xStat3:EGFP)* in WT and *il10*Mut genetic backgrounds. Scale bar = 200μm. (d) Number of GFP⁺ cells in the intestines of 5dpf *Tg(7xStat3:GFP)* WT and *il10*-Mut larvae. Dots represent individual larvae collected from 2 independent experiments. Two-way ANOVAs with Fisher LSD multiple comparison tests were used in a and b, whereas a two-tailed t-test was performed in d (** $p < 0.01$; *** $p < 0.001$).

Figure S6

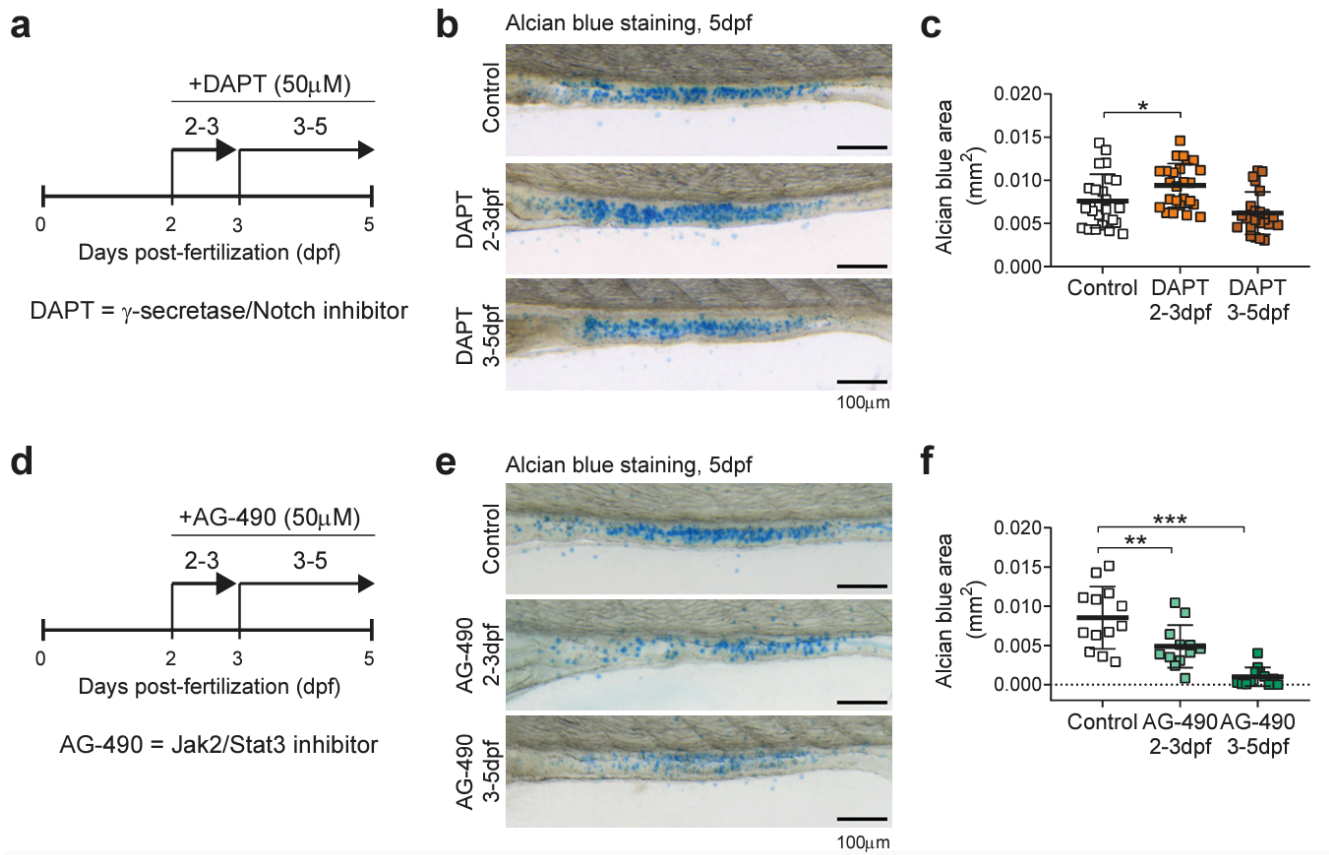


Figure S6. Early inhibition of Notch promotes alcian blue+ goblet cell expansion in WT larvae.

(a) Diagram showing the times of treatments with the γ -secretase/Notch inhibitor DAPT in WT zebrafish larvae. (b) Alcian blue staining of DAPT-treated zebrafish larvae at 5dpf. Scale bar = 100 μ m. (c). Automatic quantifications of the ab-stained area of DAPT-treated larvae at 5dpf (1 dot = 1 larva). (d) Schematics for the treatment of WT larvae with the Jak2/Stat3 inhibitor AG490. (e) Alcian blue staining of AG-490-treated zebrafish larvae at 5dpf. Scale bar = 100 μ m. (f). Automatic quantifications of the ab-stained area of DAPT-treated larvae at 5dpf (1 dot = 1 larva). One-way ANOVAs were performed in c and f.

Figure S7

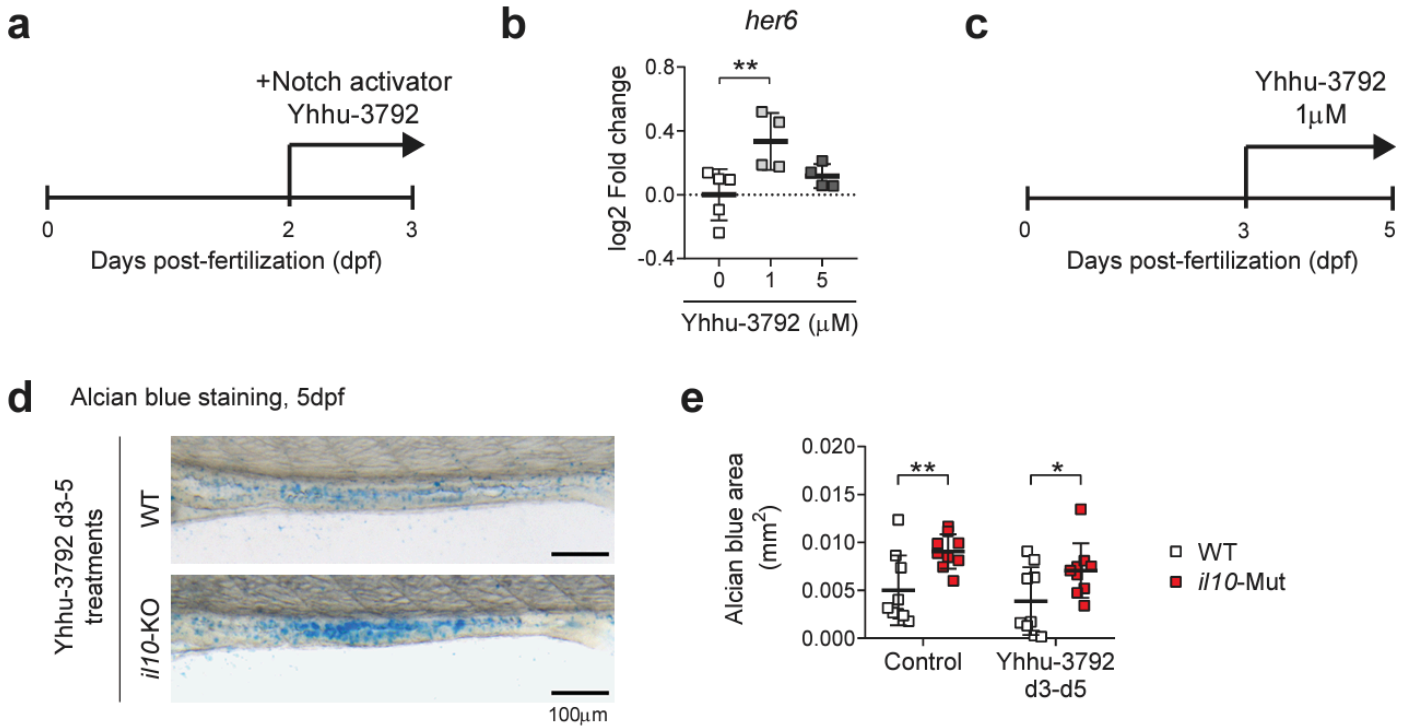


Figure S7. Validation of the Notch activator Yhhu-3792 in zebrafish larvae.

(a) Strategy to test the activity of Yhhu-3792 in zebrafish larvae. Different doses of Yhhu-3792 were used to treat larvae between 2-3dpf. (b) Expression of the Notch target gene *her6* in 3dpf zebrafish larvae after treatments with different concentrations of Yhhu-3792. Each dot represents a pool of 10 larvae collected from 2 independent experiments. (c) Strategy to test for Notch rescue experiments in *il10Mut* larvae between 3-5dpf. (d) Representative alcian blue stainings of 5dpf WT and *il10-Mut* zebrafish after treatments with Yhhu-3792 between 3-5dpf. Scale bar = 100μm. (e) Quantifications of the ab-stained area in the intestines of 5dpf WT and *il10-Mut* larvae after treatments. One-way ANOVA was performed in b, while Two-way ANOVA was performed in e (*p<0.05; **p<0.01).

Figure S8

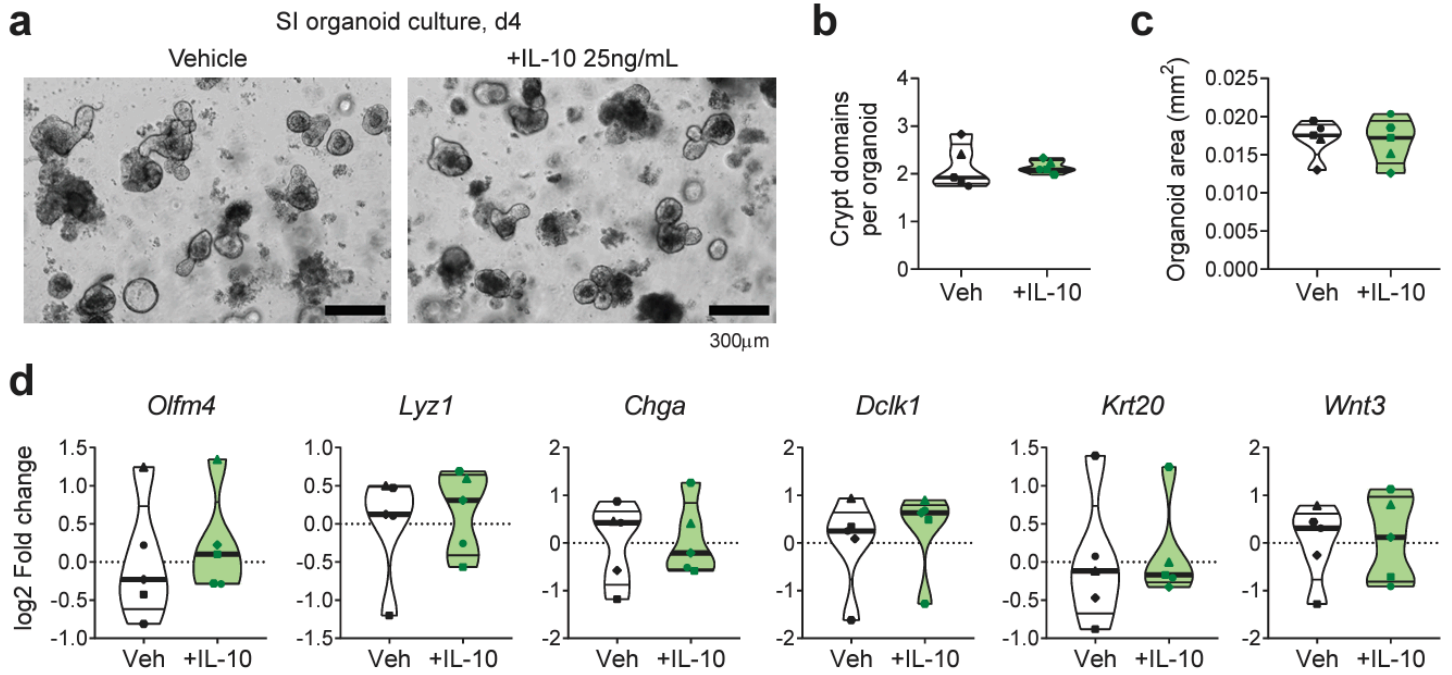
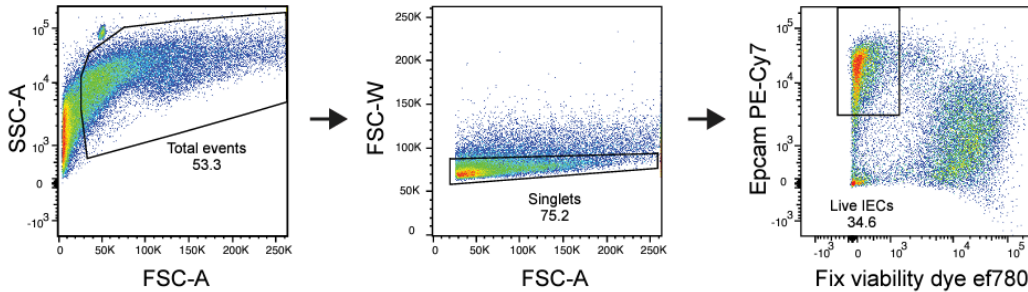


Figure S8. Analysis from mouse SI organoids.

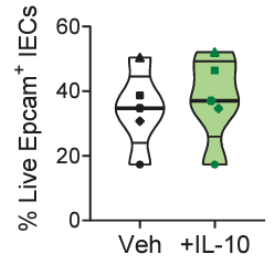
(a) Bright-field images from the organoids grown after 4 days of treatment with 25ng/mL of recombinant murine IL-10. Scale bar = 300 μ m. (b) Quantification of the crypt domains per organoid and organoid areas in control and IL-10-treated organoids at d4 of culture. The average of each independent experiment is shown. (c) Transcriptomic analysis of markers for stem cells, secretory cells, and Wnt members by qRT-PCR. In b and c, each dot represents an independent organoid culture started from the crypts of 1 mouse. Paired two-way t-tests were performed in both b and c.

Figure S9

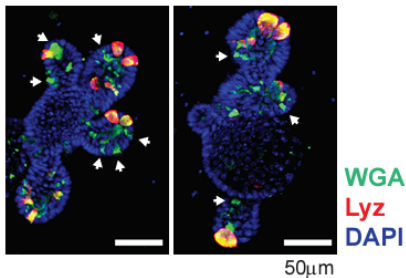
a FACS gating strategy



b



c Vehicle +IL-10



d

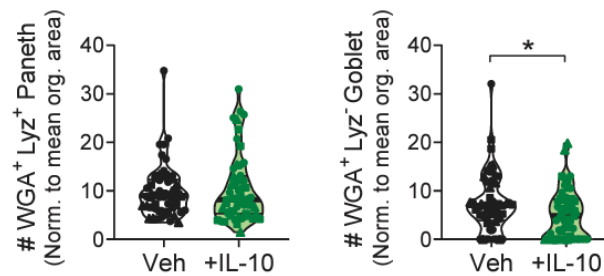


Figure S9. FACS and immunofluorescence staining of SI organoids after IL-10 treatments.

(a) Gating strategy for the selection of live Epcam⁺ intestinal epithelial cells (IECs). (b) Frequency of live Epcam⁺ IECs after treatments (1 dot = 1 independent mouse). (c) Whole-mount immunofluorescence pictures of d4 SI organoids stained with WGA and Lysozyme (Lyz) after IL10 treatments. Goblet cells (WGA⁺ Lyz⁻) are indicated with white arrows. (d) Quantification of Paneth (WGA⁺ Lyz⁺) and goblet (WGA⁺ Lyz⁻) cells per organoid. Dots represent measurements from individual organoids collected in 3 independent experiments. Scale bar = 50µm. A paired two-tailed t-test was performed in b, whereas unpaired two-tailed t-tests were used in d.

a. Oligos used for CRISPR/Cas9 mutagenesis

| Mutation | sgRNA sequence (5' - 3') (PAM in red) | Generated mutation |
|------------------------------|---------------------------------------|--|
| <i>il10^{ju1751}</i> | GGGCTTTCCTTTAAGACTGAGGG | -4bp (4bp deletion) |
| <i>il10^{ju1762}</i> | GGATTCTTCTGAAGAGCTGTTGG | +14bp insertion (3bp deletion,+17bp insertion) |

b. Primers for genotyping

| Mutation | Forward primer (5' - 3') | Reverse primer (5' - 3') |
|------------------------------|---|--------------------------|
| <i>il10^{ju1751}</i> | CTCTGGAGTCATCCTTTCTGCT | AGCTGGAGATCAGTCCAAGTGT |
| <i>il10^{ju1762}</i> | CCAAGTTCAGTGACTAAAGGATTC (WT) CATTCTGCCAACAGCTCTTCAG (KO2) | CCAAAACACTTACGCATTTCCACC |

c. Primers used for cloning of cDNA sequences

| Gene | Forward primer (5' - 3') | Reverse primer (5' - 3') |
|-----------------|-----------------------------------|-------------------------------------|
| <i>il10</i> | ATGATTTTCTCTGGAGTCATCCTTTCTGCAAAA | TTAGTGCTTAACCCTCTTTGAGGCTAGATACTGC |
| <i>il10ra</i> | AACTGCTTGGGTGCTACAGA | CCTTCACCGTATTTGGCCCT |
| <i>il10rb</i> | TCCAGTGTGACTCTGCGTTC | CCTCTGGGATGTGTGTGTCC |
| <i>il17a/f3</i> | ATGCGGCTCTCACGGGTTTTTC | CTAGTTCTGAGGCAAAACAATTGGTAAAACACATG |

d. Primers used for microbiota analysis

| Target | Forward primer (5' - 3') | Reverse primer (5' - 3') |
|------------------------------------|--------------------------|--------------------------|
| Universal 16S bacterial rRNA | ATTACCGCGGCTGCTGGC | ACTCCTACGGGAGGCAGCAGT |
| Zebrafish Genomic (<i>il23f</i>) | AGACATACTGAACGCTGTGG | TTTCTGTGTTGCAGAGCGTAAT |

Table S1. List of oligos used for mutagenesis, cloning and DNA analysis.

Zebrafish

| Gene | Forward primer (5' - 3') | Reverse primer (5' - 3') |
|----------------------|--------------------------|--------------------------|
| <i>eef1a1 (ef1a)</i> | ACCTACCCTCCTCTTGGTCG | GGAACGGTGTGATTGAGGGAA |
| <i>il1b</i> | ATCAAACCCCAATCCACAGAGT | GGCACTGAAGACACCACGTT |
| <i>tnfa</i> | GGAGAGTTGCCTTTACCGCT | TTGCCCTGGGTCTTATGGAG |
| <i>il17a/f3</i> | CGCCTTGGACATACACAACCTT | AGTAAATGGGTTGGGACTCCA |
| <i>ifng1</i> | GCTATGGGCGATCAAGGAAA | GCCGTCTCTTGC GTTCTTT |
| <i>il22</i> | CGATGACTGATACAGCACGA | TGTGCTCGTCTGATTCCAAG |
| <i>cldn15la</i> | TGTTTGATCGGCTGGGTTCT | AGCCTGAATGTGAACTGGCAA |
| <i>fabp2</i> | TGGGCGTCACCTTTGACTAT | GCGTGTCTCCCTCTATGACC |
| <i>il10 (FR)</i> | TCACGTCATGAACGAGATCC | CCTCTTGCATTTACCATATCC |
| <i>il10 (SE)</i> | TAAAGCACTCCACAACCCCA | GACCCCTTTTCTTCATCTTTTC |
| <i>il13</i> | GAAGTGTGAGCATGATTATTTT | CTCGTCTTGGTGGTTGTAAG |
| <i>il4</i> | CATCCAGAGTGTGAATGGGA | TTCCAGTCCCGGTATATGCT |
| <i>her6</i> | AGAGCGAGAATCAACGAAAGC | TTTAGGGCAGCGGTCATTTG |
| <i>her9</i> | GTTCAGATGAGCGCAGCCTT | GCCAAATGAGCCTGTTGAGC |
| <i>cyp26a1</i> | GATGCTCTGGAGCACTACATTC | GTTCTTGCTCGTCCGTCTTTAT |
| <i>atoh1a</i> | TCCTGTCAAAGTACGCGAGC | CAATCCGTGCATTCTTCGCC |
| <i>ascl1a</i> | TGAGCGTTCGTAAAAGGAAACT | TGGCTCTTTGACACTCGGAC |
| <i>olfm4.2</i> | ACAGTCTCTACGCTGAGATTCAC | GCTTGAGTCCTTTCCCATC |
| <i>sox9b</i> | GCCCAGACGGAGGAAATCAG | TGAGACTGACCGGAGTGTCT |
| <i>muc2.1</i> | TGCAACCTCAGCACATGGAT | TCCAGCTGTGACAAACTCC |
| <i>agr2</i> | AGTGCTCTTGGTCATGGTGG | AGGGGCTTGTCTTGGATCG |

Mouse

| Gene | Forward primer (5' - 3') | Reverse primer (5' - 3') |
|--------------|--------------------------|--------------------------|
| <i>B2m</i> | ACCGTCTACTGGGATCGAGA | TGCTATTTCTTCTGCGTGCAT |
| <i>Hes1</i> | GAAAAATTCCTCCTCCCGGT | GGCTTTGATGACTTTCTGTGCT |
| <i>Atoh1</i> | TCCCACAGAAGTGACGGAGA | GGGATATTTGTACGGGGCT |
| <i>Muc2</i> | CAAGTGATTGTGTTTCAGGCTC | TGGAGATGTTCTTGGTGACG |
| <i>Rgcc</i> | GTCACTCCTCGGAAAGCCAA | AGCTTCACTCTCCGAACTGC |
| <i>Wnt3</i> | TGGAAGTGTACCACCATAGATGAC | ACACCAGCCGAGGCGATG |
| <i>Chga</i> | CAGCAGCTCGTCCACTCTTT | GACGCACTTCATCACCTTGG |
| <i>Lyz1</i> | CTGACTGGGTGTGTTTAGCTCAG | AATTGATCCACAGGCATTCTT |
| <i>Dclk1</i> | CAGGAGTTTCTGTAATAGCAACCA | CCGAGTTCAATTCCGGTGGA |
| <i>Olfm4</i> | TGCTCCTGGAAGCTGTAGTCA | TGTATTCAAAGGTGCCACCCA |
| <i>Krt20</i> | GTCCACCTCAGCATGAAAGA | TCTGGCGTTCTGTGTCACTC |

Table S2. List of primers used for qRT-PCR.

DISCUSSION

1. General conclusion and personal impression

When I started my PhD, the prevailing knowledge about cytokines of the IL-10 superfamily, such as IL-10 or IL-22, focused mainly on their immunological functions in adults. However, some studies had begun to suggest that IL-22, for example, was central in physiological processes in the gut, particularly in the regulation of lipid metabolism in adult mammals²⁶⁴. In addition, evidence had emerged suggesting that disruption of IL-10 receptor signaling, particularly within IECs, resulted in a notable bias in IEC differentiation, favoring goblet cells over absorptive enterocytes³³⁶. This observation suggests that this cytokine is intricately involved in the maintenance of epithelial homeostasis. However, despite these advances in knowledge, significant uncertainties remained regarding the extent of IL-10 and IL-22 involvement in additional homeostatic processes and their potential contributions to fundamental aspects of gut maturation and functionality during the early stages of vertebrate life.

Therefore, the main aim of my research was to determine whether these cytokines truly play an important role in gut development during early stages of vertebrate life. Using the zebrafish model, we identified for the first time a role of IL-10 in intestinal goblet cells differentiation through the regulation of Notch activity and the conservation of this process in mouse organoids. I found this particularly intriguing because our findings revealed the ability of IL-10 to extend its influence beyond leukocytes and actively direct tissue development. In addition, our work confirmed the exceptional suitability of the zebrafish model to explore the development and maturation of intestinal mucosal barrier function.

As a second part of my PhD project, I discovered that *il22* is unexpectedly expressed in epithelial cells of the zebrafish developing gut. In addition, we confirmed the conservation of IL-22 target cells, signaling, and anti-bacterial function from zebrafish to mammals. Last but not least, we uncovered an unprecedented role of this cytokine in regulating gut motility during early life in zebrafish and also in young mice. Mechanistically, our investigation revealed microbiota dysbiosis, dysregulation of genes associated with neurons and muscles, as well as perturbations in the secretion of hormones by EECs. In particular, serotonin, a key regulator of gut motility in both zebrafish and mammals, was among the hormones affected.

Working on these two subjects was incredibly rewarding. It gave me the opportunity to work with world-class researchers, present my work in international conferences,

and travel abroad to learn new methods and gain insight into different aspects of biological research. This included working at the interface of immunology and developmental biology, managing zebrafish work, using cutting-edge molecular biology tools, exploring advanced imaging techniques and developing presentation skills.

Starting this PhD was particularly challenging as I was one of the first members of the laboratory when it opened in September 2019. When Pedro Hernandez started his group, I joined first working as a research assistant for two months, which were essential for setting up the laboratory. Since starting my doctoral thesis in December 2019, I had to establish most of the lab methodologies to answer new emerging questions. In the end, characterizing IL-22 role in gut development and maturation was certainly the most exciting part of my PhD, because we basically started everything from the ground up. Thanks to this, I had the opportunity to learn how to use groundbreaking techniques that had not been used in the lab before, like RNA-sequencing, HCR or 10X scRNA-seq for instance. However, deciphering the IL-22 developmental function did not come without its challenges. Indeed, this project required a high number of different transgenic and mutant zebrafish lines and we had several technical issues regarding scRNA-seq and germ-free fish generation for example that required a lot of optimization. Fortunately, our success in overcoming these challenges can be attributed to the concerted efforts of our team, the support of neighboring laboratories and collaborators.

Of course, in the field of biological research there are a large number of questions that tend to remain unanswered. During my PhD work, I have come across several intriguing observations that warrant further investigation. These observations have been grouped into 4 themes: IL-22 effect on gut physiology, the influence of microbiota, IL-22 association with body size, and its potential contribution to lymphatic development. In the following discussion, I will focus on exploring the implications of this work, the perspectives it opens for future research endeavors, and the remaining questions that have been brought to light during the course of my doctoral research.

2. Cytokines and gut physiology

In this study, we first demonstrated the role of IL-10 in regulating goblet cell numbers through the Notch pathway. This finding was an important step in demonstrating that cytokines within the IL-10 family can exert a significant influence on gut maturation during early life. Then, we unexpectedly discovered a novel role for IL-22 in controlling gut motility during early stages of zebrafish development. Our study of *il22*^{-/-} larvae revealed dysregulation in several aspects that may provide insights and explanations for this intriguing phenotype. First, we observed dysregulation in genes associated with neurons. Interestingly, most of these genes were dysregulated in 7 dpf *il22*^{-/-} larvae but not in adult guts, highlighting the early effect of IL-22 on modulating neuron activity (Figure 13).

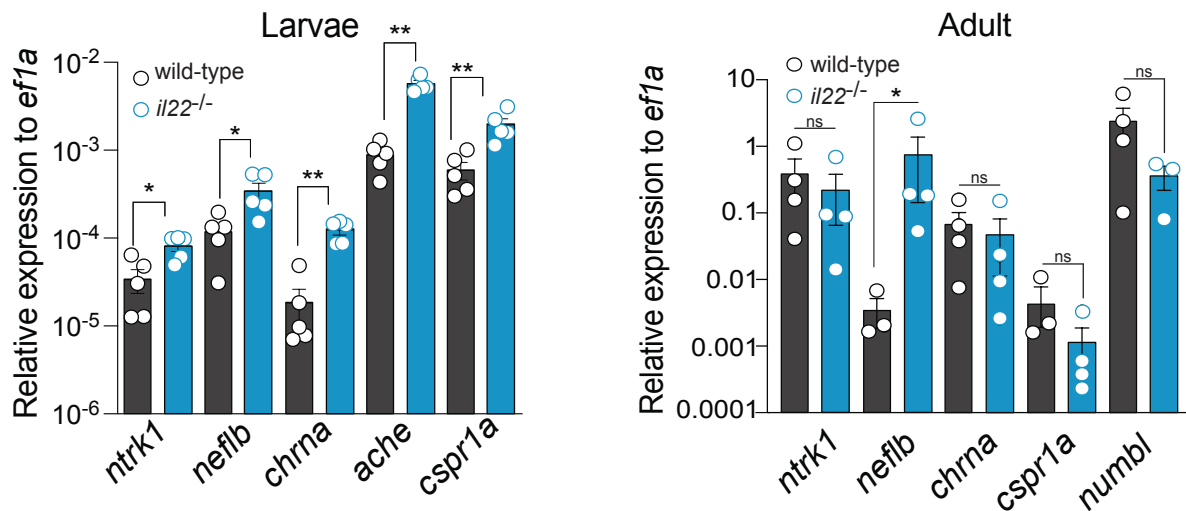


Figure 13. Most neuronal dysregulated genes in *il22*^{-/-} larvae exhibit normal expression in adult

RT-qPCR analysis of dissected guts from 7 dpf larvae (left panel) or adult WT (right panel) and *il22*^{-/-}. This data is representative of 2 independent experiments for larvae and only one in adult. Statistical analyses were performed with Mann-Whitney, ns: not significant, * $P < 0,05$, ** $P < 0,01$.

Although our quantification did not reveal significant differences in the number of neurons in the intestine of *il22*^{-/-} larvae, it is possible that our approach missed subtle changes in neuronal composition. To address this potential limitation, we performed single-nucleus RNA sequencing on dissected guts from 7 dpf WT and *il22*^{-/-} larvae. The aim was to recover enough neurons to detect changes in neuronal subtypes, if present. Unfortunately, we only obtained a limited number of neurons, but a substantial

amount of epithelial cells. We are therefore currently analyzing the dataset in the hope of identifying any meaningful changes. In addition, we observed differences in neuronal activity, and we are currently analyzing further whether the frequency is indeed impaired in the mutant. These differences in enteric neurons activity could have implications for both gut physiology and neurological disorders, highlighting the importance of understanding the underlying molecular mechanisms. Regarding smooth muscle cells, we have yet to determine whether the increased longitudinal muscle number quantified in *il22^{-/-}* is due to a dysfunctional contractile status. However, the RNA sequencing data we have obtained suggest that this may indeed be the case. These changes in muscle structure may be due to dysregulated signaling pathways targeting them.

Then, we observed a dysbiosis in *il22^{-/-}* larvae that resembled patterns often seen in mice, including an enrichment in *Enterobacteria* and a depletion of *Lactobacillus*^{239,247}. Particularly intriguing was the unexpected enrichment of species potentially involved in tryptophan and serotonin synthesis, as we observed fewer 5-HT-expressing cells in *il22^{-/-}* larvae. However, this analysis alone cannot confirm whether the expression levels of these genes are indeed impaired. To determine this, we would need to perform qPCR analysis on RNA extracted from bacteria. However, this is challenging in zebrafish larvae due to the low yield of bacterial RNA recovery (less than 4%). To overcome this difficulty, we are collaborating with the laboratory of Sylvia Brugman, a specialist in microbiota research using the zebrafish model. With her expertise, we aim to investigate whether genes associated with dysregulated processes in bacteria are also dysregulated in *il22^{-/-}* fish. This will allow us to further investigate the dysregulated pathways and understand how they may affect gut motility. Interestingly, the potential enrichment of species involved in GABA biosynthesis is an intriguing aspect of our findings. GABA is recognized as a regulator of intestinal motility. Activation of ionotropic GABA_A and GABA_C receptors usually leads to neurotransmitter release from enteric neurons, resulting in either a contractile or relaxant response of gastrointestinal smooth muscle³³⁷. This observation opens up interesting possibilities for exploring the role of GABA in gut motility regulation in the context of our study.

Another intriguing aspect of our study is the recovery of the gut motility phenotype when *il22^{-/-}* are co-housed with their WT counterparts. We hypothesized that not only microbiota but also metabolites could be directly transferred. We thus explored the role of 5-HT as a potential key player. This hypothesis was driven by our successful rescue of the gut motility phenotype of the mutant by exposure to 5-HT. However, our attempts to measure it by ELISA analysis were unsuccessful, even when pooling 100 larvae. We are therefore considering using mass spectrometry to identify the components present in the water, which may be valuable for future investigations. Nevertheless, this would require significant optimization of the protocol.

It was also surprising to observe that the WT microbiota could rescue the intestinal motility phenotype in *il22^{-/-}* zebrafish. Previous experiments in mice showed that *Il22^{-/-}* mice harboring an altered microbiota had more severe disease during experimentally induced colitis and that this altered gut microbiota could be transmitted to co-housed WT animals, increasing their susceptibility to colitis²⁴⁷. In contrast, *Il22^{-/-}* mice housed with WT mice showed no signs of improvement and continued to exhibit dysbiosis²⁴⁷. To assess whether this phenomenon also applies to the zebrafish model, we decided to co-house *il22^{-/-}* larvae with WT, while simultaneously subjecting them to DSS treatment to induce intestinal inflammation. We measured their survival over time while administering freshly prepared DSS daily for four consecutive days (**Figure 14**).

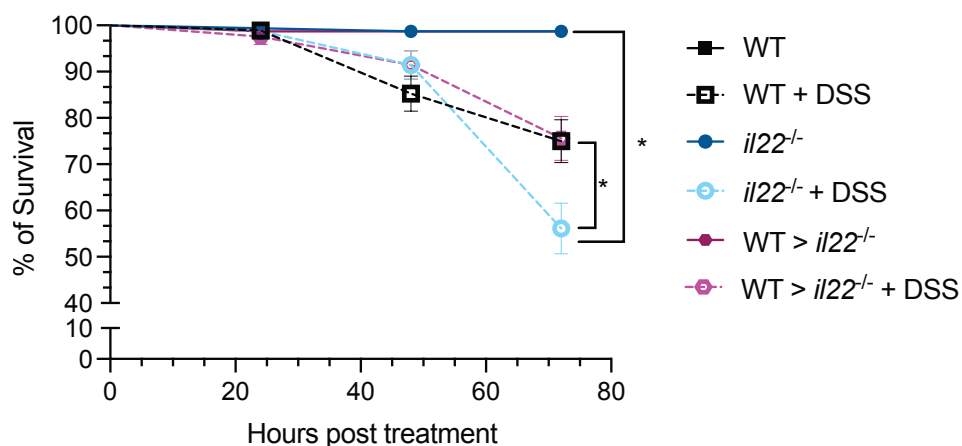


Figure 14. Co-housing with WT larvae rescues the DSS-induced inflammation susceptibility of *il22^{-/-}* larvae

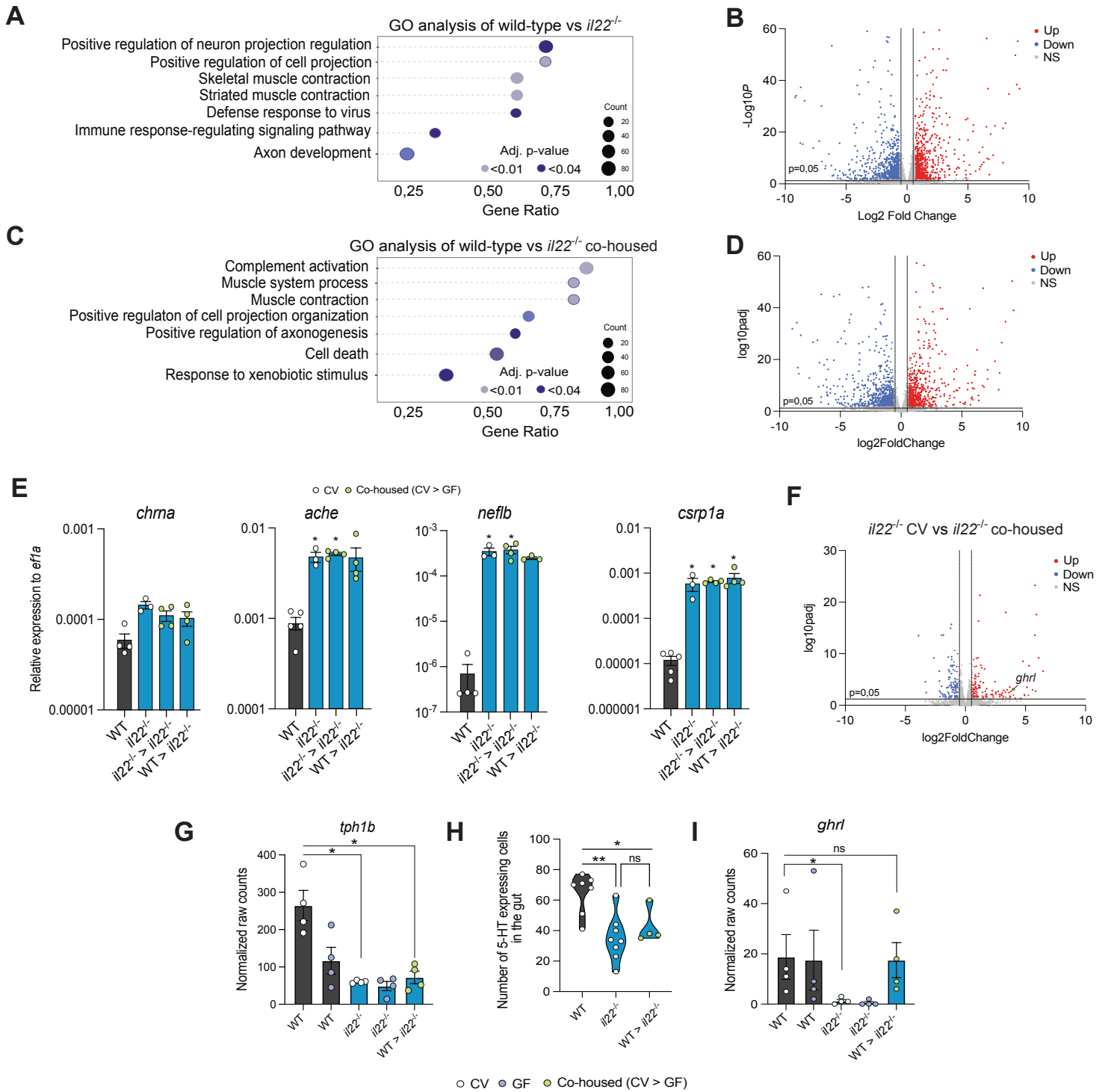
Survival curve of WT, *il22^{-/-}* or *il22^{-/-}* co-housed with WT with or without DSS treatment. This data is representative of three independent experiments. Statistical analyses were performed with multiple comparisons 2-way ANOVA * $P < 0,05$.

As expected, our experiments showed that *il22*^{-/-} zebrafish have an increased susceptibility to chemically induced inflammation. However, we found that *il22*^{-/-} zebrafish co-housed with WT larvae exhibited survival rates similar to those of WT zebrafish alone. This suggests that the transfer of WT microbiota alone is sufficient to protect the gut from DSS-induced inflammation. The exact mechanisms underlying this improvement are not yet fully understood and need further investigation. One possible hypothesis stems from the known involvement of 5-HT in gut immunity, in particular its role in modulating cytokine expression¹³³. If we consider 5-HT as the transferred molecule or a key factor synthesized during microbial transfer, it may play a role in this protective effect. In addition, we could hypothesize that the transferred WT bacteria might increase the expression of anti-microbial factors, compensating for the reduced peptides and mucin levels seen in the absence of IL-22. We could speculate that dysregulated anti-microbial peptide and mucin expression in *il22*^{-/-} larvae could alter the location of commensal bacteria, bringing them closer to the epithelium, a phenomenon that is normally prevented. This altered location may influence the course of DSS-mediated colitis in *il22*-deficient zebrafish. Thus, co-housing with WT zebrafish may provide them with a more beneficial microbiota, contributing to their improved condition.

As mentioned above, we observed that exposure to 5-HT can restore the intestinal motility phenotype of *il22*^{-/-} zebrafish. However, it is important to note that this does not imply that 5-HT is the only factor responsible for the observed defect. In fact, we found a dysregulation of other hormones expressed by EECs that are also involved in gut motility, such as PYY for example. To gain deeper insights into the changes that occur during co-housing of *il22*^{-/-} with WT zebrafish, we recently performed bulk RNA sequencing analyses on dissected guts from WT and *il22*^{-/-} 7 dpf larvae, both CV, GF, or co-housed. In our analysis, we found similar GO terms than our first RNA-seq when comparing WT and *il22*^{-/-} conditions, as expected (Figure 15A, B). These terms were associated with axon development, immunity and muscle contraction, reinforcing the consistency and robustness of our sequencing-based observations. Then, when we compared WT and *il22*^{-/-} zebrafish that had been co-housed, we detected a comparable number of differentially expressed genes, still with enrichment of GO terms related to immunity, muscle contraction and neuronal development (Figure 15C, D). These

results suggest that these factors are not significantly improved by co-housing. Consistently, our qPCR analysis confirmed that the expression levels of neuronal genes previously identified as dysregulated remain unchanged after co-housing (Figure 15E). Furthermore, when we compared the *il22^{-/-}* condition with *il22^{-/-}* co-housed zebrafish, we found less than 100 genes with different expression levels (Figure 15F). Notably, we did not observe the recovery of *tph1b* expression, which encodes an essential enzyme for serotonin synthesis (Figure 15G). This suggests that EECs do not re-express normal levels of this enzyme, which is consistent with our immunostaining results using a 5-HT antibody in co-housed larvae, which showed no restoration of protein expression in these cells (Figure 15H). Although these observations were somewhat disappointing, we did discover that the *ghrl* gene, which encodes ghrelin - a hormone that regulates satiety, food intake and intestinal motility - was among the genes showing differential expression (Figure 15I). In conclusion, it might be that ghrelin plays an important role in the regulation of intestinal motility in *il22^{-/-}* and might even be a more important factor than 5-HT. Further studies are needed to confirm this promising observation and in the coming weeks we will try to rescue the gut motility phenotype with ghrelin protein similar to 5-HT to determine the importance of this factor.

Figure 15. Ghrelin expression is rescued upon co-housing WT with *il22^{-/-}* (below)
A. Gene ontology analysis comparing WT and *il22^{-/-}*. GO terms are associated with immunity, neuronal development and muscle contraction. **B.** Volcano plot showing the downregulated genes (blue), upregulated genes (red) and unchanged genes (gray) of WT and *il22^{-/-}* CV. **C.** Gene ontology analysis comparing WT and *il22^{-/-}* co-housed with WT. GO terms are also associated with immunity, neuronal development and muscle contraction. **D.** Volcano plot showing the downregulated genes (blue), upregulated genes (red) and unchanged genes (gray) of WT compared to *il22^{-/-}* (GF) co-housed with WT (CV). **E.** RT-qPCR analysis of the neuronal dysregulated genes initially found by RNA-sequencing on WT CV, *il22^{-/-}* CV, *il22^{-/-}* co-housed with their GF counterparts and *il22^{-/-}* (GF) co-housed with WT (CV). **F.** Volcano plot showing the downregulated genes (blue), upregulated genes (red) and unchanged genes (gray) of *il22^{-/-}* CV with *il22^{-/-}* CV co-housed with *il22^{-/-}* GF. **G.** Normalized raw counts of the *tph1b* gene. **H.** Quantification of the number of 5-HT positive cells in the gut of WT CV, *il22^{-/-}* CV and *il22^{-/-}* (GF) co-housed with WT (CV). **I.** Normalized raw counts of *ghrl* and *pyyb* genes expression. Statistical analyses were performed using Mann-Whitney test: ns: not significant, * $P < 0,05$, ** $P < 0,01$.



Last but not least, we were able to confirm the conservation of the intestinal motility defect in 3-4 week old *Il22^{-/-}* mice, demonstrating the usefulness of the zebrafish model to study early physiological processes in the vertebrate intestine. However, we still need to determine whether the underlying mechanisms driving this defect are conserved at this stage of development in the mouse model. To address this crucial question, we have already established a collaboration with Fabian Guendel from the Gerard Eberl laboratory at the Institut Pasteur. We are currently collecting samples for qPCR analysis to assess whether there is also dysregulation of muscle and neuronal genes during this stage of development in mice. In addition, we plan to use gut samples to label 5-HT expression in the gastrointestinal tract of these young mice, to determine whether serotonin dysregulation is also a conserved feature. In the near future, we could perform 16S microbiota sequencing on 3-4 week old mice to investigate the presence of dysbiosis at this stage. This investigation may allow us to establish potential links between specific bacterial species and the observed food transit defect. Interestingly, we have observed that the food transit impairment does not take place beyond 3-4 weeks of age, coinciding with the weaning process in mice (introduction of solid food after milk feeding). This weaning event is associated with a significant shift in microbiota composition and with a strong immune response³³⁸. We could hypothesize that IL-22 may play a key role in regulating intestinal motility in mice prior to or during this change, providing a plausible explanation for the subsequent disappearance of the phenotype.

3. Microbiota and IL-22 expression

3.1 IL-22 induction by Trpa1b receptor activation ?

Although several tryptophan metabolites, including IAld, can act as AhR agonists²³⁵, the effects of indole on AhR activation are conflicting^{339–341}. However, it has been shown that indole activates the Trpa1 receptor in zebrafish¹³¹ which in turn activates gut motility. In our study, we have observed that the injection of indole leads to increased expression of *il22* by EECs, likely through Trpa1 activation. Unfortunately, we do not yet have access to the *trpa1b*^{-/-} nor *ahr*^{-/-} line to definitively establish if *il22* expression is dependent on the Trpa1 receptor and not Ahr. However, we plan to conduct this experiment as soon as possible.

As mentioned above, the *trpa1b* mutant has previously been shown to exhibit gut motility defects¹³¹. Previous research has indicated that it induces serotonin secretion, which subsequently activates cholinergic enteric nerves and, thus, increases gut motility¹³¹. This finding has led us to hypothesize that the induction of *il22* by the Trpa1b receptor may play a role in the observed gut motility impairment in the zebrafish receptor knock-out. From an evolutionary perspective, it is noteworthy that in mammals, dietary-derived tryptophan metabolites serve as ligands for the Ahr, contributing to IL-22 expression in ILC3²³⁵. Interestingly, despite changes in the cellular sources expressing this cytokine, IL-22 is still regulated by tryptophan metabolites. This regulation potentially still plays a role in maintaining host-microbial symbiosis and homeostasis at mucosal surfaces.

3.2 Who is expressing 5-HT ? What are the downstream effects ?

As mentioned above, 5-HT has the ability to restore normal intestinal motility in *il22*^{-/-} zebrafish. However, we do not know yet what are the target cells of this hormone in our model. Indeed, 5-HT can target both neurons and muscles, in addition to the vagal sensory nerve which communicate with the central nervous system. The precise mechanisms by which 5-HT restores gut motility in our model will require more investigations in the future.

In addition, gut motility is rescued in *il22*^{-/-} co-housed with WT larvae but we did not observe a recovery of 5-HT expression by EECs in co-housed larvae, nor did we observe an improvement in *tph1b* expression. Interestingly, previous research has

shown that in addition to modulating host serotonin biosynthesis³⁴², bacteria can also produce serotonin themselves³⁴³. The exact mechanisms by which bacteria synthesize this hormone are not yet fully understood, but two metabolic pathways have been proposed³⁴⁴: decarboxylation of tryptophan to tryptamine followed by hydroxylation (the plant-like pathway); and hydroxylation to 5-hydroxytryptophan followed by decarboxylation (the animal-like pathway). Interestingly, our analysis of 16S metagenomic datasets revealed an enrichment of bacterial species with the potential to synthesize serotonin, suggesting that *il22^{-/-}* zebrafish may harbor a higher abundance of such species. This finding was unexpected because the mutant has fewer EECs expressing serotonin, and a gut motility defect which we thought would be due to a decreased amount of this hormone. However, we could hypothesize that due to the reduced 5-HT expression in the intestine of *il22^{-/-}* zebrafish, bacteria sense this dysregulation and adjust their growth and metabolism in an attempt to compensate. Another hypothesis is that even if bacteria in the mutant express more 5-HT, which could maintain proper gut motility, we do not yet know if the amount of available tryptophan is the same in WT and *il22^{-/-}* larvae. This could also be a limiting factor explaining why gut motility is still impaired despite the presence of more bacteria potentially able to synthesize 5-HT. Tryptophan metabolites could be measured by HPLC-HRMS (high-performance liquid chromatography-high-resolution mass spectrometry), but this would require intense optimization to adapt the existing protocols to zebrafish larvae samples and is therefore unlikely to be performed in this study.

To gain a better understanding of 5-HT expression by bacteria in zebrafish larvae, we recently started a collaboration with Alvaro Banderas, a microbiologist at the Institut Curie. Recently, we cultured bacteria from the zebrafish gut, isolated them based on their phenotype and then analyzed them using ELISA to measure 5-HT expression in the supernatant. This research is ongoing, and if we are successful in detecting bacterial species that express 5-HT, our goal is to sequence the genome of the bacteria and then do mutagenesis in order to identify the genes responsible for serotonin biosynthesis in future studies.

Finally, it is interesting to note that 95% of 5-HT production occurs in the gut, yet this hormone is known to regulate behavioral and neuropsychological processes such as mood, perception, reward, anger, aggression, appetite, memory, sexuality and

attention, in addition to gut motility. In addition to EECs and bacteria, very few neurons are also able to synthesize this hormone and most neurons express its receptors. 5-HT is a perfect example of the importance of the gut-brain axis, as it can also communicate with the vagal nerve, which will communicate directly with the central nervous system. Since there is a defect in the EECs expressing 5-HT in *il22^{-/-}*, we might wonder whether there would be side effects on brain function such as depression which can be analyzed in zebrafish by behavioral testing including approach-avoidance tests for example. Interestingly, IL-22 has previously been linked to anxiety, with studies demonstrating its ability to reduce anxiety in mice under stressful conditions³⁴⁵. In addition, elevated levels of IL-22 have been found in the serum of women with severe anxiety and depression³⁴⁶. The precise role of this cytokine in modulating behavioral disorders is still not fully understood, making it an interesting subject for further investigation. As the zebrafish model is already used in translational research in neuroscience, it could provide valuable insights to explore this intriguing link between IL-22/5-HT and anxiety/depression.

4. Us to IL-22 : “How can you play so many roles ?!”

4.1 IL-22 and body size

As previously mentioned in the introduction, IL-22 appears to play a significant role in the regulation of lipid transporter expression. Notably, Mao et al. have demonstrated that an overexpression of IL-22 can result in a significant reduction in the expression of lipid transporters such as *Cd36*, *Npc1l1*, *Fabp1*, and *Fabp2* in the gut, accompanied by a decrease in serum lipid levels²⁶⁴. Interestingly, other studies investigating IL-22 involvement in lipid metabolism have suggested that it may, in contrast, promote lipid transporter expression in IECs within the small intestine²⁶⁵. Despite these seemingly opposing observations, both sets of studies have recognized this cytokine role in lipid metabolism. One plausible explanation for these differences could be attributed to variations in experimental methodologies, as sustained high-level expression of IL-22 may result in different outcomes compared to low-level expression. Additionally, IL-22 has also been associated with glucose metabolism, specifically in insulin resistance and glucose intolerance²⁶³, although the precise underlying mechanisms remain unclear. Moreover, little is known about the influence of this cytokine on the regulation of other types of transporters. Furthermore, the impact of IL-22 on body growth during development remains unexplored, even though it is possible that this cytokine could play a role in this aspect.

Intriguingly, our RNA-seq data analysis comparing WT and *il22^{-/-}* at 7 dpf revealed an upregulation of several transporters including *fabp1b.1*, which is the ortholog of the mice *Fabp1* gene, and other lipid transporters like *fabp6* (Figure 16A). This finding suggests the conservation of IL-22 role in regulating the expression of lipid transporters in zebrafish. These results support the observation made by Mao et al, who reported a suppressive effect of IL-22 on lipid transporters expression. Moreover, besides lipid transporters, we also observed the overexpression of other type of transporters, specifically those involved in amino acids or sugars transport. For instance, the *slc2a8* gene, known for its involvement in glucose transport, exhibited increased expression levels. Collectively, these findings suggest that beyond its established role in lipid metabolism, IL-22 might also play a role in other metabolic processes, potentially involving amino acids or sugar.

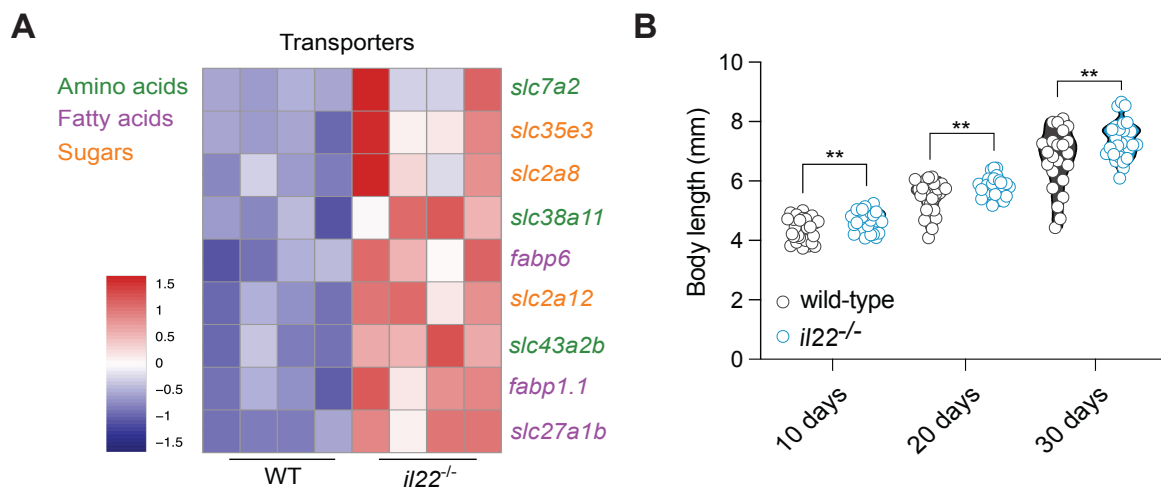


Figure 16. Dysregulated expression of transporters and increased body length in $il22^{-/-}$

A. Heatmap of RNA-sequencing data depicting expression levels of selected genes encoding for transporters in WT and $il22^{-/-}$ 7dpf larvae dissected guts. Four independent samples for each condition were used for RNA-sequencing experiment.

B. Quantification of WT and $il22^{-/-}$ zebrafish body length at different developmental stages : 10, 20 and 30 days. This data is representative of two independent experiments. Statistical analyses were performed using two-tailed unpaired t-tests ** $P < 0,01$.

Given the importance of these components for body growth, we raised the possibility of a developmental growth abnormality in $il22^{-/-}$. As a reminder, our previous results showed no significant difference in body or gut length between WT and $il22^{-/-}$ larvae at 5 dpf (as shown in supplementary Figure 2). It is worth noting that the zebrafish relies on the nutritional content of the yolk, as the lipids maternally deposited within it serves as the primary energy source during the initial developmental stages, up until 5 dpf when the larvae start feeding³⁴⁷. Hence, we hypothesized that the absence of differences observed at 5 dpf may be due to this factor. Consequently, we decided to analyze further and follow the growth of larvae at different developmental stages after feeding with dry food. Interestingly, we observed an increased body size at 10 dpf, 20 dpf and 30 dpf in the mutant (Figure 16B). This observation was intriguing and is raising several questions : Are the transporters still dysregulated at the observed developmental stages ? Is this difference due to this increased transporters expression in the mutant ? Does this anomaly persist into the adult stage in zebrafish ? Unfortunately, we did not perform yet the required experiments to address these

questions. Further research is needed to confirm these findings and to determine the precise mechanisms by which IL-22 regulates metabolism and growth. However, this area of research is very promising and could potentially provide new insights into (i) the broader impact of IL-22 on development beyond its gut-related functions and (ii) expanding our understanding of the role of IL-22 in metabolism more generally. These findings may further provide evidence that IL-22 could be a promising target for the treatment of metabolic disorders.

4.2 A novel role of IL-22 in lymphatic vessels development ?

While IL-22 or the activation of its receptor has previously been linked to angiogenesis in cancer³⁴⁸, as far as I know, there have been no reported findings regarding its involvement in lymphangiogenesis. The lymphatic system plays crucial roles in immune responses, fluid balance, fat absorption, and is implicated in various disorders such as tumor spread and lymphedema^{349,350}. In addition, the lymphatic system is closely linked to the immune system. The lymphatic vessels transport immune cells throughout the body, including lymphocytes, macrophages, and dendritic cells. Similar to blood vessels, lymphatic vessels create an intricate yet highly predictable and evolutionarily conserved network. While the lymphatic system is distinct from the circulatory system, larger lymphatic vessels (LV) and blood vessels (particularly arteries) often align together^{351,352} (Figure 17A).

In mice, key molecules that regulate lymphatic specification and differentiation include *Sox18*, *Prox1*, *VegfC*, *Vegfr3*, and *Nrp2*^{349,350}. The zebrafish offers a well-defined lymphatic vascular system, sharing morphological, molecular, and functional traits with LV in other vertebrates³⁵³. Briefly, in zebrafish the cardinal vein (blue) serves as a cell source for those having a lymphatic fate (green). This process gives rise to parachordal cells (PC), fundamental elements of the fish lymphatic system, then PC will migrate along the arteries and form lymphatic vessels (Figure 17A, B). Despite extensive studies that have identified crucial molecular regulators of lymphatic development and function, our understanding is still limited.

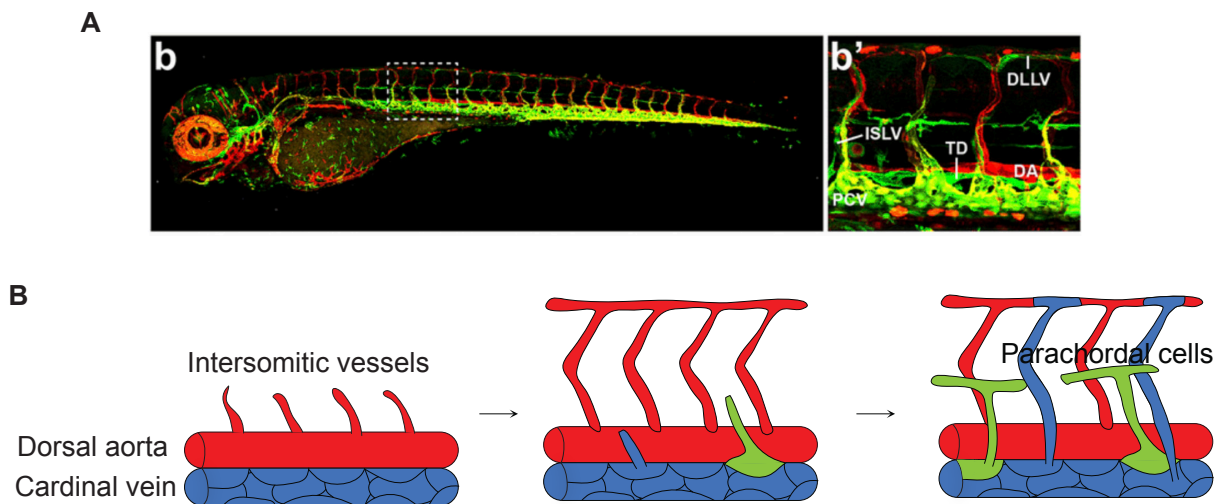


Figure 17. The zebrafish lymphatic system development

A. b Confocal imaging of a 5 dpf *Tg(mrc1a:eGFP)*, *Tg(kdr1:mcherry)* zebrafish line labelling blood vessels in red, veins in yellow and lymphatic vessels in green. **b'** A close-up of the trunk area (b) reveals the major blood vessels (dorsal aorta (DA)) and lymphatic vessels (dorsal longitudinal lymphatic vessel (DLLV), intersegmental lymphatic vessel (ISLV), and thoracic duct (TD)) in the region. *Image from Greenspan et al, 2021* **B.** Scheme representing lymphatic vessel development. First, there is formation of arterial sprouting and intersegmental vessels (in red), followed by lympho-venous sprouting (here label in green). Then, the arterio-venous patterning start and parachordal cells start accumulating at the horizontal myoseptum (right panel). Adapted from Jonathan Semo et al, 2016.

Unexpectedly, during the GO analysis of our RNA-seq data comparing WT and *il22^{-/-}*, a significant and surprising enrichment term emerged: "lymph vessel development" (Figure 18A). Interestingly, upon closer examination of the dysregulated genes, we observed a significant downregulation of *lyve-1a* (Lymphatic Vessel Endothelial Receptor 1) (Figure 18B). This gene is recognized as a marker for lymphoid tissues and lymphangiogenesis. It encodes a transmembrane receptor that interacts with hyaluronan, a common glycosaminoglycan polymer. This interaction has been suggested to facilitate the entry of dendritic cells into lymphatic vessels³⁵⁴. Another gene of note is *stab1* (Figure 18B), which encodes Stabilin-1. This molecule is involved in controlling the movement of lymphocytes within lymphatics and assisting the entry of leukocytes to inflamed sites³⁵⁵. Importantly, our analysis revealed changes in the expression of the *flt4* gene (Figure 18B), responsible for encoding vascular endothelial growth factor receptor 3 (VEGFR3). As mentioned above, *Vegfr3* is involved in lymphatic specification and differentiation and mutations in this gene have been

associated with Milroy disease, a condition linked to lymphatic defects³⁵⁶. Interestingly, *flt4* zebrafish mutant lack lymphatic vessels and the defect in juvenile zebrafish has been shown to result in the replacement of blood vessels in the vascularization of the caudal fin³⁵⁷. We don't yet know whether the dysregulation of *flt4* is conserved in *il22*^{-/-} juvenile/adult fish, but these observations suggest a possible strong developmental defect and/or functional impairment of lymphatic vessels in *il22*^{-/-} with a possible replacement of their function by blood vessels in certain regions.

The genes cited above and more were all down-regulated, while, *cxc12b* was strongly upregulated in the mutant (Figure 18B). CXCL12 and its receptor CXCR4 have well-described functions in promoting angiogenesis and establishing the pattern of embryonic blood vessels³⁵⁸. Interestingly, Cha et al. discovered that the expression of *cxc12b* within the dorsal aorta and arterial intersomitic vessels governs the coordinated movement of developing lymphatic vessels along these specific paths³⁵⁹. The signaling between *Cxcl12b* and *Cxcr4* plays a crucial role in guiding the dorsal and ventral migration of lymphatic precursors that sprout from the posterior cardinal vein, facilitating the formation of the intersomitic lymphatic vessels in conjunction with anterior intersomitic vessels³⁵⁹.

In zebrafish, several double transgenic lines have been developed to differentiate between blood vessels from lymphatic vessels such as *Tg(fli1a:egfp)y1*, *Tg(kdr1:mcherry)*³⁵⁹. Although these genetic markers don't exclusively identify lymphatic vessels, they help distinguish between blood and lymphatic vessels. More recently, researchers have made models that specifically highlight lymphatic vessels in zebrafish, like *Tg(lyve1:egfp)nz15*³⁶⁰, or *Tg(flt4BAC:mCitrine)hu7135*³⁶¹.

These new tools are very useful to study lymphatic structures in zebrafish, however, as we did not have the lines we utilized another approach: Hybridization chain reaction (HCR) which enables the visualization of multiple RNAs in tissues with high signal-to-background ratio and precise subcellular localization in zebrafish. We first wanted to determine if the increased *cxc12b* expression identified through RNA-seq could be detected using HCR and if this might cause alterations in angiogenesis pattern. Interestingly, when we used a probe designed to specifically target this mRNA, we were not able to detect it in the gut (Figure 18C). However, we noted distinct structural changes in the trunk in the mutant, characterized by an increased sprouting of arteries in *il22*^{-/-} and *crfb14*^{-/-} (Figure 18C, D). Given that lymphatic vessels usually mirror the

arrangement of arteries, these findings support the idea that the lymphatic vessel structure might be impaired in larvae lacking *il22*.

Further research is needed to confirm these findings and to determine the precise mechanisms by which IL-22 may regulate lymphatic development and function. However, this research has the potential to provide new insights into the development of lymphatic disorders and to identify new targets for therapeutic intervention. We are currently optimizing the HCR method for probes that label lymphatic vessels. Antibodies have also not been very effective. Therefore, we will import the transgenic lines described above and further investigate whether IL-22 affects lymphatic or blood vessels.

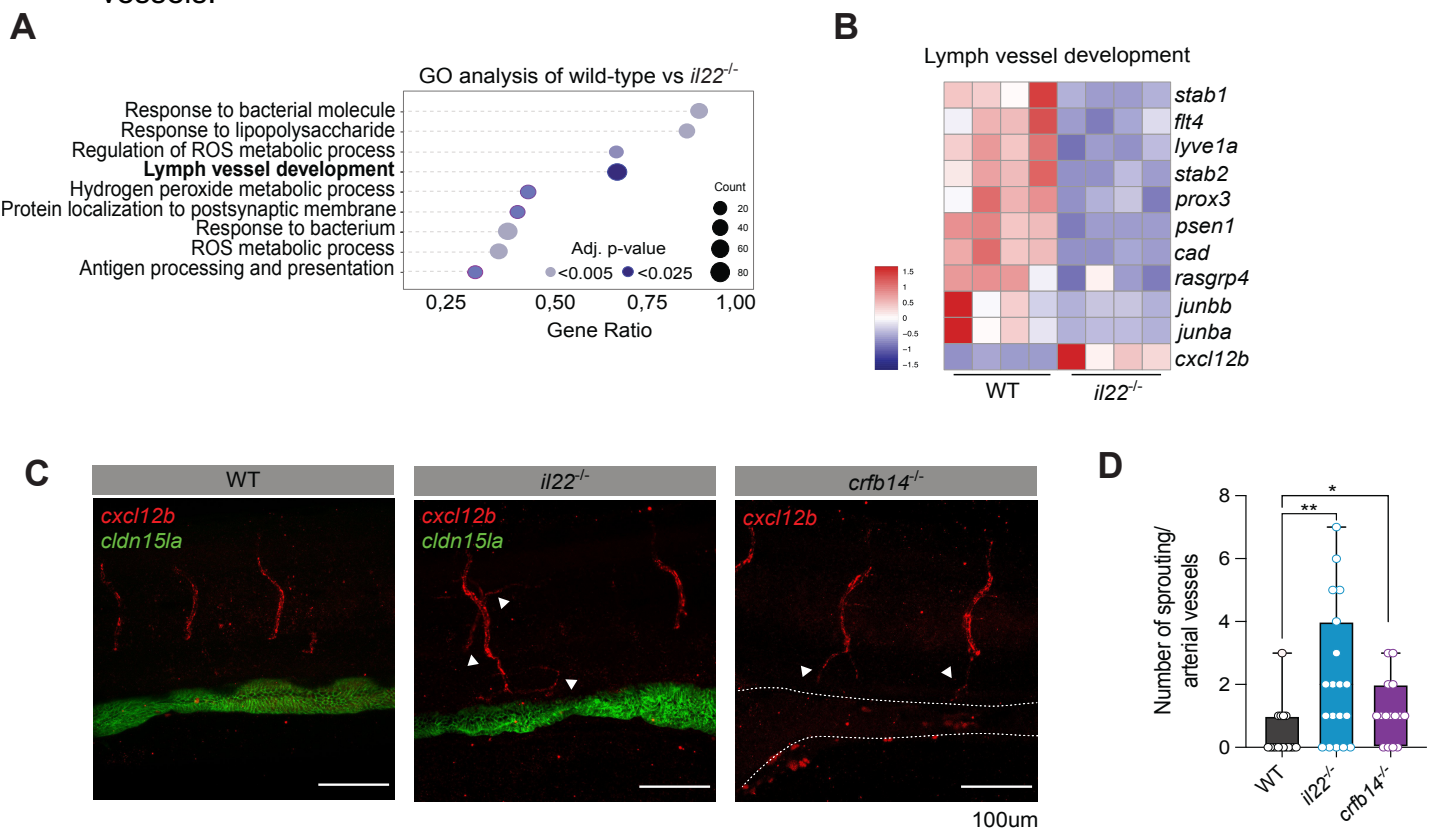


Figure 18. Dysregulation of lymphatic vessel-associated genes in *il22*^{-/-}

A. GO analysis of down-regulated genes from the RNA-sequencing data in WT and *il22*^{-/-} 7dpf larvae dissected guts. **B.** Heatmap of RNA-sequencing data showing expression levels of genes associated with GO: lymph vessel development in WT and *il22*^{-/-} 7dpf larvae dissected guts. Four independent samples for each condition were used for RNA-sequencing experiment. **C.** Images of HCR staining with anti-*cxcl12b* probe in *cldn15la*:GFP WT, *il22*^{-/-} and *crfb14*^{-/-} (the latter is non-transgenic) 7 dpf larvae. Scale bar is 100µm. **D.** Quantification of the sprouting numbers of arterial vessels stained with the *cxcl12b* probe on WT, *il22*^{-/-} and *crfb14*^{-/-} 7 dpf larvae. One dot corresponds to one artery in the aforementioned genetic background. These data are representative of two independent experiments. Statistical analyses were performed using a two-tailed Mann-Whitney test * $P < 0,05$ ** $P < 0,01$.

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ANNEXES

Scientific outreach

During my PhD, I had the opportunity to participate in the "En quête de Bio" scientific outreach program, led by Alison Bardin, a group leader at the Institut Curie, Paris. The program's purpose is to inspire passion for research among elementary school children, introducing them to model organisms, development processes, and scientific research.

In this project, I developed an eight-family game featuring various model organisms commonly used in research laboratories, such as mice, xenopus, zebrafish, and drosophila. As Emma Torun and I developed and created this game, it is licensed under Creative Commons.

The game has undergone multiple testing iterations during open door events at the Institut Curie as well as in classrooms. I am currently in the process of developing an English version of the game to expand its outreach internationally.

If interested, please contact me or Alison Bardin to learn more about our outreach proposal and to receive the card games or other materials that we have prepared. It would be my pleasure to support any of you in motivating young generations about scientific research. This card game generated with Emma Torun will be presented in the following pages.

1 Plante

Embryon: Jour 0

© Emma Tourn et Soyaigé Rabah

2 Plante

Embryon: Jour 3

© Emma Tourn et Soyaigé Rabah

3 Plante

Embryon: Jour 6

© Emma Tourn et Soyaigé Rabah

4 Plante

Germination: Jour 10

© Emma Tourn et Soyaigé Rabah

5 Plante

Rosette: 3 semaines

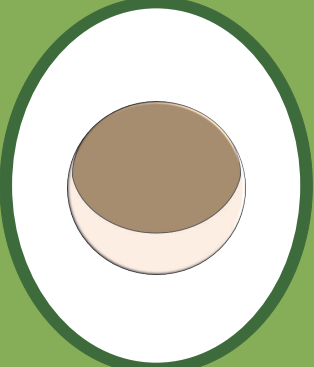
© Emma Tourn et Soyaigé Rabah

6 Plante

Adulte : 5 semaines

© Emma Tourn et Soyaigé Rabah

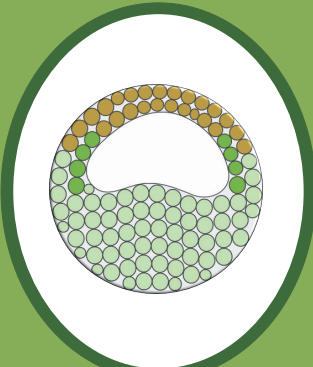
1 Grenouille



Embryon: Jour 0

© Emma Tourou et Soraya Rabah

2 Grenouille



Embryon: Heure 3

© Emma Tourou et Soraya Rabah

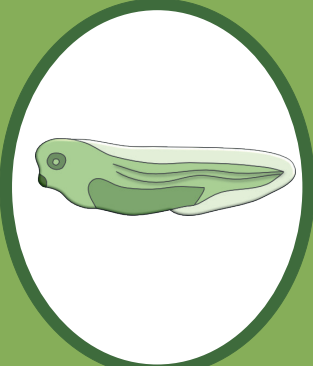
3 Grenouille



Embryon: Heure 30

© Emma Tourou et Soraya Rabah

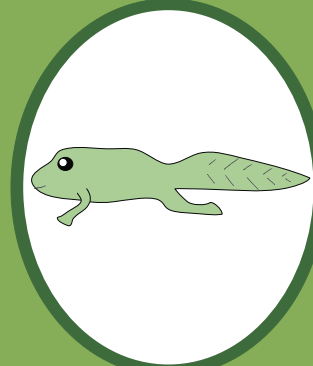
4 Grenouille



Naissance: Jour 3

© Emma Tourou et Soraya Rabah

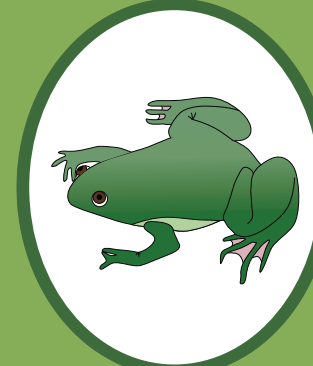
5 Grenouille



Larve : 1 mois

© Emma Tourou et Soraya Rabah

6 Grenouille



Adulte : 2 ans

© Emma Tourou et Soraya Rabah

1 Poisson zèbre

Embryon: Jour 0

2 Poisson zèbre

Embryon: Heure 4

3 Poisson zèbre

Embryon: Heure 14

4 Poisson zèbre

Naissance: Jour 3

5 Poisson zèbre

Larve : 5 jours

6 Poisson zèbre

Adulte : 3 mois

1 **Vers**

Embryon: Jour 0

2 **Vers**

Embryon: Heure 4

3 **Vers**

Embryon: Heure 8

4 **Vers**

Larve: 24 heures

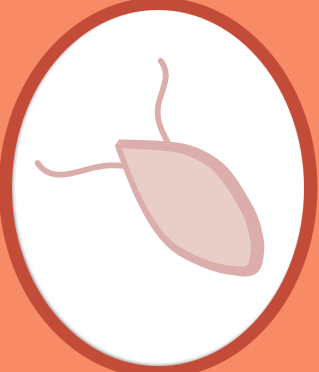
5 **Vers**

Jeune: 42 heures


6 **Vers**

Adulte: 59 heures

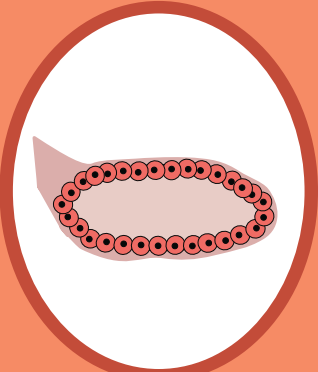
1 Mouche




Embryon: Jour 0



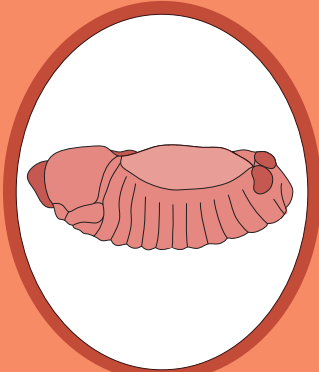
2 Mouche




Embryon: Heure 3



3 Mouche



Embryon: Heure 10




4 Mouche




Larve: Jour 3




5 Mouche




Pupe: Jour 5



6 Mouche



Adulte: 10 jours



1 Humain

Embryon: Jour 0

© Emma Toron et Sirogi Kabali

2 Humain

Embryon: Jour 5

© Emma Toron et Sirogi Kabali

3 Humain

Embryon: Semaine 10

© Emma Toron et Sirogi Kabali

4 Humain

Naissance: Mois 9

© Emma Toron et Sirogi Kabali

5 Humain

Enfant : 8 ans

© Emma Toron et Sirogi Kabali

6 Humain

Adulte : 18 ans

© Emma Toron et Sirogi Kabali

1 Souris

Embryon: Jour 0

2 Souris

Embryon: Jour 3,5

3 Souris

Embryon: Jour 10,5

4 Souris

Naissance: Jour 20

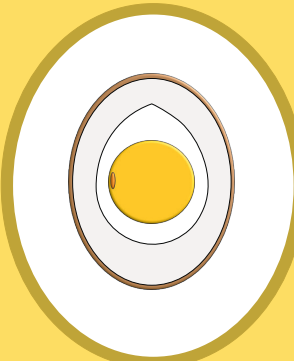
5 Souris

Jeune : 2 semaines


6 Souris

Adulte: 2 mois

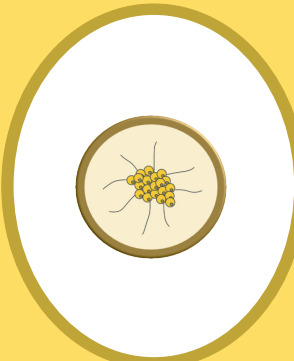
1 Poulet




Embryon: Jour 0



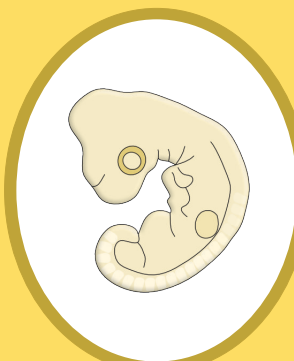
2 Poulet




Embryon: 20 heures



3 Poulet



Embryon: Heure 35



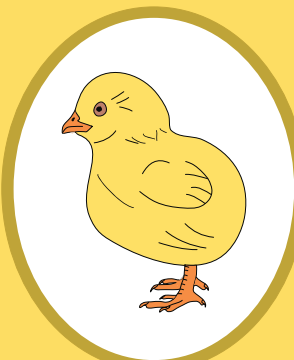
4 Poulet




Naissance: Jour 21



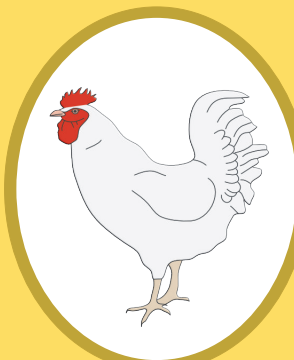
5 Poulet




Jeune : 10 semaines



6 Poulet



Adulte : 7 mois



RÉSUMÉ

Les cytokines favorisent la défense et l'homéostasie de l'intestin. Parmi les cytokines clés de l'intestin, l'interleukine-22 (IL-22) est produite par les cellules immunitaires et cible principalement les cellules épithéliales. L'IL-22 protège l'intestin contre les agents pathogènes en induisant l'expression de peptides antimicrobiens et en favorisant la réparation des tissus. Une dysrégulation de cette cytokine peut conduire à des maladies inflammatoires de l'intestin et au cancer. Au cours du développement, le tube digestif post-embryonnaire est colonisé par des micro-organismes commensaux qui favorisent la maturation de l'intestin et de son système immunitaire. Cependant, il est encore incertain si et comment l'IL-22 joue un rôle dans le développement et la maturation de l'intestin au cours de cette période critique. Le poisson-zèbre nous permet de visualiser et de manipuler les processus physiologiques *in vivo* dès le début du développement en raison de son développement externe et de sa transparence.

Au cours de ma thèse de doctorat, j'ai cherché à décrypter la fonction de l'IL-22 dans le développement de l'intestin. Mes données montrent que l'*il22* est exprimée dans les cellules épithéliales intestinales des larves avant que les lymphocytes exprimant l'IL-22 n'apparaissent dans l'intestin. J'ai identifié les cellules entéroendocrines (EEC), un sous-type de cellules épithéliales intestinales, comme la principale source d'*il22* chez les larves. De plus, j'ai révélé la conservation de la voie de signalisation de l'IL-22, sa régulation transcriptionnelle par la détection des microbes et sa fonction antibactérienne dans l'intestin. Mes données les plus récentes suggèrent que l'expression d'*il22* peut également être induite par l'activation de *Trpa1*, un récepteur connu pour reconnaître les métabolites du tryptophane chez les poissons-zèbre et la souris. Enfin, j'ai découvert un nouveau rôle de l'IL-22 dans la modulation de la motilité intestinale chez les poissons-zèbres. Mécaniquement, une dysbiose a été observée chez les *il22* mutants, ainsi que des défauts potentiels dans la production de métabolites dérivés des bactéries dans l'intestin. De plus, un dysfonctionnement a été constaté dans la fonction des EEC, caractérisé par une expression hormonale dysrégulée. Il a été intéressant de constater que le mutant exprime des niveaux plus bas de 5-HT, une hormone importante régulant la motilité intestinale chez les mammifères et les poissons-zèbres. Nous avons démontré que l'administration de celle-ci est capable de rétablir la motilité intestinale, ce qui signifie que 5-HT est suffisante pour restaurer une motilité intestinale correcte. Enfin, j'ai découvert la conservation du défaut de motilité intestinale chez les souris en bas âge, ce qui suggère la conservation de cette nouvelle fonction de l'IL-22 chez les mammifères tôt au cours du développement. Dans l'ensemble, ce projet contribue à une meilleure compréhension de la manière dont les cytokines orchestrent le développement et la maturation de l'intestin au cours des premières étapes de la vie.

MOTS CLÉS

Cytokine, cellules épithéliales, intestin, poisson-zèbre, développement

ABSTRACT

Cytokines promote gut defense and homeostasis. Among key gut cytokines, interleukin-22 (IL-22) is produced by immune cells and primarily targets epithelial cells. IL-22 protects the gut from pathogens by inducing anti-microbial peptides expression and promoting tissue repair. Dysregulation of this cytokine can lead to inflammatory bowel disease and cancer. During development, the post-embryonic gut is colonized by commensal microorganisms that promote maturation of the gut and its immune system. Nevertheless, whether and how cytokines such as IL-22 play a role in gut organ development and maturation during this critical time window remains unclear. The zebrafish allows us to visualize and manipulate physiological processes *in vivo* since early development due to its external development and transparency.

During my PhD, I wanted to decipher the function of *il22* in the developing gut. My data show that *il22* is expressed in larval gut epithelial cells before *il22*-expressing lymphocytes appear in the gut. I identified enteroendocrine cells (EECs), a gut epithelial cell subtype, as the main source of *il22* in larvae. Furthermore, I revealed conservation of the IL-22 signaling pathway, its transcriptional regulation by microbe sensing, and its antibacterial function in the gut. My latest data suggest that *il22* expression can also be induced by the activation of *Trpa1*, a receptor known to recognize tryptophan metabolites in zebrafish and mice. Finally, I found a novel role of IL-22 in modulating gut motility in zebrafish. Mechanistically, dysbiosis was observed in *il22*^{-/-}, along with potential defects in the production of bacteria-derived metabolites in the gut. Surprisingly, we found that the microbiota from wild-type larvae was able to restore the gut motility impairment of *il22*^{-/-}, highlighting the important role of the microbiota in *il22*-mediated regulation of this process. Furthermore, an impairment was found in EECs function, which are known to be sensitive and respond to microbial cues. The dysregulation of EECs was characterized by abnormal hormone expression, specifically reduced levels of serotonin (5-HT), a critical hormone regulating gut motility in both zebrafish and mammals. External administration of 5-HT successfully rescued the gut motility phenotype of *il22*^{-/-}, indicating that 5-HT is sufficient to restore proper gut motility. Finally, I found conservation of the gut motility defect in early life mice, suggesting the conservation of this novel IL-22 function in mammals. Altogether, this project contributes to a better understanding of how cytokines orchestrate gut development and maturation during the early stages of vertebrate life.

KEYWORDS

Cytokine, epithelial cells, intestine, zebrafish, development