Raquel Maia de Carvalhal Soares

Bachelor's Degree in Biology

Neuronal-Intraepithelial Lymphocyte Interactions at the Intestinal Mucosa

Dissertation submitted in partial fulfillment
of the requirements for the degree of

Master of Science in
Molecular Genetics and Biomedicine

Supervisor: Roksana Pirzgalska, PhD,
Postdoctoral Researcher, Champalimaud Research,
Champalimaud Centre for the Unknown

Co-supervisor: Henrique Veiga-Fernandes, DVM, PhD,
Principal Investigator, Champalimaud Research,
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Abstract

Intestinal Intraepithelial Lymphocytes (IELs) are located at the critical interface between the intestinal lumen and the core of the body. Besides constituting the first line of immune defence at the intestinal mucosa, IELs are also essential regulators of this organ homeostasis.

Growing amount of evidence suggest that the immune cell function can be modulated by signals from the intestinal innervation. Importantly, this neuroimmune communication has been shown to be fundamental for the protection of the intestinal mucosa against pathogens and for maintaining tissue homeostasis.

Our preliminary analysis indicated that IELs possess the machinery to integrate neuron-derived signals, therefore we hypothesized that neuronal cues can modulate the function of IELs. We further showed that specific neuronal signals alter the gene expression profile of IELs. Moreover, we also demonstrated the impact of extrinsic factors, such as diet, in shaping these regulatory responses.

These findings establish a possible new layer of local and systemic homeostatic regulation that can be crucial for health.

Keywords: Intraepithelial Lymphocytes; Neuroimmune Interactions; Metabolism; Enteric Inflammatory Diseases; Metabolic Diseases.

Resumo

Os Linfócitos Intra-epiteliais (LIEs) estão localizados na interface entre o lúmen do intestino e o interior do organismo. Para além de constituírem a primeira linha de defesa imunitária contra potenciais patogéneos na mucosa intestinal, os LIEs são também essenciais para estabelecer a homeostasia deste órgão.

Inúmeros projetos de investigação desenvolvidos recentemente têm demonstrado que a função de células do sistema imunitário pode ser modulada por estímulos neuronais com origem intestinal. Além disso, esta comunicação neuro-imune tem-se vindo a revelar fundamental para a defesa da mucosa intestinal contra patógeneos e para a manutenção da homeostasia nos tecidos.

As nossas análises preliminares indicam que os LIEs possuem mecanismos para integrar sinais de fonte neuronal. Por conseguinte, hipotetizámos que sinais provenientes de neurónios podem modular as funções dos LIEs. Conseguimos ainda mostrar que sinais neuronais afetam a expressão génica dos LIEs. Ademais, também demonstrámos o impacto de fatores extrínsecos, como a dieta, em modular estas respostas regulatórias.

Estas conclusões permitem estabelecer um novo nível de regulação homeostática local e sistémica, que pode ser crucial para a saúde.

Palavras-chave: Linfócitos Intra-epiteliais; Interações Neuro-imunes; Metabolismo; Doenças Inflamatórias Entéricas; Doenças Metabólicas.

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Acronyms

6-OHDA 6-hydroxydopamine

Ach Acetylcholine

ADRA1 α1-adrenoceptors

ADRA2 α2-adrenoceptors

ADRB β-adrenoceptors

AHR Aryl Hydrocarbon Receptor

AMP Anti-Microbial Peptides

AMPAR α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid Receptor

APC Antigen-Presenting Cell

BCR B-cell Receptor

BW Body Weight

CBG Corticosteroid-Binding Globulin

CD Cluster of Differentiation

cDNA Complementary DNA

CNS Central Nervous System

DN Double Negative

DP Double Positive

DPlowPD1hi Double-positive-low PD-1-high

ENS Enteric Nervous System

EPSP Excitatory Postsynaptic Potential

FACS Fluorescent Activated Cell Sorting

GABA Gamma Aminobutyric Acid

GAD Glutamic Acid Decarboxylase

GALT Gut-Associated Lymphoid Tissues

GLP-1 Glucagon-like Peptide 1

GTT Glucose Tolerance Test

HFD High Fat Diet

HSC Hematopoietic Stem Cell

IBD Inflammatory Bowel Diseases

IEC Intestinal Epithelial Cells

IEL Intraepithelial Lymphocyte

IFAN Intrinsic Intestinofugal Afferent Neurons

IL-17 Interleukin-17

IL-1 Interleukin-1

ILCs Innate Lymphoid Cells

ILF Isolated Lymphoid Follicles

iNKT invariant Natural Killer T cells

IPAN Intrinsic Primary Afferent Neurons

IPSP Inhibitory Postsynaptic Potential

ITLs Intraepithelial T Lymphocytes

KGF Keratinocyte Growth Factor

KR Kainate Receptor

mAChrRs Metabotropic Muscarinic Receptors

MHC Major Histocompatibility Complex

MLN Mesenteric Lymph Nodes

nAChRs Ionotropic Nicotinic Receptors

ND Normal Diet

NE Norepinephrine

NK Natural Killer

NMDAR N-methyl-D-aspartate Receptor

NMU Neuromedin U

PACAP Pituitary Adenylate-Cyclase-Activating Polypeptide

PAMPs Pathogen Associated Molecular Patterns

PCR Polymerase Chain Reaction

PNS Peripheral Nervous System

PRRs Pattern Recognition Receptors

qPCR Quantitative PCR

RT Room Temperature

RT-PCR Reverse Transcriptase Polymerase Chain Reaction

TCR T-cell Receptor

TLR Toll-like Receptors

TN Triple Negative

TNF Tumour Necrosis Factor

TP Triple Positive

UC Ulcerative Colitis

VIP Vasoactive Intestinal Peptide

Chapter 1

Introduction

1.1 An Overview of the Immune System

The human body is continuously exposed to different environmental changes and challenges: from changes in diet and temperature fluctuations to infections and microbial exposure. The need to prepare and defend the organism from possible pathological outcomes that might arise through these challenges is imperative. Due to the multitude and diversity of potential pathogenic invaders, vertebrates have evolved many immune cell types, molecules and mediators that interact with other cells and each other in a complex and dynamic network that comprise the 'immune system'. Thus, among the complex systems intrinsic to the organism, the immune system stands out as a highly adaptable interface that evolved to provide host protection from pathogens, therefore playing a crucial role in host defence [1]. However, it is important to highlight that this protection from disease caused by invading pathogens is only one of the main roles where the immune system is acting. Indeed, previous works showing the involvement of immune cells in the repair and regulation of major organs such as skin [2, 3] , lung [4] , intestine [5–7] and even adipose tissue [8], are just a few examples of how the immune system and its elements are also playing key roles in developing, regulating, maintaining and, if necessary, re-establishing tissue homeostasis in both sterile and infectious conditions [9].

When it comes to host defence, immune responses involve cellular and molecular mechanisms that protect the host in a non- or antigen-specific manner. It is therefore important to appreciate that there are two interconnected and collaborative systems of immunity based on timing of activation and specificity of the immune response: the innate and adaptive immunity [1].

1.1.1 The Innate Immunity

The innate immunity is the first and immediate line of body's immune defence and comprises external physical and chemical barriers – like skin, mucosal membranes and external mucous secretions -, humoral effector mechanisms – where the complement system, acute phase proteins and antimicrobial peptides play major roles - and cell-mediated effector mechanisms – where typical innate immune cells operate [10]. To provide this immediate defence, as it was reviewed by Medzhitov and Janeway Jr. in 2002 [11], the innate immune system utilizes three strategies based on the distinguishment of what is intrinsic (self) or extrinsic (non-self) to the body. The first strategy is based on the 'microbial nonself' recognition – where conserved structures exclusively of microbial origin termed Pathogen Associated Molecular Patterns (PAMPs) are perceived as molecular signatures of infection by specific receptors of the innate immune system termed Pattern Recognition Receptors (PRRs), thus triggering an immune response [12, 13]. The second strategy is based on the 'missing self' recognition – where constitutively expressed specialized markers exclusive to normal healthy cells of the host are recognized by specific host inhibitory receptors, thus blocking the beginning of immune responses against self [14–16]. The last strategy is characterized by the recognition of 'induced or altered self' – where markers of abnormal host cells that are induced upon cellular infection or transformation are recognized, thus being eliminated by the immune system to prevent infection spreadness and cancer development [17]. Nevertheless, although capable to rapidly discriminate signs of infection by decoding these patterns of self and non-self, the innate responses are not very specific and therefore unable to distinguish small differences in foreign antigens, many times leading to indiscriminate damage of normal tissue [18].

In order to copy with the constant demanding obligations required by the organism, the innate immunity rely on specific innate immune cells to guarantee host's health. Although all innate immune cells share their inherent ability to rapidly respond to tissue injury without memory of previous assaults or antigen specificity, and although all arise from an Hematopoietic Stem Cell (HSC) by a process termed haematopoiesis [19], they comprise a broad range of different cell types with unique morphologies and effector properties. Thus, a unique HSC has the ability to give rise to different innate myeloid cells - including dendritic cells, monocytes, macrophages, neutrophils, eosinophils, basophils and mast cells - and different innate lymphoid cells – including other dendritic cells, Natural Killer (NK) cells, some Intraepithelial T Lymphocytes (ITLs) and Innate Lymphoid Cells (ILCs) [19]. The main functions and features of each innate cell type have been extensively reviewed throughout the last years [10, 20–24].

1.1.2 The Adaptive Immunity

Although the innate immune system is many times sufficient to effectively eliminate infections, the countless possibilities of pathogens' antigenic structures and their ability to escape host detection, limit the range of common PAMPs recognized by innate immunity.

Therefore, a second and more comprehensive line of immune defence, known as adaptive immunity, is activated to overcome the struggles encountered by the innate immune system. The adaptive immunity relies mainly on specific adaptive immune cells called B and T lymphocytes that, similarly to some of the innate cells, derive from a common lymphoid progenitor that arises from a single HSC by haematopoiesis [19]. Although morphologically very similar, these two major classes of lymphocytes display different immune functions and express different antigenic-specific receptors that allow their distinguishment: B-cell Receptor (BCR) and T-cell Receptor (TCR) for B and T lymphocytes, respectively [25]. While the BCRs, membrane-bound immunoglobulin molecules, can recognize soluble or particulate antigens, the TCRs are only capable of recognizing processed pieces of antigens most of the times bound to specific cell-membrane proteins termed Major Histocompatibility Complex (MHC) molecules [26–29]. It is also important to highlight that, because lymphocytes can be divided into different subpopulations, an accurate way to distinguish them is based on their expression of specific surface proteins, often referred to by the Cluster of Differentiation (CD) nomenclature [30].

After maturation in bone-marrow and thymus, respectively, both B and T lymphocytes are considered mature naïve cells until encounter any antigen. After antigen presentation by an Antigen-Presenting Cell (APC) coming from the periphery and correspondent recognition by their BCR and TCR, B and T lymphocytes undergo proliferation and differentiation into both effector and memory cells [25]. While the effector cells will carry out specific functions to combat the pathogen, the memory cells will persist in the host to mediate a greater and quicker secondary response upon a re-challenge with the same antigen. Hence, because it relies on a primary encounter and "categorization" of antigens to adapt and better recognize, eliminate and remember the invading pathogen, the adaptive immune system is slower in responding to an infection, but much more accurate and effective. Therefore, it can be broadly described in a simplistic way by its two main characteristics: specificity and memory [25].

1.1.3 Cellular Communication in Immunity

For innate and adaptive immunity to work together to properly defend the organism and maintain tissue homeostasis, these two systems and its components must be able to quickly and efficiently communicate with one another. In a general way, this communication is achieved by both cell-to-cell interactions, where close contact of cells is required, and by soluble messengers, where communication is mediated by soluble molecules without direct cell communication [25]. Molecules that communicate among cells of the immune system are commonly referred to as cytokines. Therefore, cytokines are generally cell-secreted small proteins of low molecular weight that can bind to specific cell-surface receptors to signal these cells to alter their own or another cell behaviour. Although the result from this interaction varies and is dependent on the type of cytokine and cell involved, typically leads to changes in activation, division, movement or apoptosis of the

target cell [25, 31–33]. Cytokines can be grouped into six different families accordingly to their features or functions [1]: the Interleukin-1 (IL-1) family, the Hematopoietin family (Class I cytokines), the Interferon family (Class II cytokines), the Tumour Necrosis Factor (TNF) family, the Interleukin-17 (IL-17) family and the Chemokine family. The principal cytokines and a briefly description of each family are depicted in Table 1.1. Due to their importance in the process of inflammation, cytokines can be referred to as pro- or anti-inflammatory cytokines, depending on their role in inducing, or not, an increase in capillary permeability and leukocytes migration into the infected tissues, respectively [34].

Although cytokines' synthesis and release is intimately associated with the sensing of an infection by the immune system to trigger an effector immune response, studies showing the endocrine [35] and nervous system [36] affecting peripheral inflammatory responses or the direct impact of diet in modulating cytokine's expression [37] are just a few examples proving that the communication using these molecules is also modulated by other systems beside the immune system.

Table 1.1: The families of cytokines, their representative members and general characteristics (reproduced from [1])

Family Name	Representative Members of Family	General Characteristics
IL-1 family	IL-1α, IL-1β,IL-1Rα, IL-18, IL-33	Members of this family are generally proinflammatory mediators.
Hematopoietin / Class I Cytokines family	IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-12, IL-13, IL15, IL-21, IL-23, GM–CSF, G-CSF, Growth hormone, Prolactin, Erythropoietin/hematopoietin	Members of this family are functionally different but share a three-dimensional four-helix bundle motif organized into four anti-parallel helices.
Interferon/Class II Cytokines family	IFN-α, IFN-β, IFN-γ, IL-10, IL-19, IL-20, IL-22, IL-24	All members of this family are important modulators of immune response with the IFNs displaying important roles in anti-viral responses
TNF family	TNF-α, TNF-β, CD40L, Fas (CD95), BAFF, APRIL, LTβ	Members of this family regulate the development, effector function and homeostasis of immune, skeletal and neuronal cells, among others. Important in establishing tissue homeostasis.
IL-17 family	IL-17 (IL17-A), IL17B, C, D, and F	Members of this family are proinflammatory mediators.
Chemokine family	IL-8, CCL19, CCL21, RANTES, CCL2 (MCP-1), CCL3 (MIP-1α)	Members of this family have chemotactic activity, therefore displaying a key role in leucocyte migration.

1.2 An Overview of the Nervous System

Together with the immune system, the other main sensory interface of the body capable to perceive, integrate and respond to environmental or intrinsic changes and challenges is the nervous system. In order to reach the entire body to cope with these processes, the nervous system relies on the efficacy of two different types of specialized cells: the nerve cells (or neurons) - typically composed by an axon, a cell body and projections arising from the cell body termed dendrites - and their supporting glial cells (or glia). While the neurons are responsible to generate electric signals termed action potentials that allow them to quickly transmit information over short or long distances along the body, glial

cells display a protective and supportive role, being essential contributors to the nervous system well function [38–40].

1.2.1 The Architecture of Nervous System: the Central, Peripheral and Enteric Nervous Systems

The vertebrate nervous system can be anatomically divided into two different main halves: the Central Nervous System (CNS), that comprises the brain and spinal cord and where the acquired information is processed, and the Peripheral Nervous System (PNS), that enables the CNS to connect to every part of the body and that comprises nerves - bundles of long peripheral axons gathered - and ganglia - local accumulations of nerve cell bodies and supporting cells.

The PNS is functionally divided into two principal components: sensory components, that include sensory or afferent neurons responsible for acquiring information from the environment or the organism itself and carrying it towards the brain or spinal cord, and motor components, that include motor or efferent neurons responsible to carry such information away from the brain or spinal cord to muscle fibbers throughout the body in order to generate specific actions or responses [38]. The motor portion of the PNS can be further divided into two components: the somatic motor system – containing axons that connect the brain and spinal cord to the skeletal muscle; responsible for voluntary behaviours – and the visceral or autonomic motor system – with cells and axons that innervate smooth muscles, cardiac muscle and glands; responsible for involuntary behaviours. Lastly, the autonomic nervous system can be further sub-divided into sympathetic, parasympathetic and enteric nervous systems. While the sympathetic nervous system is associated with 'fight-or-flight' responses and its ganglia lie along or in front of the vertebral column and send their axons to different peripheral targets, the parasympathetic nervous system is associated with 'rest-and-digest' responses and its ganglia are found within or adjacent to the organs they innervate. The Enteric Nervous System (ENS) is made up of small ganglia and individual neurons scattered throughout the wall of the intestine with the main purpose of influence gastric motility and secretion [38] (for detailed information about the ENS see Chapter 1.4.3). This complex architecture of the nervous system is described in a simplistic way in Figure 1.1.

1.2.2 Chemical Communication in the Nervous System

When a certain stimulus sensed by a neuron is sufficient to trigger an all-or-nothing change in the electric potential (voltage) across the nerve cell membrane, an action potential happens and the information starts to be transmitted from one point to another along the nervous system. Therefore, the first action potential becomes a self-regenerating wave of electrical activity that propagates from its point of initiation in the cell body to the axon terminus, being then passed to the next target neuron by a process termed as

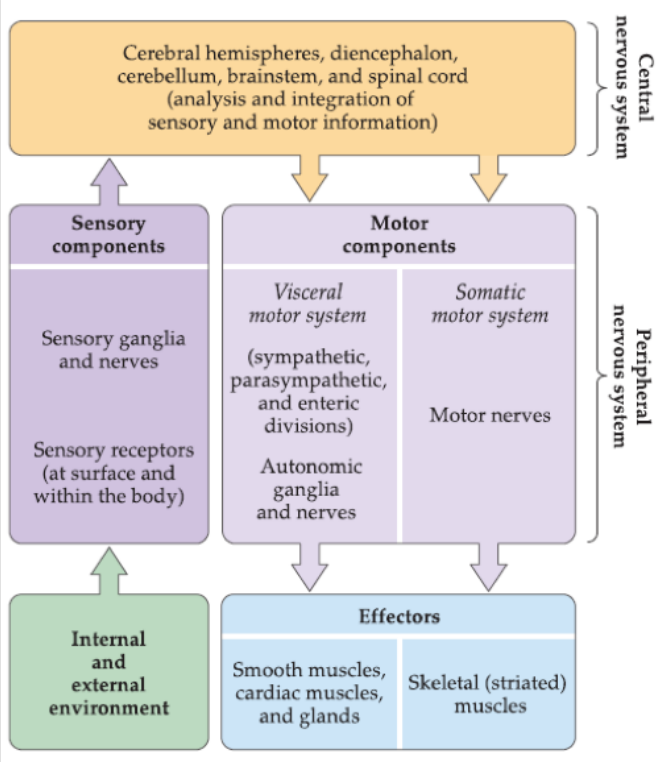

Figure 1.1: **The Architecture of the Nervous System and the Functional Relationships Between its Major Components.** Representative diagram of the major components of the nervous system and their functional relationships. Information coming from the environment or the organism itself is sensed by the sensory components of the Peripheral Nervous System (PNS) and transmitted to the Central Nervous System (CNS), which in turn process and interprets their significance and send signals to peripheral effectors in order to perform an effective response. (adapted from [38])

synaptic transmission. There are two principal types of synapses that allow this communication: electrical synapses, relatively rare and where the nerve cells are in direct contact, facilitated by gap junctions; and chemical synapses, the most abundant type of synapse and where pre- and postsynaptic components communicate via the secretion of molecules from the presynaptic terminal that bind to receptors in the postsynaptic cell. The molecules secreted in chemical synapses are called neurotransmitters, which are then going to be responsible to modify the electrical properties of the target cell [38]. Neurotransmitters can be divided into three main types according to their specific chemical structure: amino acids - including glutamate, glycine and Gamma Aminobutyric Acid (GABA) -, biogenic amines – including dopamine, norepinephrine, epinephrine and serotonin – and peptides – including substance P, neuropeptide Y, opioids, Vasoactive Intestinal Peptide (VIP), *etc.*. Besides these main types of neurotransmitters, it is important to highlight a fourth type including all the other compounds that do not fit in the previous categories but are still considered as neurotransmitters, such as purines, fatty acids, organic compounds and even gaseous neurotransmitters (including adenosine,

anandamide, acetylcholine, nitric oxide, carbon monoxide, *etc.*) [41, 42]. The neurotransmitters can be often referred to as inhibitory, when leading to an Inhibitory Postsynaptic Potential (IPSP) characterized by a postsynaptic membrane hyperpolarization through efflux of K^+ or influx of Cl^- less likely to generate an action potential, or excitatory, when leading to an Excitatory Postsynaptic Potential (EPSP) characterized by a postsynaptic membrane depolarization through influx of Na^+ more likely to generate an action potential. Nevertheless, it is important to highlight that both IPSP and EPSP signals are often simultaneously received by the postsynaptic neuron, thus being the postsynaptic end response the result of this 'competition'. The term neuromodulator is also commonly used when the neurotransmission effect of these molecules is not directly excitatory or inhibitory [38].

Due to the enormous variety of small organic molecules that can serve as neurotransmitters, the sections that follow focus only on those used for the purposes of this work.

1.2.2.1 Norepinephrine (NE)

Norepinephrine (NE) or noradrenaline is a neurotransmitter belonging to the catecholamines family that occurs in the central and peripheral nervous systems, but mainly in the sympathetic ganglia of peripheral tissues, as it is primarily released in response to acute stress reactions [43, 44]. Being a catecholamine, this 'fight-or-flight' neurotransmitter is characterized by its catechol nucleus (*i.e.* a benzene ring with two hydroxyl side groups next to each other) and an ethylamine side chain. NE is synthetized from the amino acid tyrosine by a series of enzymatic steps [45]. There are currently three known subfamilies of adrenoceptors to which NE can bind: α1-adrenoceptors (ADRA1) (subdivided in ADRA1A, ADRA1B and ADRA1D receptors), α2-adrenoceptors (ADRA2) (subdivided into ADRA2A, ADRA2B and ADRA2C receptors) and β-adrenoceptors (ADRB) (subdivided into ADRB1, ADRB2 and ADRB3 receptors) [46]. Either by its importance on cardiovascular effects in stress-responses, by its ability to directly interact with the immune system [36, 47] or by its pronounced role in modulating metabolism [48, 49], it is clear that NE is an important neurotransmitter displaying a wide range of functions among central and peripheral tissues.

1.2.2.2 Acetylcholine (Ach)

Acetylcholine (Ach) is a fast-acting small-molecule neurotransmitter and neuromodulator that occurs in the central and peripheral nervous systems, being associated with both sympathetic and parasympathetic ganglia of peripheral tissues. Its name is derived from its chemical structure, thus being characterized by an ester choline and acetic acid. Ach is synthetized from choline by a choline acetyltransferase enzyme in a single-step reaction involving acetyl coenzyme-A. There are two known major subfamilies of cholinergic receptors to which Ach can bind, being distinguished by their ability to bind their

natural nonendogenous agonists: the Ionotropic Nicotinic Receptors (nAChRs) (consisting of ligand-gated ion channels containing a cysteine loop near the extracellular transmembrane domain region, with natural ability to bind to nicotine) or the Metabotropic Muscarinic Receptors (mAChrRs) (which are coupled with G-proteins and their associated downstream signalling pathways, with natural ability to bind to muscarine) [50]. Although usually associated with motor neurons due to its well-known role in smooth muscle activation [50], this neurotransmitter has also been implicated in many other biological functions, including direct vascular effects by promoting vasodilation [51], memory- and learning-related effects [52] and immune-related effects by modulation of inflammation and response to infection [53–55].

1.2.2.3 Glutamate (L-glutamic acid)

Glutamate (many times referred to as L-glutamic acid, its protonated form) is an amino-acid that can operate as an excitatory neurotransmitter in both central and peripheral nervous systems. Glutamate can be synthetized either from amino acids such as glutamine, arginine, proline and histidine, or either from α-ketoglutarate by glutamate dehydrogenase or a variety of aminotransferases [56]. There are two known major subfamilies of glutamatergic receptors to which glutamate can bind: the ionotropic receptors, which include the N-methyl-D-aspartate Receptor (NMDAR), the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid Receptor (AMPAR) and the Kainate Receptor (KR), that, similarly to the nicotinic receptors, act as ion channels; and the metabotropic receptors (Group I, II and III receptors), that, similarly to the muscarinic receptors, are linked to intracellular second messenger systems [57]. Besides its crucial role as an excitatory neurotransmitter, glutamate is involved in many other functions related with cell energy and metabolism [58, 59]. Moreover, it is also involved in protein structure's stabilization by providing its negative charge [60, 61], is the immediate precursor of GABA, thus having a crucial role in its synthesis [56] and, paradoxically, it also displays potent neurotoxic effects [62, 63].

1.2.2.4 GABA

GABA is an amino-acid that can operate as an inhibitory neurotransmitter in both central and peripheral nervous systems. It is synthetized from glutamate by a Glutamic Acid Decarboxylase (GAD), which can exist in two different isoforms (GAD65 and GAD67). There are three known major subfamilies of GABAergic receptors to which GABA can bind: ionotropic GABA-A receptors (consisting of pentameric chloride channels constituted by a combination of different α (1-6), β (1-3), γ (1-3), δ, ϵ, θ, π or ρ subunits), metabotropic GABA-B receptors (consisting of heterodimeric receptors coupled to a G-protein) and ionotropic GABA-C receptors (consisting of pentameric chloride channels entirely composed of ρ subunits) [64]. Besides being capable of altering the excitability of neural circuits by regulating glutamatergic neurons and preventing hyperexcitation [65],

GABA also displays important roles beyond the nervous system. Previous works showed that GABA is involved in the replication and survival of the pancreatic insulin-producing β-cells [66, 67], in the inhibition of glucagon secretion in pancreas [68] or even in the regulation of cytokines' secretion by immune cells and modulation of inflammation [69, 70].

1.2.2.5 VIP

VIP is a 28-amino acid peptide originally recognized by Said and Mutt in lung and small intestine in its ability as vasodilator [71, 72] that today is known to usually operate as a neurotransmitter or neuromodulator in both central and peripheral nervous systems, where it is widely but selectively distributed. VIP belongs to the glucagon-related superfamily of peptides, thus sharing a similar structure with several other peptides such as secretin, glucagon, growth-hormone releasing hormone, Pituitary Adenylate-Cyclase-Activating Polypeptide (PACAP), among others. There are three known major types of VIPergic receptors to which VIP can bind: VPAC1, VPAC2 and PAC1 receptors, all belonging to the class II G-protein-coupled receptor family [73–75]. According to its wide distribution, VIP plays a role in numerous biological processes, including systemic vasodilatation [72, 76], smooth muscle relaxation and gastric motility [77], hyperglycaemia [72], enteric secretion of water and eletrolytes [73], *etc.*. This pleiotropic neuropeptide has also been identified as a key player in the immune system, regulating the balance between anti- and pro-inflammatory mediators [78, 79] and restoring immune tolerance by inducing dendritic cells with suppressive activity against autoreactive responses, thus acting as a potent immunomodulatory factor [80].

1.3 An Overview of the Endocrine System

Another interface of the body that works side-by-side with the immune and nervous systems to regulate many physiological processes and maintain body's homeostasis is the endocrine system. The endocrine system is comprised of numerous glands distributed throughout the body, whose main function is to synthetize and secrete hormones to the circulation to regulate the respective target organs. Some of the endocrine glands responsible to produce these signalling molecules include the thyroid gland, adrenal gland and pituitary gland. Organs such as liver, pancreas, ovaries and testicles make also part of the endocrine system, therefore contributing to this hormone-mediated communication [81, 82].

1.3.1 The Glucocorticoids

The hormones secreted by the endocrine organs and glands have different chemical structures, therefore they are divided into different classes. The corticosteroids – one subclass

of the steroids-hormones class – can be further divided into mineralocorticoids and glucocorticoids. The endogenous glucocorticoids (such as cortisol in humans and corticosterone in rodents) are synthetized from cholesterol in the adrenal cortex and released to the systemic circulation. In the extracellular space, endogenous glucocorticoids can be found in two different forms: in their inactive form, due to binding with Corticosteroid-Binding Globulin (CBG); or in their active unbound form. Because unbound glucocorticoids are lipid soluble, they are able to diffuse through cell membranes where they can bind to their cytosolic glucocorticoid receptors – receptors ubiquitously expressed by nuclear cells and encoded by the *Nr3c1* gene - as a part of a chaperone complex that will favour their translocation to the nucleus. Within the nucleus, the glucocorticoid receptor interacts with DNA and with other proteins to then exert genomic and biological effects [83].

Because endogenous glucocorticoids also bind with high affinity to mineralocorticoid receptors and are subject to endogenous inhibitors, synthetic glucocorticoids that have minimal mineralocorticoid effects, that are not inhibited by these intrinsic mechanisms and whose properties such as fat-solubility and half-life time can be modulated, are often routinely use in clinic as therapeutic drugs. Of the glucocorticoids' analogues list, beta- and dexamethasone are those showing a higher potency, a higher half-life time and lower mineralocorticoid effects [83, 84].

1.3.2 The Neuro-Immune-Endocrine Communication

The bidirectional interaction between the immune and the endocrine systems has long been appreciated. Some studies showed that several cytokines released by immune mediators are able to affect the release of hormones by acting on the hypothalamus and/or the pituitary gland [85, 86]. Other studies indicate that glucocorticoids promote macrophage phagocytosis of apoptotic neutrophils [87] or act as potent anti-inflammatory and immunosuppressive molecules [35, 88, 89], therefore demonstrating the opposite side of the coin, with the immune system being regulated by the endocrine system. In addition to this, in the same way as the nervous system is interconnected with the immune system, both the nervous and the immune systems can communicate with the endocrine system. Hence, the three biological systems are connected with each other, thus creating a neuro-immune-endocrine network of molecules, pathways and processes that regulate body's homeostasis.

1.4 The Small Intestine As a Neuro-Immune Interface

The intestine represents the largest compartment of the gastrointestinal tract responsible for food digestion and nutrient and water absorption. However, because it constitutes a wide barrier surface that separate the body from the external environment, this organ is not just crucial for digestion-related processes but is also essential for defending the

body from the microorganisms. Because intrinsic and complex relationships between the nervous and the immune systems are found in the intestine (see Section 1.4.4), this organ represents a robust interface to study these interactions. Unless specified otherwise, the factors here presented are similar in mice and humans.

1.4.1 The Anatomy and Physiology of the Intestine

The intestinal tract is composed by the small and large intestines that, together, form a continuous tube that goes from the outlet of the stomach to the anus. The small intestine – that begins at the pylorus and ends at the ileocecal valve - can be divided into three main segments: the duodenum, in the more proximal part, closest to the stomach; the jejunum, in the middle; and then the ileum, in the more distal part. In its turn, the large intestine – that begins at the caecum and finishes at the anus – can be divided into ascending (proximal) colon, transverse colon, descending (distal) colon and rectum [90]. While the small intestine is longer in length but thinner in diameter and characterized by a non-flat surface with finger-like projections known as villi extending into the lumen, the colon is wider in diameter but shorter in length and characterized by a flat surface without any villi (Figure 1.2). At the villi level, it is easy to distinguish three major layers towards the intestinal lumen: a thick muscle layer, a layer of connective tissue called submucosa and a layer called mucosa that comprises the intestinal epithelium, the underlying lamina propria and the muscularis mucosa, which is a thin muscle layer (Figure 1.2). The lamina propria is composed of loosely packed connective tissue, thus being essential for supplying the mucosa with blood, lymph and neuronal signals, while simultaneously sustaining the villi [90]. The different anatomies found within the different regions of the intestine are due to their distinct physiological functions: while the upper part of the intestine (*i.e.* duodenum and jejunum) has long thin villi extended to the lumen to increase the surface area available for the digestion and absorption of metabolites from the diet, the distal part of the intestine (*i.e.* caecum, colon and rectum) has no villi, as the large intestine as little or no intrinsic digestive function. Therefore, the villi become progressively shorter and broader going down the length of the intestine, which is consistent with the lower rates of absorption that occur in these regions [90].

In addition to this, another similar but antagonistic difference happens at the microbiome site. The idea that the human intestine is home to trillions of commensal microorganisms that are essential for life is no strange concept today. Indeed, it is known that the intestine is the major source of commensal bacteria - containing 10^{14} microorganisms of more than 500 different species – and that these bacteria use complex polysaccharides and undigestible dietary fibres as energy sources to produce essential metabolites for the host, such as vitamin k and short-chain fatty acids [91, 92]. Moreover, besides having energetic and metabolic roles, these bacteria can also modulate the expression of host genes [93], contribute to gut epithelial homeostasis [94] and compete with other potential pathogenic invaders while shaping the immune system, therefore being also crucial for

host protection and immunity [91, 95, 96]. In the same way that anatomical and physiological differences are found along the intestinal tract, the distribution of commensal bacteria is neither linear or similar within the different gut regions, as the number of bacteria generally increases going down the length of the gastrointestinal tract, ranging from 10^5 per mL in the upper small intestine to 10^{12} per mL in the colon, in humans (Figure 1.2) [90]. It is therefore easy to notice that the antigenic content provided by the dietary nutrients and by the commensal microorganisms have opposite distributions along the length of the intestinal tract: while the antigens from diet decrease in a proximal-to-distal manner (*i.e.* are more frequent in the duodenum and jejunum), the antigens from commensal microorganisms decrease in a distal-to-proximal manner (*i.e.* are more frequent in the ileum and large intestine) (Figure 1.3).

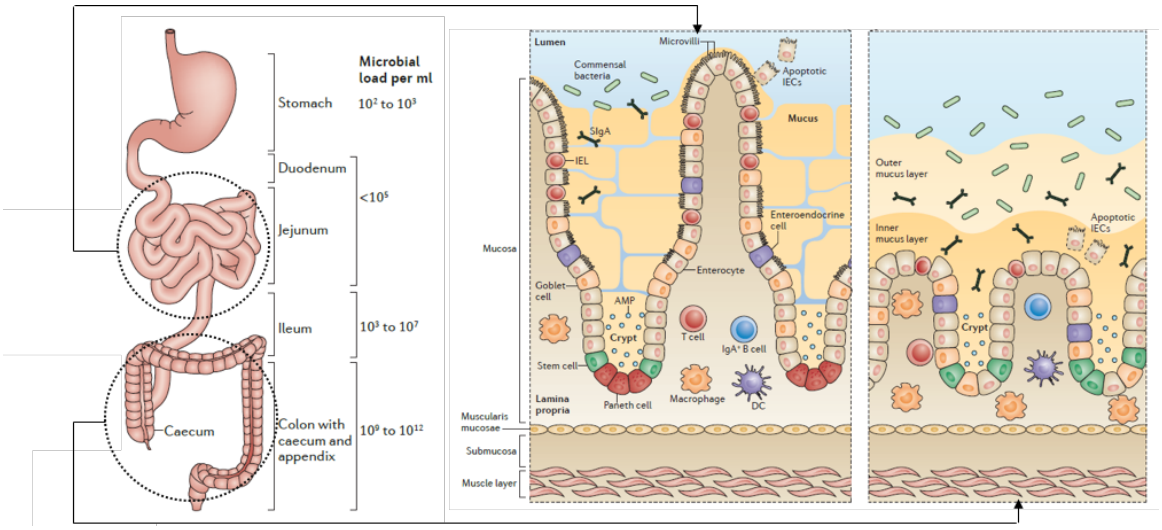

Figure 1.2: **The Gastrointestinal Tract and the Anatomy of the Intestinal Mucosa.** The intestinal tract comprises the small and large intestines, each one anatomically and functionally different from the other. The upper small intestine – comprised by the duodenum and jejunum – is characterized by long thin villi covered by a surface epithelium that contain absorptive enterocytes with microvilli containing digestive enzymes, which increases the area available for digestion. Besides these enterocytes, other specialized epithelial cells can be found, such as mucus-secreting goblet cells, AMP-producing Paneth cells and enteroendocrine cells, all arising from stem cells in the crypts. Still in the surface epithelium, a specific type of immune cells is found lying between epithelial cells: the Intraepithelial Lymphocytes (IELs). Going down the length of the intestine, the villi and microvilli start to become progressively shorter and broader, goblet cells become more numerous and the mucus layer become thicker, which is consistent with the progressive lower rates of digestion and higher numbers of commensal microorganisms. In the large intestine, the villi are completely absent. Towards the intestinal lumen, three main layers can be found within both small and large intestines: the muscle, submucosa and mucosa layers. Except for the IELs, the majority of intestinal immune cells are found in the lamina propria of the mucosa layer. AMP, antimicrobial peptide; SIgA, secretory immunoglobulin A; DC, dendritic cell. (adapted from [90])

1.4.2 Cellular Components of the Small Intestine

Different types of cells with diverse functions can be found in the mucosa layer of the small intestine. In a simplistic way, they can be classified within one of the two categories of cells: immune cells and epithelial cells.

1.4.2.1 Immune Cells

The intestine contains the larger number of immune cells than any other tissue in the body. There are two main effector sites of the intestine where these immune cells can be found: the lamina propria, where the majority of intestinal immune cells – such as B and T cells and numerous innate immune cell populations, including dendritic cells, macrophages, eosinophils and mast cells – are found; and the epithelial layer itself, where Intraepithelial Lymphocyte (IEL) - primarily T cells - are found (Figure 1.2) [90]. Although an extensive characterization of the immune cell types found in the small intestine is not within the purposes of this work, a more detailed list of these cells and some of their main features are listed below [90]:

- **Lamina propria T cells:** including $CD4^+$ (T_H1 cells, T_H2 cells, T_H17 cells and $FOXP3^+$ T_{Reg} cells) and $CD8^+$ T cells found at an approximate ratio of 2/1, most displaying an effector memory phenotype; in mice, T_{Reg} and T_H17 cells have opposite distributions throughout the intestine, as the first tend to progressively increase from the proximal to the distal small intestine in number, while the latter tend to decrease [97].
- **Lamina propria B cells:** naturally occurring in the healthy intestine, they include mainly IgA-, but also IgM-producing plasma cells.
- **Innate Lymphoid Cells:** including ILC1, ILC2 and ILC3 subsets; while in both mice and humans ILC1 and ILC2 numbers tend to be very similar, ILC3 numbers tend to progressively increase from the proximal to the distal small intestine [98].
- **Invariant T cells:** including invariant Natural Killer T cells (iNKT), a minor subsets of T cells that recognize glycolipid antigens presented by the non-classical MHC class I molecule CD1d [99]; iNKT only account for 0.5% of jejunum lamina propria in humans.
- **Intestinal Macrophages:** the most abundant leukocytes in the lamina propria showing high expression of MHC class II; in humans, their numbers tend to be very similar along the length of the small intestine [97, 100].
- **Intestinal Dendritic cells:** including $CD103^+$ $CD11b^+$, $CD103^+$ $CD11b^-$, $CD103^-$ $CD11b^-$, $CD103^-$ $CD11b^+$ subsets; in the mouse, the first subset shows a tendency to progressively decrease along the length of the small intestine and makes up most of dendritic cells [97].

- **Eosinophils:** naturally abundant in the healthy intestine; in mice, their numbers are higher in the duodenum and tend to progressively decrease along the length of the intestine [101].
- **Mast cells:** naturally abundant in the healthy intestine and also found in the submucosa and epithelium [102]; in mice, their numbers tend to progressively decrease along the length of the intestine [103].
- **Intraepithelial Lymphocytes (IELs):** including different subpopulations of mainly T cells located in the intestinal epithelial layer; these cells are extensively reviewed in Chapter 1.5.

As just described, the distribution of intestinal immune cells is not similar along the length of the small intestine, as these cells show regional differences in their frequency in response to the constantly-changing dietary and microbial antigens. The impact of microorganisms and diet on the distribution of immune cells along the intestine has been shown in several studies. It was shown that bacterial overgrowth can reverse the dominant isotype of IgA in the upper part of the intestine in humans [104]. It was also demonstrated that the small intestine's colonization with a single commensal bacteria is sufficient to induce IL-17- and IL-22-producing $CD4^+$ T helper cells [96] and that the lack of specific nutrients in a diet can shape ILC-mediated immune responses [105]. Nevertheless, regardless of their specific location, all these immune cells act to ensure gut defence and homeostasis.

Because the principal locations for intestinal antigens up-take and presentation by antigen-presenting cells are the Gut-Associated Lymphoid Tissues (GALT) and draining lymph nodes, it becomes logical to mention these lymphoid structures side-by-side with the immune cells. Thus, GALT are subepithelial lymphoid structures associated with the gut mucosa that regulate lymphoid function and contribute to the control of inflammatory or tolerant immune responses. These structures comprise the Peyer's patches, cryptopatches and Isolated Lymphoid Follicles (ILF) [90, 106, 107]. Peyer's Patches are constituted by several B cell follicles with germinal centres, which are flanked by small T cell areas, and their size and density increase from the proximal to the distal part of the small intestine, being particularly concentrated at the distal part of the ileum [90]. Humoral responses triggered in these regions are usually mediated by IgA production [108]. Cryptopatches are structures of clustered ILC3s and that usually mature to ILFs as microbiota colonizes the intestine [109]. ILFs consist of B cells with no clear T cell zone, but still containing germinal centres, and are involved in T cell independent IgA class-switching in mice, therefore promoting humoral responses [110, 111]. Besides the GALT, intestinal draining lymph nodes are also important sites for gut priming adaptive immune cell responses. The series of lymph nodes responsible for draining the jejunum and ileum are called the Mesenteric Lymph Nodes (MLN), while the duodenum is drained by the duodenopancreatic lymph nodes [90].

1.4.2.2 Epithelial Cells

Besides the immune cells, the mucosa layer also contains functionally and anatomically different specialized epithelial cells distributed along the intestinal epithelium. Because this epithelium serves as a semi-permeable barrier restricting the movement of water, ions and macromolecules from the gut lumen, while simultaneously blocking the entry of pathogens, the epithelial cells found within it have to be displayed and to function according to this selectivity. In a general way, the different types of epithelial cells are the following (depicted in Figure 1.2):

- **Multipotent Stem cells:** located in intestinal invaginations known as crypts of LieberKühn, these cells are responsible to give rise to the several different types of epithelial cells [90, 112].

- **Absorptive epithelial cells:** also referred to as just 'epithelial cells', these cells comprise almost all the surface epithelium that covers the thin villi of the small intestine, consisting of absorptive enterocytes that are covered by their own layer of microvilli (or brush border) that contain nutrient transporters and enzymes needed to digest dietary components. Therefore, the surface area available for digestion, which is already increased by the long villi, is even more enhanced with the presence of this brush border in each epithelial cell of the upper small intestine. Newly formed immature Intestinal Epithelial Cells (IEC) produced from the multipotent stem cells start to maturate while they move from the bottom of the crypts to the tip of the villi, from where they are extruded after 4-5 days [90, 112]. These cells are connected by specialized molecular structures such as tight junctions to avoid paracellular traffic and are also equipped with several PRRs, such as Toll-like Receptors (TLR), to recognize specific molecules of bacteria to mount an immune response or promote tolerance [113–115]. Hence, these epithelial enterocytes display both barrier and absorptive functions.

- **Goblet cells:** these cells are specialized epithelial cells that comprise less than 10% of all epithelial cells in the small intestine, although their numbers progressively increase going down the length of the gastrointestinal tract [90]. Under the control of certain immune mediators [116], these cells are responsible to produce glycosylated mucin proteins (*i.e.* mucus) that coat the small intestine mucosa in a thin and loose layer termed glycocalyx. This mucus layer not only acts as physical barrier but is also responsible to provide a scaffold to which antibodies and antimicrobial peptides can adhere [117].

- **Paneth cells**: these cells are long-lived specialized epithelial cells located immediately below the stem cells in the intestinal crypts and that are exclusive of the small intestine, being particularly concentrated in the ileum. Contrary to all the others epithelial cells, newly-formed Paneth cells migrate downwards to the bottom

of the crypt while they maturate [90, 112]. In response to certain stimuli, such as IL-22 or TLR stimulation, these cells produce apical cytoplasmic dense granules containing Anti-Microbial Peptides (AMP), such as defensins and lysozyme, and further release them to the crypt lumen, therefore displaying potent antibacterial functions [118]. Besides this, Paneth cells are also important to maintain normal crypt stem cell niche activity [119].

- **Tuft cells:** these cells are long-lived specialized epithelial cells that only make up 1% of the epithelium. They depend on innervation and cholinergic signalling to survive and have a crucial role in the initiation of type 2 immune responses [112].
- **Enteroendocrine cells:** these flask-shaped cells are specialized epithelial cells scattered throughout the intestinal epithelium. These cells typical have microvilli-covered apical processes, that allow them to sense the luminal content, and basolateral granules containing molecular mediators (peptides or amines) that are released in response to certain stimuli and can further act as paracrine regulators of neighbouring cells, hormones activating distant cells or neurotransmitters modulating local or distant responses. These enteroendocrine cells possess a pseudopodal extension, the neuropod, that is closely associated with enteric nerve terminals and display molecular components of synaptic release machinery. Some of the intestinal enteroendocrine cells' populations in the small intestine include cells expressing secretin (S-cells), cholecystokinin (I-cells), neurotensin (N-cells), glucose-dependent insulinotropic polypeptide (K-cells) and proglucagon (L-cells)[112, 120].

1.4.3 Neuronal Innervation of the Small Intestine

To correctly orchestrate the pathways, molecules and signals involving all the previous described cells and to perform the enormous variety of tasks related with food digestion, nutrient absorption and mucosa defence, the intestine must be able to sense and quickly adapt to the intrinsic and extrinsic demands of a constantly-changing gut environment. To accomplish this, the gastrointestinal tract is highly innervated by a complex network of different neurons that guarantee a fast and accurate response to changes. Therefore, although the gut-innervating neurons are still part of the cellular components of the small intestine, it becomes logical to mention them in a separate section, as they belong to a highly relevant and complex platform of gut regulation.

The small intestine is innervated by extrinsic and intrinsic neurons. While the extrinsic innervation is comprised of sympathetic and parasympathetic axons of afferent and efferent peripheral neurons, the intrinsic innervation comprises the neurons that are exclusive to the gut to form the ENS [121, 122].

The ENS is composed of two major ganglionated plexuses in which almost all intrinsic nerves reside (Figure 1.4A): the myenteric plexus, that represents the outer of the two and is formed by a network of neurons and glial cells located between the outer muscle

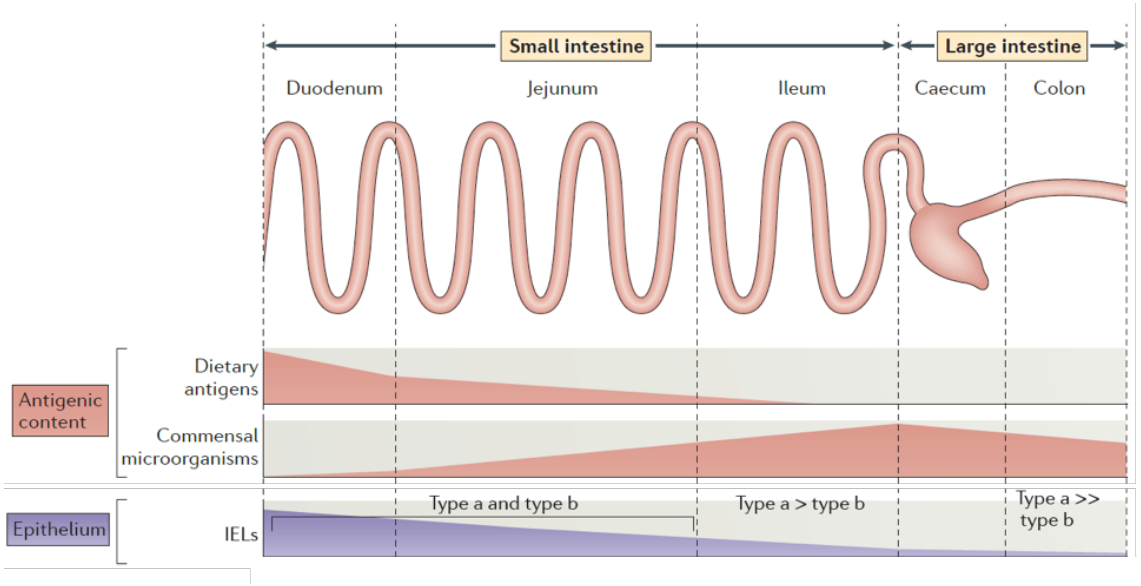

Figure 1.3: **The Antigenic Content and the Numbers of Intraepithelial Lymphocytes Variation Along the Intestinal Tract.** While the frequency of commensal microorganisms increases along the length of the intestinal tract, the frequency of dietary antigens shows an opposite behaviour, being higher in the upper part of the small intestine. Interestingly, the frequency/distribution of Intraepithelial Lymphocytes (IELs) is very similar to the dietary antigens. (adapted from [90])

layers of the gastrointestinal tract, and the submucosal plexus, that represents the inner plexus and is located in the submucosa layer [121, 122]. While the myenteric plexus extends the full length of the gastrointestinal tract and is primarily involved in the initiation and control of smooth muscle for peristaltic movements, the submucosal plexus is prominent only in the small and large intestines and coordinates reflexes such as secretion, absorption and some motor control of smooth muscles [121, 123]. Besides these two major plexuses, it is also possible to find delicate nerve and glial networks in the mucosa layer that, collectively, form the mucosal plexus, which extends to the lamina propria beneath the surface epithelium and therefore is in contact with the transmitters released by the existing enteroendocrine cells [121]. In a simplistic way, there are three broad types of neurons in the ENS distributed along these ganglionated plexuses: the motor neurons, the sensory neurons and the interneurons. Within each type, different subsets can generally be categorized by their function and connectivity. Thus, the intestinal intrinsic motor neurons are responsible to induce effector functions and can be divided in: muscle motor neurons (excitatory or inhibitory), that can be found in the myenteric, submucosal and mucosal plexuses and respond to signals initiated by mechano- and tension receptors; and (non-)secretomotor/(non-)vasodilator neurons, that can be found in both myenteric and submucosal plexus and manage fluid and molecular exchange between the gastrointestinal vasculature, tissue and lumen [122, 123]. The intrinsic sensory neurons contribute to a CNS-independent control of the intestine, as they perceive information regarding the gut condition through specific receptors and convey it to effector neurons

within the ENS. These sensory neurons can be divided in: Intrinsic Primary Afferent Neurons (IPAN), that consist of large multi-axonal neurons existing in both myenteric and submucosal plexuses that are responsible for detecting molecular and mechanical aberrations of the gastrointestinal tract and transmit the correspondent signals to any other type of enteric neuron; and Intrinsic Intestinofugal Afferent Neurons (IFAN), that exist mainly in the myenteric plexus and are responsible to send neuronal impulses from the gastrointestinal tract to extrinsic ganglia, with sympathetic impulses being then sent back to the ENS [122, 123]. Finally, the interneurons, which are responsible for connecting both sensory and motor neurons to propagate the neuronal impulse, can be found in the myenteric plexus and can be divided in ascending, descending (divided in three types) or supplying secretomotor interneurons [122, 123]. Some of the primary and secondary transmitters of each intrinsic motor, sensory and interneuron subsets are described in Table 1.2 [123, 124].

Table 1.2: Principal transmitters of enteric intrinsic motor neurons, sensory neurons and interneurons (reproduced from [123]; reviewed in [122])

Type of neuron	Primary transmitter	Secondary transmitters
Excitatory muscle motor	Ach	Tachykinins, Calretinin, GABA
Inhibitory muscle motor	NO	VIP, ATP, Carbon Monoxide, PACAP, Opioid peptides
Cholinergic secretomotor	Ach	Calretinin, NPY
Noncholinergic secretomotor	VIP	PACAP, NPY
IPAN	Ach, CGRP, Tachykinins	Calretinin, Calbindin
IFAN	Ach	VIP, GRP, CCK, Opioid peptides
Ascending interneuron	Ach	Tachykinins, ATP, Calretinin
ChAT, NOS descending interneuron	ATP, Ach	NO, VIP
ChAT, 5-HT descending interneuron	Ach	5-HT, ATP
ChAT, somatostatin, descending interneuron	Ach	Somatostain
Interneurons supplying secretomotor neurons	Ach	5-HT, ATP

Abbreviations: Ach, acetylcholine; CCK, cholecystokinin; ChAT, choline acetyltransferase; CGRP, calcitonin gene-related peptide; GRP, gastrin releasing peptide; NPY, neuropeptide Y; NO, nitric oxide; NOS, nitric oxide synthase; PACAP, pituitary adenylyl-cyclase activating peptide; VIP, vasoactive intestinal peptide; 5-HT, 5-hydroxytryptamine

Some specific enteric neurons' receptors are crucial to mediate important intestinal functions. These receptors are expressed by neuronal and enteroendocrine cells and can be divided in three main types: mechanoreceptors, which are responsive to mucosal abrasion, tension receptors, which are responsive to stretch, and chemoreceptors, which are responsive to various chemical stimuli in the lumen (including pH, osmolarity and nutrients) [122, 123].

Regarding the intestinal extrinsic innervation, there are two broad types of neurons that allow the connectivity from the CNS to this organ (Figure 1.4B): motor or efferent neurons, that convey information from the CNS to the gut, and sensory or afferent neurons, that convey information regarding the gut condition to the CNS. Both types

of neurons are composed of sympathetic and parasympathetic nerve fibers [122, 125]. Both sympathetic and parasympathetic extrinsic motor neurons can reach the intestine directly - by synapse directly to the gastrointestinal tract - or indirectly - through via enteric circuits and intrinsic motor neurons - by different vagal, sympathetic and pelvic pathways. Regarding the small intestine, its extrinsic innervation is made by motor post-ganglionic sympathetic neurons that are originated through sympathetic preganglionic fibers emerging from motor neurons in the spinal column and by motor postganglionic parasympathetic vagal neurons that are originated through vagal preganglionic fibers emerging from motor neurons in the dorsal nucleus of the vagal nerve (*i.e.* in the brain-stem). The extrinsic sensory neurons that convey the information to the CNS may follow the vagal route, which comprises the vagal afferent neurons in the nodose and inferior jugular ganglia, or the spinal route, which comprises the spinal afferent neurons in the dorsal root ganglia. Here, the principal transmitter is NE and Ach for the sympathetic and parasympathetic neurons, respectively. It is important to highlight that neurons in sympathetic prevertebral ganglia receive both CNS and ENS inputs [122, 125].

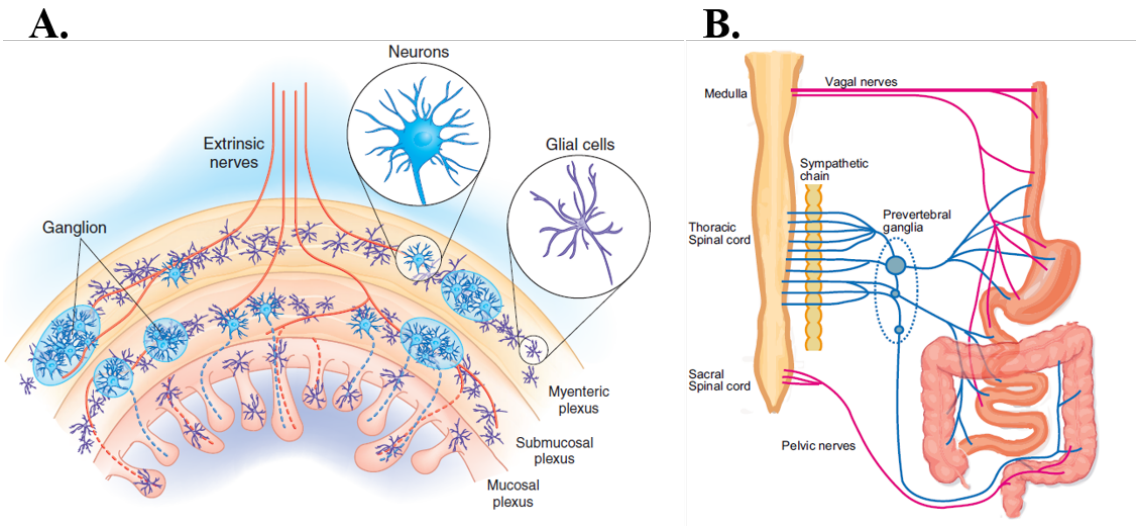

Figure 1.4: **Intrinsic and Extrinsic Innervation of the Gastrointestinal Tract. (A)** Schematic representation of the enteric nervous system (ENS) and its different ganglionated plexuses. Extrinsic neurons innervating the different intestinal layers are also represented (image from [121]). **(B)** Schematic representation of the extrinsic motor innervation of the gastrointestinal tract. Information from the central nervous system (CNS) reach the enteric nervous system (ENS) or the gastrointestinal tract effector tissue directly through vagal, sympathetic and pelvic pathways. Extrinsic afferent neurons follow spinal and vagal routes. Neurons in sympathetic prevertebral ganglia receive both ENS and CNS inputs. (image from [125]).

1.4.4 Neuro-Immune Interactions at the Intestinal Mucosa

The immune and the nervous systems are in constant communication with each other at the intestinal mucosa [36, 47, 122]. Indeed, besides controlling the absorption, secretion,

circulation and movements of the gut, the nervous system also plays a role in modulating the intestinal immune function. As an indirect example of this association, stress was shown to modulate several functions of the gut mucosa, such as intestinal permeability, mucin and IgA secretion and even microbial composition [126–128]. However, the immune system can also be modulated directly at the level of central, peripheral and enteric nervous systems. One example was shown by McLean et al., 2015 [129], when mice deficient in type 3 muscarinic Ach receptors showed a leaky intestinal barrier and higher basal levels of IFN-γ, IL-17a and TNF-α, resulting in the delayed clearance of *C. rodentium*. Another example was shown by Abad et al., 2003 [130] and Gomariz et al., 2005 [131], where VIP was demonstrated to mitigate intestinal inflammation and to inhibit TLR expression in macrophages, dendritic cells and lymphocytes in a mouse model of colitis. Moreover, Cardoso et al., 2017 [132] also showed the importance of the neuropeptide Neuromedin U (NMU) in regulating type 2 innate immunity in ILC2s to confer an immediate mucosal protection. In addition, efferent vagal nerves signals were shown to attenuate macrophage activity and led to anti-inflammatory responses in the intestine [133, 134]. On the other hand, the immune system also regulates the nervous system. One example of this was the loss of up to 50% of the enteric neurons in an inflammatory induced-mouse model of colitis, which was further reversed by a treatment with an anti-inflammatory topical steroid [135].

Although these are just a few examples of this bi-directional communication in the intestine, it is easy to conclude that the connection between these two systems play a fundamental role in controlling homeostasis of the gut. Therefore, a deregulation at one or both levels can lead to gastrointestinal disorders and imbalance of the intestinal mucosa.

1.5 The Intestinal Intraepithelial Lymphocytes (IELs)

The IELs comprise a population of mainly T cells that are found in the epithelial layer of mucosa linings, such as the respiratory and gastrointestinal tract. The following sections will focus only on the IELs found within the intestinal mucosa: the intestinal IELs.

1.5.1 The Classification of IELs

As it was previously seen here, and contrary to most of the other immune cells that are found in the lamina propria, the intestinal IELs are located in the epithelial layer. Therefore, these cells are long-lived tissue-resident effector immune cells that, as the name says, are in direct contact (*i.e.*, interspersed) with the epithelial cells. Although they exist along the entire length of the intestine (with ~1 IEL per 10 IECs), their distribution is not uniform, as at least 10-times more IELs can be found in the small intestine in comparison with the colon and as, within the small intestine itself, a gradual decrease

in their numbers in a proximal-distal manner can be verified in both mice and humans (Figure 1.3) [90, 136].

Despite their shared properties and location, intestinal IELs comprise a wide diversity of lineages, being classified in different ways. One of the ways to classify IELs is according to their TCR expression profile. Based on it, IELs can be either TCR positive or TCR negative. The TCR positive IELs represent the vast majority of IELs and, according to the mechanism by which they are activated and to the cognate antigens they recognize, they can be further divided into two major subsets: natural and induced IELs (Figure 1.5) [136]. Natural IELs - also termed as type B, thymic or unconventional IELs - directly differentiate from pre-committed thymic precursors and acquire their effector properties in the presence of self-antigens. They can be either TCR$\alpha\beta^+$ or TCR$\gamma\delta^+$ and do not express CD4 or CD8$\alpha\beta$ co-receptors on their surface. Although their majority express the homodimer CD8$\alpha\alpha$, some of these natural lymphocytes can be negative for CD8$\alpha\alpha$ [136, 137]. Induced IELs – also termed as type A, peripheral or conventional IELs - are the progeny of conventional CD4$^+$ or CD8$\alpha\beta^+$ T cells that express the TCR$\alpha\beta$, therefore being, respectively, MHC-class II or MHC-class I restricted. However, many induced IELs can also become positive for the homodimer CD8$\alpha\alpha$ upon entry into the intestinal epithelium [138]. These cells are selected in the thymus and activated in response to non-self-antigens in the periphery [136, 137]. The TCR negative IELs represent the minority of the IELs and can be also divided into different subsets, including group-1 innate lymphoid cells, ILC1-like cells, ILC3-like cells, cells expressing intracellular CD3 chains (iCD3) and CD8α innate IELs (iCD8α) [139] (Figure 1.5).

Besides varying along the length of the intestine, the density and composition of IEL subsets also varies greatly with age and antigen exposure: while induced IELs are scarce early in life and tend to increase with age in response to peripheral antigens, natural IELs are the first type of antigen-experienced T cells to colonize the gut. The first IELs appear even before birth [140]. The subsets and subpopulations of IELs also differ among species. Although both murine and human intestines, for example, are characterized by progressive decreasing number of IELs along their length, the first contains fewer induced IELs while the latter contains fewer natural IELs. Moreover, the murine induced IELs exist in higher numbers in the colon, while the human ileum and colon are more enriched with natural IELs. In addition, most IELs in the human jejunum are induced CD8$\alpha\beta^+$. Lastly, contrary to the murine intestine, natural TCR$\gamma\delta^+$ T cells remain a rather minor proportion of human intestinal IELs, which does not change along its length [90].

1.5.2 IELs Development and Migration to the Intestine

Although the development of both natural and induced IELs occurs in the thymus, the developmental pathways involved are different within each subset.

The development of unconventional or natural TCR$\alpha\beta^+$ IELs occurs in the thymus,

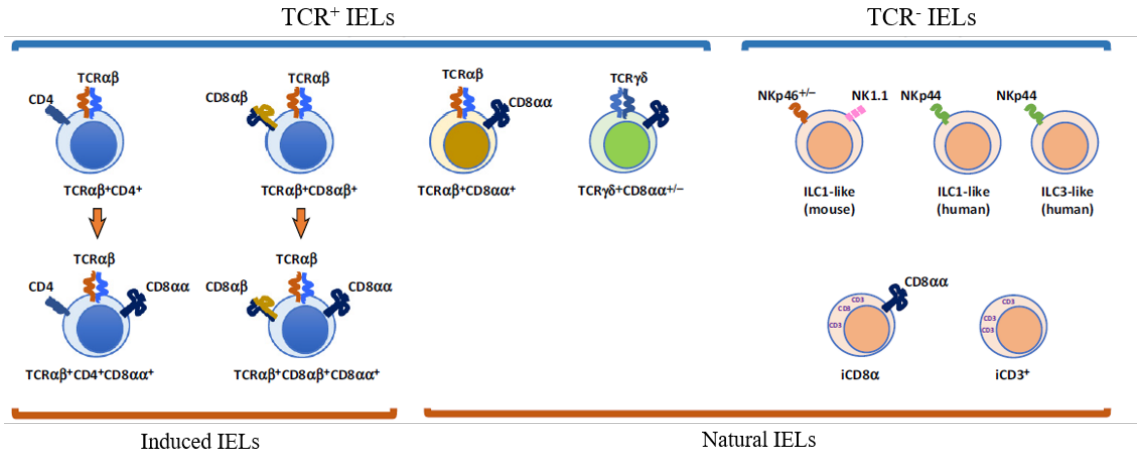

Figure 1.5: **The Classification of Intestinal Intraepithelial Lymphocytes (IELs).** IELs can be classified according to their T cell receptor (TCR) expression profile in TCR^+ or TCR^- IELs. TCR^+ IELs represent the most abundant type of IELs and comprise natural ($TCR\alpha\beta^+$ or $TCR\gamma\delta^+$) and induced (always $TCR\alpha\beta^+$, but $CD4^+$ or $CD8\alpha\beta^+$) IELs. Induced IELs can become positive for $CD8\alpha\alpha$ upon entry into the intestinal epithelium. Some differences in the prevalence and phenotype of mouse versus human IELs are indicated. (adapted from [139])

where a $CD4^+CD8\alpha\beta^+$ Double Positive (DP) cell acquire the expression of the homodimer $CD8\alpha\alpha$, becoming a $CD4^+CD8\alpha\beta^+$ $CD8\alpha\alpha^+$ Triple Positive (TP) cell [141]. $CD8\alpha\alpha$ expressing cells but not $CD8\alpha\beta$ are strong ligands for the thymic leukaemia antigen tetramer, a non-classic MHC class I molecule abundantly expressed in intestinal epithelial cells [142]. This TP thymocyte expressing a self-reactive TCR undergo an alternative positive selection, termed agonist selection - where, contrary to the regular clonal deletion process, cells with a high self-reactive TCR are selected instead of being killed by apoptosis [143, 144] - and differentiate into a Double-positive-low PD-1-high (DPlowPD1hi) cell by downregulating the expression of CD4 and $CD8\alpha\beta$, while expressing $TCR\alpha\beta$ and high levels of PD-1. Indeed, the high expression levels of PD-1 and CD69, for example, are indicative of high TCR signalling, which can provide the survival signals to these cells [137]. Still in the thymus, this DPlowPD1hi cell, the main precursor population of the natural $TCR\alpha\beta^+$ IELs, will continue to downregulate the expression of CD4 and $CD8\alpha\beta$, acquire its effector programme and start to upregulate the expression of gut-homing molecules (see Section 1.5.2.1), therefore functionally differentiating into a mature $CD4^-CD8\alpha\beta^-$-double-negative $TCR\alpha\beta^+$ ($DNTCR\alpha\beta^+$) effector cell. Hence, these cells leave the thymus already as Double Negative (DN) effector T cells that are programmed to go directly to the intestinal epithelium [137] (Figure 1.6). The development of unconventional or natural $TCR\gamma\delta^+$ IELs also occurs in the thymus, but in a slightly different way of the $TCR\alpha\beta^+$ IELs. Here, a $CD4^-CD8\alpha\beta^-CD8\alpha\alpha^-$ Triple Negative (TN) cell acquires the expression of the homodimer $CD8\alpha\alpha$ while simultaneously loses the expression of CD4 and $CD8\alpha\beta$, becoming a naïve $CD4^-CD8\alpha\beta^-$ DN T cell that expresses a self-reactive $TCR\gamma\delta$.

Although still naïve, this TCR$\gamma\delta$-expressing DN T cell already expresses gut-homing molecules (see Section 1.5.2.1) and can therefore leave the thymus, through the blood circulation, to the GALT [136, 137]. Once in the GALT, the TCR$\gamma\delta$-expressing DN naïve T cell will divide and acquire its effector programme after exposure to self-ligands expressed by epithelial cells [145] and, after priming, it will recirculate through the lymph back to the blood and selectively home to the gut epithelium as a DN effector T cell [137]. However, it has been shown that TCR$\gamma\delta$-expressing DN naïve T cells can also migrate directly to the gut epithelium after leaving the thymus, therefore acquiring their effector properties directly there [146] (Figure 1.6). Hence, although both TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ IELs respond to self-ligands rather than foreign ligands, TCR$\alpha\beta^+$ IELs have their effector programme induced in the thymus and leave this organ as effector T cells directly to the gut epithelium, while TCR$\gamma\delta^+$ IELs have their effector programme induced in the periphery (gut or GALT) and leave the thymus as naïve T cells. Despite these differences, natural IELs share some features regarding their maturation, maintenance and survival. Indeed, both natural IELs require IL-15 for these processes [146, 147], and it has been shown that the development of CD8$\alpha\alpha^+$ IELs is also dependent on the expression of T-bet by IEL precursors [147]. Moreover, T-bet is required for the IL-15-dependent activation, differentiation and expansion of IEL precursors in the periphery [147, 148]. Besides this, the Aryl Hydrocarbon Receptor (AHR), a ligand-dependent transcription factor whose ligand is mainly obtained through diet, showed to be crucial for the maintenance of both natural IELs in the intestine, although it is not needed for their development [149].

It is important to highlight that the developmental pathways described above should not be seen as a certainty, but yet as a hypothesis supported by prevailing evidence. Indeed, the ontogeny of these subsets of IELs has been subject of considerable debate, including some groups suggesting an extrathymic origin for both natural IELs [145, 150, 151].

Regarding the conventional or induced CD8$\alpha\beta^+$ IELs, a CD4$^+$CD8$\alpha\beta^+$ DP immature thymocyte undergo positive thymic selection in the thymus and differentiate into CD4$^+$ or CD8$\alpha\beta^+$ naïve T cells expressing the TCR$\alpha\beta^+$. These naïve T cells exit the thymus and migrate to the periphery where, in response to foreign antigens presented in the GALT, they start to proliferate, to upregulate the expression of gut-homing molecules (see Section 1.5.2.1) and to acquire their cytolytic effector programme to differentiate into effector CD4$^+$ or CD8$^+$ T cells. These effector T cells then migrate through the lymph to reach the blood circulation in order to selectively home to the gut epithelium, where they become incorporated [136, 137] (Figure 1.6).

1.5.2.1 The Gut-Homing Molecules

The selective expression of chemokine receptors, chemokines and adhesion molecules have a very important role in T cell homing. Important gut-homing receptors expressed by lymphocytes are the αEβ7, CCR9 and α4β7 receptors, which lead to the lymphocyte

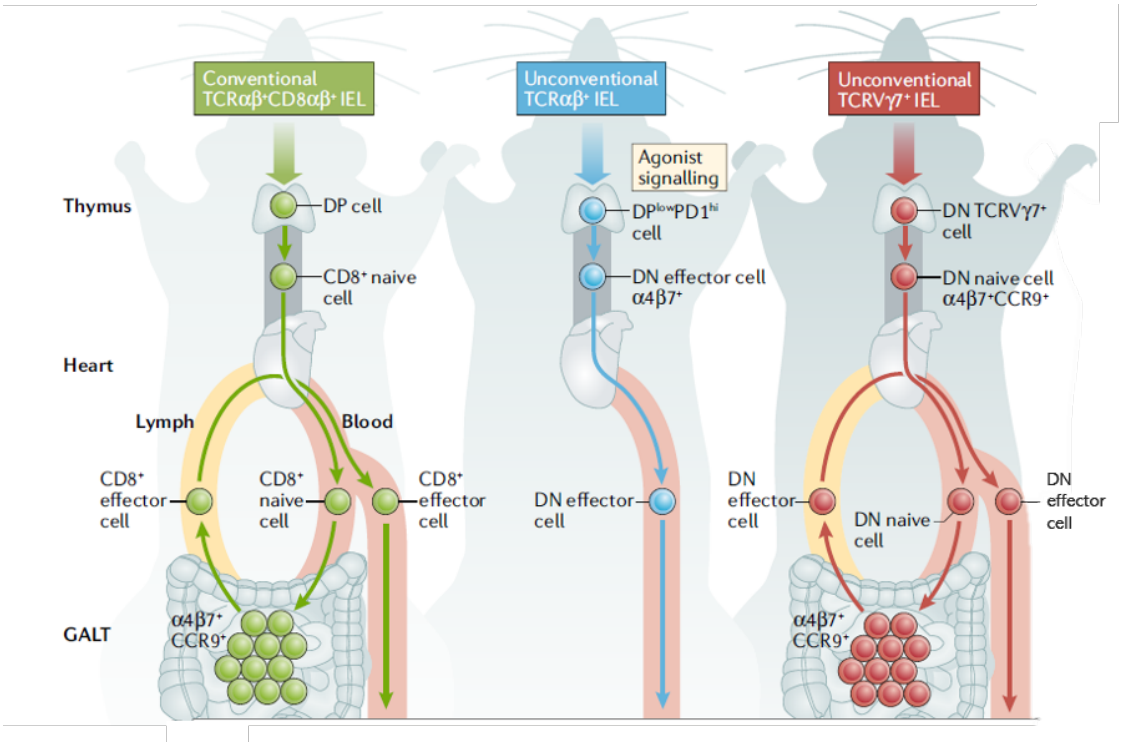

Figure 1.6: **The Developmental Pathways of the Three Main Subpopulations of IELs.** Both natural and induced Intraepithelial Lymphocytes (IELs) subsets are represented. Induced IELs (here represented by their major subpopulation, CD8$\alpha\beta^+$, in green) are originated from naïve T cells that are primed by foreign antigens in the gut-associated lymphoid tissues (GALT) to divide, acquire their cytolytic effector programme and up-regulate the expression of gut-homing molecules, therefore becoming effector T cells that home the gut epithelium. Natural IELs (here represented by their two major subpopulations, TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$, in blue and red, respectively) acquire their effector programme in response to self-antigens. Natural TCR$\alpha\beta^+$ IELs acquire it in the thymus through agonist selection and leave this organ as effector T cells already programmed to home the gut epithelium. Natural TCR$\gamma\delta^+$ IELs leave the thymus as naïve T cells that already express gut-homing molecules, but only acquire their effector programme in the gut epithelium or GALT. (adapted from [137])

entry in the epithelium upon contact with the respective ligands expressed by intestinal epithelial cells [32, 152, 153]. The integrin αEβ7 is expressed on more than 90% of intestinal IELs and promote the adhesion of T cells to the intestine through binding to E-cadherin, which is selectively expressed by epithelial cells [154]. CCR9 is a chemokine receptor functionally and selectively expressed in small-intestine lymphocytes that, by interacting with the CCL25 ligand, which is constitutively expressed in small-intestine epithelial cells, leads to the recruitment of circulating lymphocytes to the intestine. The majority of CCR9$^+$ cells co-express the α4β7 integrin, which is also involved in the lymphocytes' migration to the gut [32, 155]. This integrin binds to its ligand MAdCAM-1, which is expressed in postcapillary venules of mucosal tissues, to promote lymphocyte adhesion and gut homing [32]. It has been shown that the CCL25/CCR9 pair can influence

the early induction of CD103 (αE integrin), which indicates that this pair can regulate lymphocyte-epithelial interactions through $\alpha E\beta 7$ in the small intestine [156].

1.5.3 The Function of IELs

Because intestinal IELs are strategically located between the external environment and the core of the body, they constitute one of the first lines of intestinal immune defence. However, the exact function of each IEL subset is still not clear.

The IELs are antigen-experienced T cells with an effector programme that allows them to contribute to protective immunity. Both natural and induced IELs have the potential to produce both pro- and anti-inflammatory cytokines such as TNF-α, IFN-γ and IL-10, immunoregulatory chemokines and molecules associated with innate cell functions to regulate other immune and non-immune cells [138, 157–159]. IELs can also secrete antimicrobial factors to directly mediate the death of potential pathogens in order to avoid their entry and spreading through the intestine [160] and also have the ability to release cytotoxic granzymes (being GzmB the most abundant type) to induce apoptosis of target-cells [158, 161]. The natural IELs also express the CD95 ligand (also known as FasL), which can trigger the apoptosis of a target cell after binding to its extracellular receptor Fas [158]. Moreover, TCR$\gamma\delta^+$ IELs also have the ability to change their behaviour and motility-pattern in response to infection by communicating with epithelial cells [162]. The CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ IELs can indirectly contribute to regulate immunity by providing help for B cells and by promoting their production of IgE [163, 164]. They were also shown to trigger the maturation of dendritic cells [165–167]. In addition, CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ IELs also display immune suppressive roles by controlling the numbers and activation of CD8$\alpha\beta^+$ induced IELs and by reducing the expression of the activating NK cell receptor [168].

Nevertheless, IELs display many functions that go beyond host defence, including regulatory and homeostatic functions. Indeed, TCR$\gamma\delta^+$ natural IELs, for example, can secrete several factors that have direct or indirect roles in protecting the integrity of the epithelium, such as TGFβ1, TGFβ3 and prothymosin β4 [158], which are responsible for the control of epithelial cell growth and turnover [169, 170]. Moreover, their ability to secret Keratinocyte Growth Factor (KGF) shown to be crucial to restore the integrity of the epithelium in response to physical and inflammatory damage [5, 171, 172]. Finally, CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ IELs express other molecules associated with regulation, including TGFβ, fibrinogen-like 2, prothymosin β4 and several killer cell immunoglobulin-like receptors [173].

The strategic location of IELs near the enterocytes responsible for nutrient absorption, especially in the upper part of the intestine, and the fact that they are metabolically prepared for action by expressing genes related with metabolism [162], make one to predict another possible role for IELs in metabolism. Consistent with this, recently this year, He et al. [174] published a work showing that IELs can regulate the levels of a

specific metabolism-related hormone: the Glucagon-like Peptide 1 (GLP-1); therefore giving a novel metabolic role for these so-far considered 'typical' immune cells. GLP-1 is a 30 amino-acid peptide hormone constitutively secreted at low basal levels by intestinal enteroendocrine L-cells by differential processing of their proglucagon gene [175, 176]. In response to food, neuronal stimulation, microbiome and gut barrier dysfunction, the secretion of this hormone by L-cells is stimulated and a quick and drastic increase in its levels is verified within minutes [175, 176]. Because it is a pleiotropic hormone, once it is released, GLP-1 can act at different levels and in many organs and tissues of the body, including the brain, heart and blood vessels, kidney, skeletal muscle, adipose tissue, liver, gut, pancreas, stomach and even on the immune system itself (Figure 1.7), therefore displaying metabolic, energetic, neuronal and inflammatory roles [177]. Some of the best elucidated physiological roles of GLP-1 are related with metabolism, as this hormone is capable of reducing the levels of glucose in the blood - either directly by inducing insulin release from pancreatic β-cells or indirectly by stimulating the proliferation and inhibiting the apoptosis of these cells -, of activating the degradation of fat by brown adipose tissue cells, of slowing the rate of digestion by slowing gut motility and the emptying of the stomach and of activating the neural signalling pathways related with satiety [175]. By using integrin-β7-deficient mice that lack natural IELs, He et al. [174] verified that, when fed a normal diet, these mice were metabolically hyperactive and exhibit improved glucose tolerance and, when fed a high-fat and high-sugar diet, they were protected from cardiovascular disease. Moreover, by measuring circulating GLP-1 levels and L-cell numbers in the integrin-β7-deficient mice, they verified that the levels of the hormone and the numbers of cells were elevated compared with those in wild-type mice, which suggested that the differences between the mice and the protection phenotype observed had a hormonal basis. Authors then proposed that IELs can act as a 'GLP-1 sink' and control its bio-availability either by regulating the production of new L-cells, either by directly capturing the released GLP-1 through their GLP-1 receptors. The new metabolic role for intestinal IELs can be crucial for the control of present-day diseases related with overabundance of diets rich in fat and sugar.

Altogether, these findings suggest that, more than just typical immune cells that avoid the entry of pathogens, the intestinal IELs also play a part in establishing intestine's integrity and in controlling whole-body metabolism and homeostasis.

1.6 Enteric and Metabolic Diseases and the Need for Therapeutic Solutions

Changes in human ecology such as diet, physical activity, stress and microbial exposure, have dramatically shifted the spectrum of human diseases over the past years. Although modernization has increased lifespan, many enteric and metabolic diseases are still the cause of many deaths and contribute to a loss of quality of life many times translated

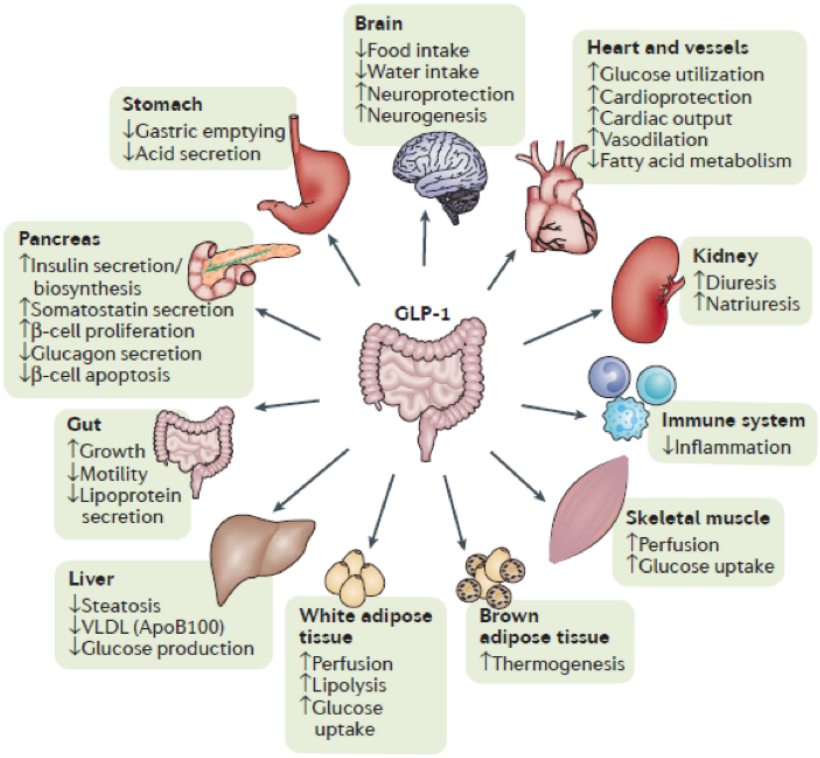

Figure 1.7: **Putative Actions of Glucagon-like Peptide 1 (GLP-1).** Schematic representation of the principal physiological roles driven by the intestine-derived pleiotropic hormone GLP-1 in different organs and tissues. (image from [177])

to further deeper problems. Interestingly, the intestinal IELs seem to be involved in the pathophysiology of some of these diseases.

Among the inflammatory diseases of the intestine, coeliac disease and inflammatory bowel diseases are the most relevant. The coeliac disease is a T cell mediated immune disorder restricted to the duodenum and upper small intestine induced by an immune response against dietary gluten, being developed in genetically susceptible individuals that express HLA-DQ2 and/or HLA-DQ8 MHC class II molecules [178]. These molecules bind gluten peptides with strong affinity and, under inflammatory conditions, are greatly induced on enterocytes, which then become targets for gluten-specific $CD4^+$ effector T cells, ultimately leading to the destruction of the intestinal epithelium and villous atrophy [18, 178]. Although their exact mechanisms of action remain elusive, induced IELs have been shown to exacerbate this disease, as TCR-activated $CD8\alpha\beta^+$ induced IELs caused severe villous atrophy by targeting epithelial cells expressing stress-induced MHC class I polypeptide-related sequence antigens [179, 180] and a gluten-dependent infiltration of activated cytotoxic IELs is the hallmark of this disease [181]. Moreover, it is likely that these induced IELs, through the secretion of pro-inflammatory cytokines, can promote the development of small intestinal inflammation in patients with coeliac disease, therefore contributing to its development [178].

On the other hand, Inflammatory Bowel Diseases (IBD) reflect aberrant T-cell mediated inflammatory responses to commensal bacteria and comprise two major disorders: Ulcerative Colitis (UC) and Chron's Disease. UC is characterized by erythema, superficial ulceration and pseudopolyps in response to an inflammation of the mucosa and sub-mucosa tissues of the large intestine [182]. Chron's Disease can affect all regions of the gastrointestinal tract, although is more common in the terminal ileum and colon, and is characterized by a transmural inflammation, strictures and granuloma formation [182]. In addition to the increasing incidence (with a prevalence of up to 1 in 198 persons for UC and 1 in 310 persons for Chron's Disease) [183] and morbidity associated with IBD, they have a markedly negative impact on people's quality of life. Both natural and induced IELs have been implicated in IBD. Direct evidence showing that TCR$\gamma\delta^+$ IELs promote immunopathology in various mouse models of IBD, for example, suggest a pro-inflammatory role for the natural IELs in these diseases [157, 184, 185]. In addition, a direct correlation between the numbers of TCR$\gamma\delta^+$ cells in the intestinal mucosa and disease severity was observed in patients with IBD [186, 187]. Induced CD8$\alpha\beta^+$ cells have also been implicated in the development of IBD [188].

Besides IELs, there is also evidence that the sympathetic nervous system can modulate intestinal inflammation and control the development and progression of these enteric inflammatory diseases. Stimulation of the vagus nerve, for example, has been shown to reduce inflammation in animal models of and post-operative ileus [189, 190] and chemical ablation of the sympathetic nerves by 6-hydroxydopamine (6-OHDA) treatment in a mouse model of colitis was shown to attenuate inflammation [191]. Moreover, catecholamines can also influence the composition of the microbiome and its interactions with epithelial cells [192], which is also an important determinant for the immune activation within the gut. On the other hand, patients with IBD in clinical remission exhibited increased and decreased indices of sympathetic and parasympathetic activities, respectively [193], and functional changes in the ENS have been described in patients and animal models of IBD [135, 194], suggesting effects of the gastrointestinal tract inflammation on sympathetic and enteric nerve dysfunction.

Despite the need to search for therapeutic approaches for the enteric inflammatory diseases, there is also a need to search for solutions to the so-called 'modern human diseases', such as obesity [195]. In 2015, 107.7 million children and 603.7 million adults worldwide were estimated to be obese [196]. These numbers are significantly higher in comparison to previous years [195]. Diets high in calories, especially from refined sugars and saturated fatty-acids, and sedentary lifestyles have been contributing to the epidemic of obesity; and this rapid rise in obesity is accompanied by a similar increase in cardiovascular diseases and insulin resistance or type 2 diabetes [197]. Because the diet clearly shapes these diseases and the small intestine is one of the primary organs responsible for diet-derived nutrient absorption, it makes sense to see it as a logical checkpoint for solving these disorders. Indeed, studies showing that gut dysfunction and metabolic endotoxemia are associated with obesity or insulin resistance in mice and

humans [198–200], or studies showing the role of the microbiome in shaping an obesity-like phenotype [201] and inducing chronic inflammation [198, 200], a common feature of obesity and type 2 diabetes [202], make one to understand the intestine's importance in these diseases.

1.7 *Mus musculus* as an Experimental Model

The mammal model *Mus musculus* has been used in research for the last hundred years. Since then, a lot has been discovered in mice and extrapolated to other model organisms: from formulating new therapeutic drugs to testing dietary supplements, mice have been playing a critical role in developing new medical wonders. Besides being small, easily housed and maintained and with a relatively quick reproductive cycle, *M. musculus* share a high degree of homology with humans [203, 204]. Indeed, their genetic, biological and physiological characteristics closely resemble those of humans and many symptoms of human conditions can be replicated in mice, which make them extremely useful to create different disease's models and to unravel biological processes and pathways that can lead to new therapeutic solutions. Although showing some differences regarding IELs subsets and distribution (depicted in Chapter 1.5), mice share the same general anatomical, physiological and cellular features as humans when it comes to the small intestine and its immunity.

Chapter 2

Hypothesis and Aims

IELs constitute one of the first lines of immune defence in the intestinal mucosa by constantly patrolling the gut environment to avoid potential pathogens' invasion. In addition, IELs are strategically located mainly in the upper part of the intestine side-by-side with absorptive enterocytes [136] and it has been recently shown that they also display metabolic functions [174]. In the case of natural TCR$\gamma\delta^+$ IELs, they have the machinery to integrate specific neuronal signals, as they differentially express genes for specific neurotransmitters and neuropeptides receptors [162].

Because IELs display both inflammatory and metabolic functions while simultaneously expressing the genes to perceive specific neuronal signals, our hypothesis is that neuronal cues can modulate the inflammatory and/or metabolic profile of IELs as a mechanism to achieve local or systemic homeostatic responses.

With this, our objective is to use neuron-derived cues to induce a tissue protective or a metabolic-related response in the main IEL subpopulations to further establish the basis for future therapeutic approaches against inflammatory enteric- and/or metabolic diseases.

Chapter 3

Materials and Methods

3.1 Mice Husbandry and Strains

Wild-type C57BL/6 (B6) mice were purchased from Charles River, Nr3c1$^{flox/flox}$ mice were purchased from Jackson Laboratory and IL7Rα-Cre mice were obtained in-house. Nr3c1$^{flox/flox}$ and IL7Rα-Cre lines were interbred in-house to obtain the strain IL7Rα-Cre.Nr3c1$^{flox/flox}$. All mice used were male between 7 and 9 weeks-old unless stated otherwise. All mice were fed *ad libitum* with free access to food and water. All mice were maintained in a temperature-controlled room (22°C) on a 12-hour light cycle at the Champalimaud Centre for the Unknown facility under specific pathogen free conditions. All animal procedures and experiments were performed accordingly to national and institutional ethical guidelines.

3.2 Buffers and Media

RPMI complete was prepared by supplementing RPMI 1640 (Corning) with 10% (v/v) Fetal Bovine Serum (Corning) heat inactivated for 30 minutes at 56°C, 1% (v/v) Penicillin/Streptomycin (Corning), 10mM HEPES Buffer (Corning), 1 mM Sodium Pyruvate (Corning) and 50μM β-mercaptoethanol (GIBCO).

3.3 Isolation of Intraepithelial Lymphocytes (IELs) from Small Intestines

Mice were euthanized using CO_2 until the absence of any paw reflex, pinned down with the ventral part facing up and sprayed with Ethanol 70%. An incision on the ventral

part was done using sharp-straight scissors. The distal part of the oesophagus was cut to facilitate the stomach's exposure and subsequent intestine removal. Pointed forceps were used to separate intestines from mesentery and remove excess of fat while it was simultaneously collected. The small intestines were collected to a 12-well plate containing ice-cold complete RPMI media. For the analysis of intestinal segments, the upper part (5 cm) of the small intestine was taken as duodenum, the most distal part (5 cm) as ileum and the middle part (10 cm) as jejunum. Each segment was collected to a 12-well plate containing ice-cold complete RPMI media. The intestines or intestinal segments were placed on the top of a Petri dish and carefully flushed of faecal content with cooled PBS 1x using a 20 mL syringe. Using sharp-straight scissors, Peyer's patches were removed, and the intestines or intestine segments were opened longitudinally. Each intestine or intestine segment was cut in small pieces of 0.5 cm length and placed inside a 50 mL falcon tube containing 20 mL or 10 mL of complete RPMI with 1mM DL-Dithiothreitol (DTT, Sigma-Aldrich). The falcon tubes containing the intestinal pieces were vortexed for 30 seconds (s) at maximum speed and shaken at 180 rpm for 20 minutes (min) at 37°C. Subsequently, the tubes were vortexed (30s, maximum speed) and passed through a 100 μM cell strainer. These steps were repeated one more time: the intestinal segments were added to a complete RPMI media with 1mM DTT, shaken, vortexed and filtered as described above. Intraepithelial cells were pelleted by centrifugation at 300xg for 5 minutes at 4°C. The cell pellet was layered over a discontinuous 40/80% Percoll (GE Healthcare) gradient and centrifuged at 2400 rpm for 25 min at Room Temperature (RT). Cells from the 40/80% interface were carefully collected, washed and resuspended in complete RPMI media and pelleted by centrifugation (300xg, 5 min, 4°C). The red blood cells were lysed using BD PharmLyse Lysing Buffer, diluted in MilliQ water according to the manufacturer's instructions, for 3 to 5 minutes at RT and protected from light. The cells were washed with 3 mL of complete RPMI, transferred through a 70 μM strainer to a new 50 mL falcon tube and pelleted by centrifugation at 300xg for 5 min at 4°C. The cells were counted and transferred to a 96-well plate, incubated for 10 minutes at 4°C with 30 – 50 μL of Anti-Mouse CD16/CD32 (eBioscience) and washed with FACS Buffer (PBS 1x, 2% FBS) prior to the antibody staining.

3.4 Cell Sorting

Intestinal IELs' cell suspensions were stained for 25 min at 4°C or RT (when anti-Ccr9 and anti-$\alpha 4\beta 7$ where present) and protected from light. Subsequently, cells were washed twice with Fluorescent Activated Cell Sorting (FACS) Buffer and pelleted by centrifugation at 700xg for 2 min at 4°C. Cells were then resuspended in FACS Buffer. Cells were sorted using BD FACSAria Fusion Cell Sorter equipped with an 85 μM nozzle. Cells were sorted as singlets, live [(negative for a viability dye Live/Dead Aqua (LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit, Life Technologies)] and CD45$^+$. The three IEL subpopulations were sorted as follows: CD8$\alpha\alpha^+$ CD4$^-$ TCR$\alpha\beta^+$ for CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ natural IELs;

CD8$\alpha\alpha^+$ CD4$^-$ TCR$\gamma\delta^+$ for CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ natural IELs; and CD8$\alpha\beta^+$ for CD8$\alpha\beta^+$ induced IELs. The gating strategy used for sorting is depicted in Annex I, Figure I. Cells for *ex vivo* stimulations were sorted to complete RPMI medium (5mL FACS collection tubes). Cells for RNA extraction were sorted directly to Buffer RLT (QIAGEN) or Buffer RLT Plus (QIAGEN) in 1.5 or 2mL Eppendorf tubes, respectively, and stored at -80°C. Biological replicates were always sorted into separate tubes for all populations. Cell sorting results' analysis was performed using FlowJo Software (vX.0.7). All information for primary antibodies used for cell suspensions' immunostaining is depicted in Table 3.1.

Table 3.1: List of antibodies used for immunostaining of cell suspensions.

Antibodies	Conjugated	Recognize	Host	Concentration	Supplier	Cat. Number
Primary	Alexa Fluor 647	CD45.1	Mouse	1:100	Biolegend	110720
	APC-Cy7	CD45.2	Mouse	1:100	Biolegend	109824
	BV 421	CD4	Rat	1:100	Biolegend	100437
	BV 711	CD8α	Rat	1:100	Biolegend	100748
	PE	CD8β	Rat	1:200	Biolegend	126607
	PE-Cy5	TCRβ	Hamster	1:200	Biolegend	109210
	PE-Cy7	TCR$\gamma\delta$	Hamster	1:200	eBioscience	25-5711-82
	APC	LPAM-1 ($\alpha4\beta7$)	Rat	1:100	Biolegend	120607
	FITC	CD199 (CCR9)	Mouse	1:100	Biolegend	128706

3.5 *Ex vivo* Stimulations Assay

For *ex vivo* experiments, purified IELs' subpopulations were pelleted by centrifugation at 300*x*g for 5 min at 4°C and transferred to 96-well plates under sterile conditions (50 000 cells/well). Cells were then incubated in complete RPMI or RPMI containing a desire neurotransmitter or its agonist, neuropeptide or glucocorticoid receptor agonist for 1 hour at 37°C + 5% CO_2. For stimulations, the following concentrations were used: Norepinephrine (100 μM, L-()-Norepinephrine (+)-bitartrate salt monohydrate, Sigma-Aldrich, A9512-250MG), Denopamine (25 μM, R(-)-DENOPAMINE, Sigma-Aldrich, D7815-5MG), Clenbuterol (10 μM, Clenbuterol hydrochloride, Sigma-Aldrich, C5423-250MG), Acetylcholine (5 mM, Acetylcholine chloride, Sigma-Aldrich, A6625-25G), L-glutamic Acid (25 μM, L-Glutamic acid monosodium salt hydrate, Sigma-Aldrich, G1626-100G), GABA (10 μM, Gamma-Aminobutyric Acid, Sigma-Aldrich, A2129-25G), VIP (5 μM, Vasoactive In-testinal Peptide, Phoenix, 064-30), Dexamethasone (0.1 μM, Dexamethasone powder, Sigma-Aldrich, D4902). Drug stock solutions were prepared in MILIQ water (for Norepinephrine, 1000 mM; Denopamine, 100mM; Acetylcholine, 500mM; L-glutamic acid, 50mM; GABA, 10mM; VIP, 1mM; and Dexamethasone, 1.5M) or DMSO (Dimethyl sulfoxide, Fisher Scientific) for Clenbuterol, 50mM. Intermediate dilutions of drugs were prepared in complete RPMI.

After the stimulation, IELs were washed with complete RPMI, lysed using Buffer RLT Plus (QIAGEN) and stored in 1.5 mL eppendorfs at -80°C for further RNA extraction. For all *ex vivo* stimulations the entire small intestine was used.

3.6 Intestinal Epithelial Organoid Cultures

Mouse intestinal organoids were done using IntestiCult™ Organoid Growth Medium (OGM) Mouse kit (STEMCELL, 06005), according to manufacturer's protocol. In brief, small intestine was isolated, washed with cold PBS 1x, opened longitudinally and cut into small pieces. Intestinal pieces were washed with a pre-wetted 25 mL serological pipette until the supernatant was clear and incubated with 15 mL Gentle Cell Dissociation Reagent (Corning, 07174) on a rocking platform at 50 rpm for 15 min at RT. Intestinal pieces were settled by gravity, supernatant was removed and 10 mL of cold PBS with 0.1% Bovine Serum Albumin (BSA) was added. Intestinal pieces were settled by gravity and supernatant was passed through a 70 μm filter into a new 50 mL falcon tube labelled as 'Fraction 1'. These resuspension and filtration steps were repeated 3 times to generate Fractions 2-4. All fractions were pelleted by centrifugation at 290xg for 5 min at 4°C and each pellet was resuspended in 10 mL of cold PBS + 0.1% BSA and further transferred to fresh 15 mL falcon tubes labelled with the corresponding fraction number. All fractions were pelleted by centrifugation at 200xg for 3 min at 4°C and each pellet was resuspended in 10 mL of cold DMEM/F12 with 15mM HEPES (STEMCELL) + 1% Penicillin/Streptomycin (Corning). An inverted microscope was used to assess the quality of the suspensions and the fraction with the greatest enrichment for desirable intestinal crypts was chosen. For the selected fraction, the number of crypts in a 10 μL aliquot was counted with a microscope and the number of crypts per mL of the fraction further calculated (e.g. 15 crypts in 10μL x 100 = approximately 1500 crypts per mL). The selected fraction was aliquoted in 15 mL falcon tubes in volumes containing approximately 3000 crypts which were further pelleted by centrifugation at 200xg for 3 min at 4°C. Each pellet was resuspended in 150 μL of IntestiCult™ OGM followed by 150 μL of undiluted Matrigel® Matrix (Corning). The 3000-crypt suspension was plated (50 μL) as domes into separated wells of a pre-warmed 24-well plate and incubated for 10 minutes at 37°C for Matrigel Matrix solidification. 500 μL of IntestiCult™ OGM was added to each well and the culture medium was exchanged every 3-4 days by removing the existing medium and replacing it with fresh IntestiCult™ OGM. The plates were incubated at 37°C + 5% CO_2. All techniques for intestinal organoids were performed under sterile conditions.

3.7 Co-Culture of IELs with Intestinal Epithelial Organoids

Intestinal organoids were prepared following the protocol described above. On day 7 of the organoid culture, natural IELs were isolated as described previously (CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ and CD8$\alpha\alpha^+$TCR$\alpha\beta^+$) and stimulated with VIP (5 μM) for 1 hour. During the stimulation time, 500μL of Cell Recovery Solution (Corning) was added to the organoids cultures which were further placed at 4°C for 30 min. After stimulation, IELs were washed with complete RPMI and pelleted by centrifugation at 200xg for 2 min at 4°C. The organoids suspensions were transferred to 15mL falcon tubes and IELs were transferred to

the corresponding 15 mL falcon tubes containing the organoids. The tubes were washed with 10mL of DMEM/F12 with 15mM HEPES (STEMCELL) + 1% Penicillin/Streptomycin (Corning) and pelleted by centrifugation at 200xg for 3 min at RT. 100μL of complete IntestiCult™ OGM followed by 100μL of Matrigel Matrix (previously thawed) were added to each pellet. After mixing the content of each tube, 50μL of each suspension was placed in the centre of a well as described previously (50 000 IELs were co-cultured with around 100 organoids). After incubation at 37°C + CO_2 for 10 minutes, 500μL of complete IntestiCult™ OGM was added to each well. The stimulated IELs and the intestinal organoids were co-cultured for 16 hours at 37°C + CO_2. At the end of the co-culture, the IntestiCult™ OGM was removed and 500μL of Gentle Cell Dissociation Reagent (Corning) was added on the top of the dome of each well. The organoids were broken by pipetting up-and-down and all the content of each well was transferred to 1.5mL eppendorf tubes. The tubes were incubated on a rocking platform at 120 rpm for 10 min at RT and further pelleted by centrifugation at 290xg for 5 min at 4°C. The pellets were immediately resuspended in 350μL of RLT Plus Buffer (QIAGEN). The tubes were vortexed and stored at -80°C for further RNA extraction. All techniques for intestinal organoids, IELs stimulations and co-culture were performed under sterile conditions.

3.8 RNA Extraction

For cells from the small intestine, intestinal segments (duodenum, jejunum) and for intestinal organoids, total RNA was extracted using RNeasy Plus Mini Kit (QIAGEN), according to manufacturer's protocol. In brief, cells were disrupted and homogenised in Buffer RLT Plus by vortexing. Homogenized lysate was then transfer to gDNA columns for all-DNA removal. To guarantee RNA's binding proper conditions to the RNeasy spin columns, precipitation of the genomic material was done using ethanol 70%. Washes of the contaminants were done using specific Buffers provided by the kit. RNA was eluted in 30μL RNase-free water. Due to low number of cells from the distal intestinal segment (ileum), total RNA was extracted using RNeasy Micro Kit (QIAGEN), according to manufacturer's protocol. In brief, cells were homogenised in Buffer RLT and ethanol 70% was added to this homogenized lysate to guarantee RNA's binding proper conditions to the RNeasy MinElute spin columns. DNase digestion for all-DNA removal was done on-column for 15 minutes using DNase I (QIAGEN). Washes of the contaminants were done using specific buffers provided by the kit. RNA was eluted with 15μL RNase-free water.

RNA concentration and quality were determined afterwards using a Nanodrop™ 2000 Spectrophotometer. For determining RNA quality, the ratio of A260/A280 measured by the Nanodrop was used: because an absorbance at 260 nm provides total nucleic acid content while an absorbance at 280 nm determines sample purity [205], a ratio of 2 was considered pure; therefore, RNA samples which A260/A280 ratio value was between 1.95 and 2.05 were considered pure and used for further complementary DNA synthesis.

3.9 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

All RNA samples were used to synthetize Complementary DNA (cDNA) by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) using High Capacity RNA-to-cDNA kit (Applied Biosystems), according to manufacturer's protocol. In brief, RT-PCR was performed in a SimpliAmp 96-well Thermocycler (ThermoFisher Scientific) by an incubation step at 37°C for 60 minutes followed by a period of 5 minutes at 95°C to stop the reaction; The final volume for each reaction was 20μL, consisting of 10μL of 2x RT Buffer Mix, 1μL of 20x Enzyme Mix and 9μL of total RNA.

To test for gDNA contamination in the RNA samples, all the samples used for cDNA synthesis were simultaneously used in a parallel reverse transcriptase negative control reaction in which the reverse transcriptase enzyme was replaced by Nuclease-free water. To test for RNA contamination in the RNase-free water used for the extraction, a single internal negative control reaction was made in which the RNA sample was replaced by RNase-free water, while maintaining the other reaction conditions. All the synthetized cDNA that was not immediately used after the RT-PCR reaction, was stored at -20°C.

3.10 Pre-Amplification PCR

The cDNA samples were pre-amplified using TaqMan PreAmp Master Mix (Applied Biosystems), according to manufacturer's protocol. In brief, the pre-amplification reaction was performed in a SimpliAmp 96-well Thermocycler (ThermoFisher Scientific) with one initial step at 95°C for 10 minutes for enzyme activation and 14 cycles of a denaturation step at 95°C for 15 seconds followed by an annealing step at 60°C for 4 minutes. The final volume for each reaction was 50μL, consisting of 25μL of 2x TaqMan PreAmp Master Mix, 0.125μL of each 20x specific TaqMan Probe (Applied Biosystems) + Tris-EDTA Buffer solution 1x (hereafter, TE Buffer) (Corning) until reaching the volume of 12.5μL, and 12.5μL of cDNA sample. All the pre-amplified cDNA that was not immediately used after the pre-amplification Polymerase Chain Reaction (PCR) reaction was stored at -20°C. The pre-amplification PCR was performed for all cDNA samples except those derived from dexamethasone-stimulated IELs and intestinal organoids.

3.11 Quantitative PCR (qPCR)

Both pre-amplified and non-pre-amplified cDNA were diluted 1:10 in TE Buffer 1x (Corning). Gene expression levels were quantified by Real-Time Quantitative PCR (qPCR) using TaqMan Gene Expression Master Mix and specific TaqMan Probes from Applied Biosystems, according to manufacturer's protocol. In brief, the qPCR reaction was performed in a 384-well QuantStudio™ 5 Real-Time PCR System (Applied Biosystems) with

one initial step at 50°C for 2 min for uracil-N-glycosylase incubation to prevent false-positive amplification, a second step at 95°C for 10 min for polymerase activation and 40 cycles of a denaturation step at 95°C for 15 s followed by an annealing step at 60°C for 60 s. The final volume for each reaction was 10μL, consisting of 5μL of 2x TaqMan Gene Expression Master Mix, 0.5μL of 20x specific TaqMan Probe, 2μL of Nuclease-free water and 2.5μL of diluted pre-amplified or non-pre-amplified cDNA sample. All TaqMan Probes used for qPCR are listed in Table 3.2.

Table 3.2: List of probes used for Real-time PCR (all from Applied Biosystems)

Gene	Reference
Hprt	Mm01545399_m1
EpCam	Mm00493214_m1
Adra2a	Mm00845383_s1
Adrb1	Mm00431701_s1
Adrb2	Mm02524224_s1
Apoe	Mm01307193_g1
Ccr9	Mm02528165_s1
Fabp2	Mm00433188_m1
Gabbr1	Mm00444578_m1
Gcg	Mm00801714_m1
Glp1r	Mm00445292_m1
Grina	Mm00458212_g1
Gzmb	Mm00442837_m1

Gene	Reference
Ifng	Mm01168134_m1
IL10	Mm00439614_m1
IL15	Mm00434210_m1
IL18	Mm00434225_m1
Itga4	Mm01277951_m1
Itgb7	Mm01296188_m1
Ltb	Mm00434774_g1
Nr3c1	Mm00433832_m1
Slc2a1	Mm00441480_m1
Tnfa	Mm00443260_g1
Vipr1	Mm00449214_m1
Vipr2	Mm01238618_g1

An internal negative control for all qPCR reactions was done in a similar manner as described for the RT-PCR. For all qPCRs, three technical replicates were done for each biological sample. Cycle Threshold (hereafter, Ct) value of each biological sample was calculated as mean value of the Ct values of its technical replicates. The relative expression of each gene was normalized either to *Hprt* (for IELs) or *EpCam* (for ECs) reference genes' expression. Expression values were calculated according to the comparative CT method ($2^{-\Delta Ct}$) in which $\Delta\text{CT}_{gene\ of\ interest} = \text{CT}_{gene\ of\ interest} - \text{CT}_{reference\ gene}$ was employed. When fold change comparison between samples was required, the comparative ΔCT method ($2^{-\Delta\Delta Ct}$) was applied [206].

3.12 High-Fat Diet (HFD) Feeding

When B6 mice reached 8 weeks of age, Normal Diet (ND) was replaced with High Fat Diet (HFD) (Ssniff, Spezialdi.ten, Soest, Germany, E15742-347). Analyses in Figures 4.7 and 4.8 were performed when mice achieved a 40% increase in body weight and glucose intolerance (after around 16 weeks of an HFD feeding). Control group of age-matched males was maintained on ND for the same amount of time.

3.13 Glucose Tolerance Test (GTT)

Prior to the Glucose Tolerance Test (GTT), mice were fasted for 16-hours. Subsequently, mice were weighted and tail blood was assayed for glucose levels with a Glucometer (ACCU-CHEK Aviva). Glucose (D-(+)-Glucose, Sigma-Aldrich) was dissolved in MiliQ water and injected intraperitoneally (i.p.) (2g/kg body weight). Glucose levels were measured 15, 30, 45, 60, 90 and 120 min after glucose i.p. injection. For each time-point, 2 to 3 glucose measurements were done, and an average was further calculated for each mouse.

3.14 Statistical Analysis

Statistical analysis was performed with GraphPad Prism software (San Diego, CA) using non-parametric Mann-Whitney-test (two-tailed) when two groups were being compared and Kruskal-Wallis Test or One-way ANOVA when several groups were being compared. For multiple comparisons, the mean rank of each column was compared with the mean rank of every other column or with the mean rank of a control column. Normality was tested prior with the D'Agostino-Pearson Test for all samples' groups. A p-value <0.05 was considered statistically significant. Results were scored as * when $p<0.05$, ** when $p<0.01$, *** when $p<0.001$, and **** when $p<0.0001$. Data are represented as mean ± SEM.

Chapter 4

Results

4.1 The Three Major IELs' Subpopulations Differentially Express Neuronal- and Metabolism-related Receptors' Genes

Our preliminary analysis based on the Illumina NextSeq 500 (*Mus musculus*) dataset (available online with the GEO accession number GSE97184) [162] indicated that the CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ IEL subpopulation have the machinery to integrate specific neuronal signals by expressing specific neurotransmitters- and neuropeptides-receptors' genes. Moreover, the analysis of the same dataset also indicated that these IELs are metabolically prepared for action by expressing genes associated with metabolism (such as *GLP1r*, *Apoe*, *Slc2a1*). However, no similar information was available for the other subpopulations and no literature addressing the neuronal receptors in IELs is available. To investigate whether the three major subpopulations of IELs could perceive neuronal-derived molecules while simultaneously playing a role in metabolic processes, we examined the basal expression levels of neuronal- and metabolism-related receptors' genes for each IEL subpopulation. To this end, we purified CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ and CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ natural IELs and CD8$\alpha\beta^+$ induced IELs from the small intestine of B6 mice by FACS (as depicted in Annex I, Figure I.1) and further quantified the expression of neuronal- and metabolism-related genes by real-time qPCR (Figure 4.1A).

Based on the neurotransmitters' genes with a higher expression found in the above-mentioned dataset for CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ IELs, we performed qPCR for norepinephrine-, Acetylcholine-, L-glutamic acid- and GABA-receptors' genes. Regarding these neurotransmitters receptors' genes (Figure 4.1B), no significant differences were found for the adrenergic receptor gene *Adra2a* among the three IELs' subpopulations. The expression

of *Adrb1* was significantly higher in the natural CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ subpopulation while *Adrb2* was significantly higher in the induced CD8$\alpha\beta^+$ subpopulation. The cholinergic receptor gene *Chrnb1* had a higher basal level of expression in the CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ subpopulation when compared with the induced CD8$\alpha\beta^+$ subpopulation. For the GABAergic receptor gene *Gabbr1*, the expression showed to be higher in the induced CD8$\alpha\beta^+$ subpopulation when compared with CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ and, for the glutamatergic receptor gene *Grina*, all three IELs' subpopulations seemed to have similar expression levels.

It has been shown that VIP is an important neuropeptide in regulating the expression of anti- and pro-inflammatory cytokines in immune cells [78, 79]. Moreover, this neuropeptide has a crucial function in the digestion process as it promotes enteric secretion of water and electrolytes and enteric smooth muscle relaxation, increases gut motility and has been shown to stimulate the pepsinogen-mediated break down of proteins in the stomach [73, 207]. Due to its dual regulatory role in inflammation and metabolism, we also tested the expression of the receptors for this peptide in IELs: *Vipr1* and *Vipr2*. For these neuropeptides receptors' genes, natural and induced IELs showed opposite expression levels. *Vipr1* had significantly lower and *Vipr2* significantly higher basal levels of expression in both CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ natural IELs (Figure 4.1B).

Because a relationship between the immune and endocrine systems has long been appreciated [83, 87, 88] and it is known that endocrine-derived hormones have multiple effects in metabolism [75, 177, 208], we also performed qPCR analysis for the glucocorticoid receptor *Nr3c1* in IELs. The expression of the glucocorticoid receptor gene *Nr3c1* was significantly enhanced in both natural IELs (Figure 4.1C).

GLP-1 is an example of a hormone with major metabolic impacts that exerts glucose control and induces the break-down of fat by brown-fat cells [177]. Because it has been recently shown that IELs can regulate the bio-availability of this hormone through their GLP-1 receptor (GLP-1r) [174], we also quantified the expression of this receptor gene in all IELs' subpopulations. *Glp1r* showed to be statistically upregulated in the CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ natural IELs when compared with the CD8$\alpha\beta^+$ induced subpopulation, and a tendency for a higher basal expression level in the natural CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ IELs, when comparing with the CD8$\alpha\beta^+$ subpopulation, was also noticed (Figure 4.1C).

Thus, these results show that the neurotransmitters-, neuropeptides- and metabolism-related receptors' genes tested are differentially expressed among the three major IELs' subpopulations. Therefore, both natural and induced IELs naturally express the machinery to differentially perceive neuronal signals while likely displaying different metabolic roles.

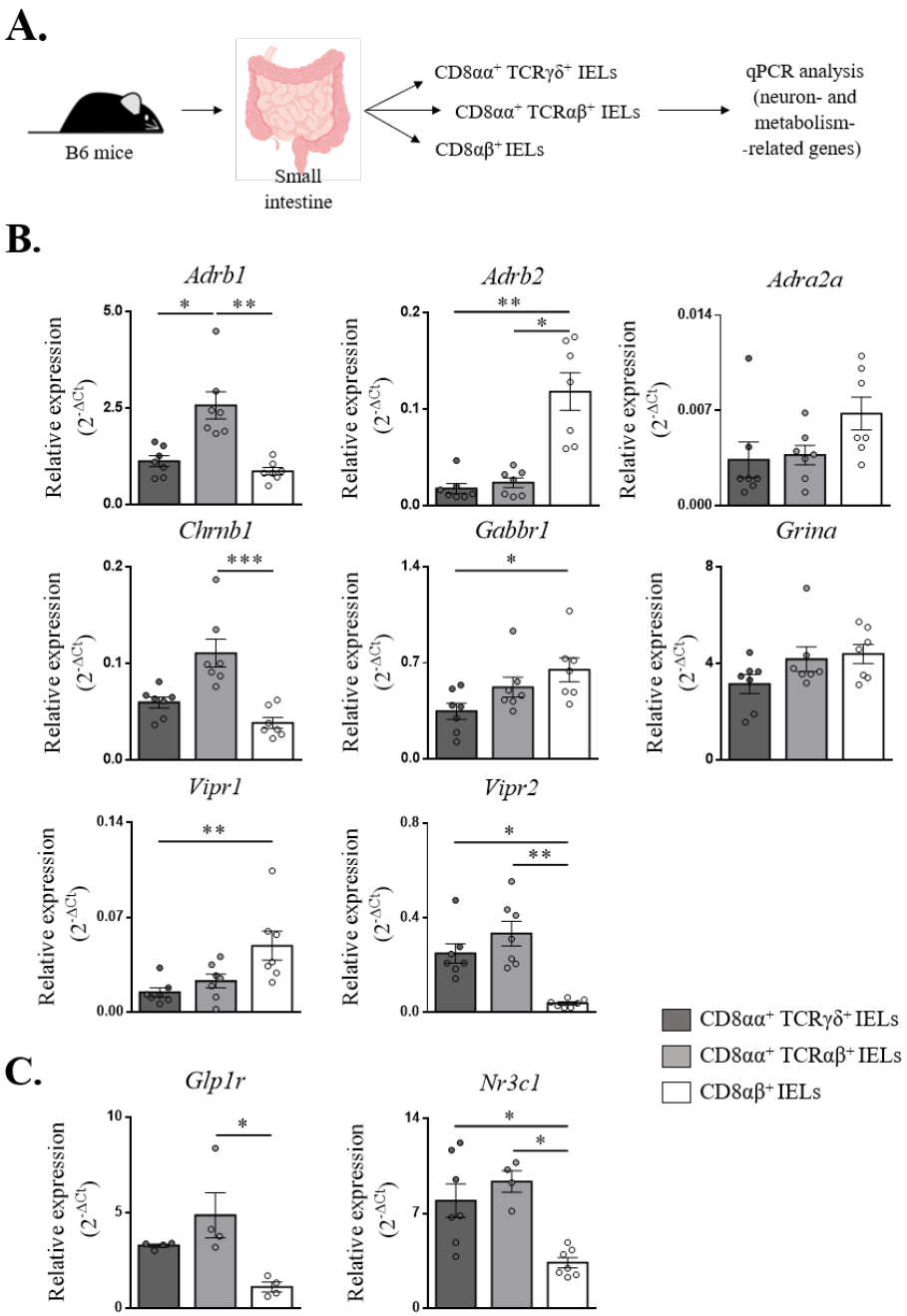

Figure 4.1: **The three major subpopulations of IELs differentially express neurotransmitters-, neuropeptides- and metabolism-related-receptors' genes. (A)** Representative scheme of the experimental strategy. CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$, CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ and CD8$\alpha\beta^+$ main subpopulations of intestinal IELs were purified by sorting from 7 to 9 weeks old B6 male mice and qPCR was performed for neurotransmitters-, neuropeptide- **(B)** and metabolism-related **(C)** genes. Results were normalized to *Hprt* expression. n = 4 or n = 7 per experimental group. Data represented as mean ± SEM. Kruskal-Wallis Test was applied. *P<0.05, **P<0.01 and ***P<0.001.

4.2 *Ex vivo* Stimulation with Neuron-Derived Cues Induces Different Metabolic and Inflammatory Responses in IELs' Subpopulations

To explore the responsiveness of IELs to neuronal cues, we stimulated each IEL subpopulation with the neurotransmitter or neuropeptide recognized by the abovementioned receptors. To this end, we purified CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ and TCR$\alpha\beta^+$ natural IELs and CD8$\alpha\beta^+$ induced IELs from the small intestine of B6 mice as described above and performed *ex-vivo* stimulation assays. Following cell sorting, cells were stimulated for 1 hour with the following neurotransmitters or their agonists: Norepinephrine (100 μM); Clenbuterol (10 μM), a specific agonist of beta-2 adrenergic receptor [209]; Denopamine (25 μM), a specific agonist of beta-1 adrenergic receptor [210]; Acetylcholine (5 mM); L-glutamic acid (25 μM); and GABA (10 μM). For the neuropeptide's receptors, *ex-vivo* stimulation was made with VIP (5 μM). After stimulation, the expression of genes involved in metabolism (*Glp1r* for glucose and lipid metabolism, *Apoe* for lipid metabolism [211], *Slc2a1* for glucose metabolism [212] and *Nr3c1* for metabolism involving corticosteroids [83]), genes for intestine-homing markers (*Itga4*, *Itgb7* and *Ccr9*) [32, 152] and genes for cytolytic enzymes and cytokines associated with Type I and Type III inflammatory responses [1, 136] (*Ltb*, *Gzmb*, *Ifng*, *TNFa* and *IL10*) were quantified by real-time qPCR.

Glp1r showed high responsiveness to both L-glutamic acid and VIP stimulations in the CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ IEL subpopulation, as its expression significantly increased ($P < 0.001$) upon both neuronal stimuli (Figure 4.2B). Although no statistical difference was found for this gene for the CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ subpopulation, the *Glp1r* gene also showed to be upregulated upon stimulations with both L-glutamic acid and VIP. The *Apoe* gene was downregulated in the CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ IEL subpopulation upon stimulations with L-glutamic acid or GABA but, in the CD8$\alpha\beta^+$ subpopulation, it was upregulated upon stimulation with VIP. Regarding the glucose transporter Slc2a1 (Figure 4.2C), the only significant difference occurred in the induced CD8$\alpha\beta^+$ IEL subpopulation upon stimulation with L-glutamic acid, which led to an upregulation of this gene. The expression of the glucocorticoid receptor gene *Nr3c1* (Figure 4.2D) was found to be upregulated in the CD8$\alpha\beta^+$ IEL subpopulation upon stimulations with Norepinephrine, Denopamine, L-glutamic acid, GABA or VIP. In addition, the expression of *Nr3c1* gene in both natural CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ and TCR$\alpha\beta^+$ IELs' subpopulations was significantly reduced upon stimulation with GABA and Acetylcholine, respectively.

For the intestine-homing markers-coding genes (Figure 4.3), the stimulation with GABA led to the downregulation of both *Itga4* and *Itgb7* in the CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ IEL subpopulation. *Itgb7* expression was significantly higher in the CD8$\alpha\beta^+$ induced IEL subpopulation upon stimulations with L-glutamic acid, GABA and VIP. Regarding the Ccr9 gene, its expression only changed in the natural IELs and it was significantly lower

upon stimulations with L-glutamic acid or VIP in the CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ subpopulation, and lower in the CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ subpopulation upon all stimulations but VIP.

For the cytolytic enzymes- and cytokines-coding genes (Figure 4.4), *Ltb* showed to be downregulated in both natural IELs upon all stimulations but L-glutamic acid and VIP (for CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ IELs) and Clenbuterol, GABA and VIP (for CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ IELs). *Gzmb* was upregulated upon stimulation with Acetylcholine and GABA in the CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ and CD8$\alpha\beta^+$ IEL subpopulations, respectively, while downregulated in the CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ subpopulation upon stimulation with L-glutamic acid and VIP. *Ifng* was downregulated in both CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ and TCR$\alpha\beta^+$ IEL subpopulations upon stimulation with Acetylcholine and VIP, respectively. *Tnfα* was downregulated in both CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ and TCR$\alpha\beta^+$ IEL subpopulations upon stimulation with GABA and VIP, respectively. No changes were observed in the expression of the anti-inflammatory cytokine *Il10*, for any of the IELs' subpopulations.

Altogether, these results show not only that all three IELs' subpopulations can differentially and directly respond to neuron-derived signals, but also that these responses have potential to trigger or modify their effector and functional programmes to regulate inflammatory and metabolic pathways involving these immune cells.

4.3 Natural and Induced IELs' Subpopulations Display Regional Gene Expression Profile

Because it is known that IELs abundance varies greatly along the length of the intestine [90], we further investigated whether this could be also reflected in IELs' subpopulations by differential expression of specific genes. To this end, we purified CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ (CD8$\alpha\alpha^+$ CD4$^-$ TCR$\gamma\delta^+$) and TCR$\alpha\beta^+$ (CD8$\alpha\alpha^+$ CD4$^-$ TCR$\alpha\beta^+$) natural IELs and CD8$\alpha\beta^+$ induced IELs from B6 mice small intestine's segments - duodenum, jejunum and ileum - by FACS (as depicted in Annex I, Figure I.1). We further quantified the expression of the same Norepinephrine-, Acetylcholine-, L-glutamic acid-, GABA- and VIP-receptors' genes used in 4.1 for each subpopulation in each intestinal segment by qPCR, as previously described.

No consistent regional differences were found for the *Adrb1*, *Adrb2*, *Adra2a*, *Chrnb1*, *Gabbr1*, *Grina* and *Vipr1* genes among the three subpopulations of IELs (Figure 4.5A and B). However, a zonal pattern of expression was observed for *Vipr2* (Figure 4.5B), as the expression of this gene decreased in a proximal-to-distal manner for all the three IELs' subpopulations.

We further verified whether the metabolism-related gene *Glp1r* could also show a zonal expression profile. A tendency for *Glp1r* to be upregulated in the proximal part of the small intestine, the duodenum, was evident, but not significant, among the natural IELs (Figure 4.5C).

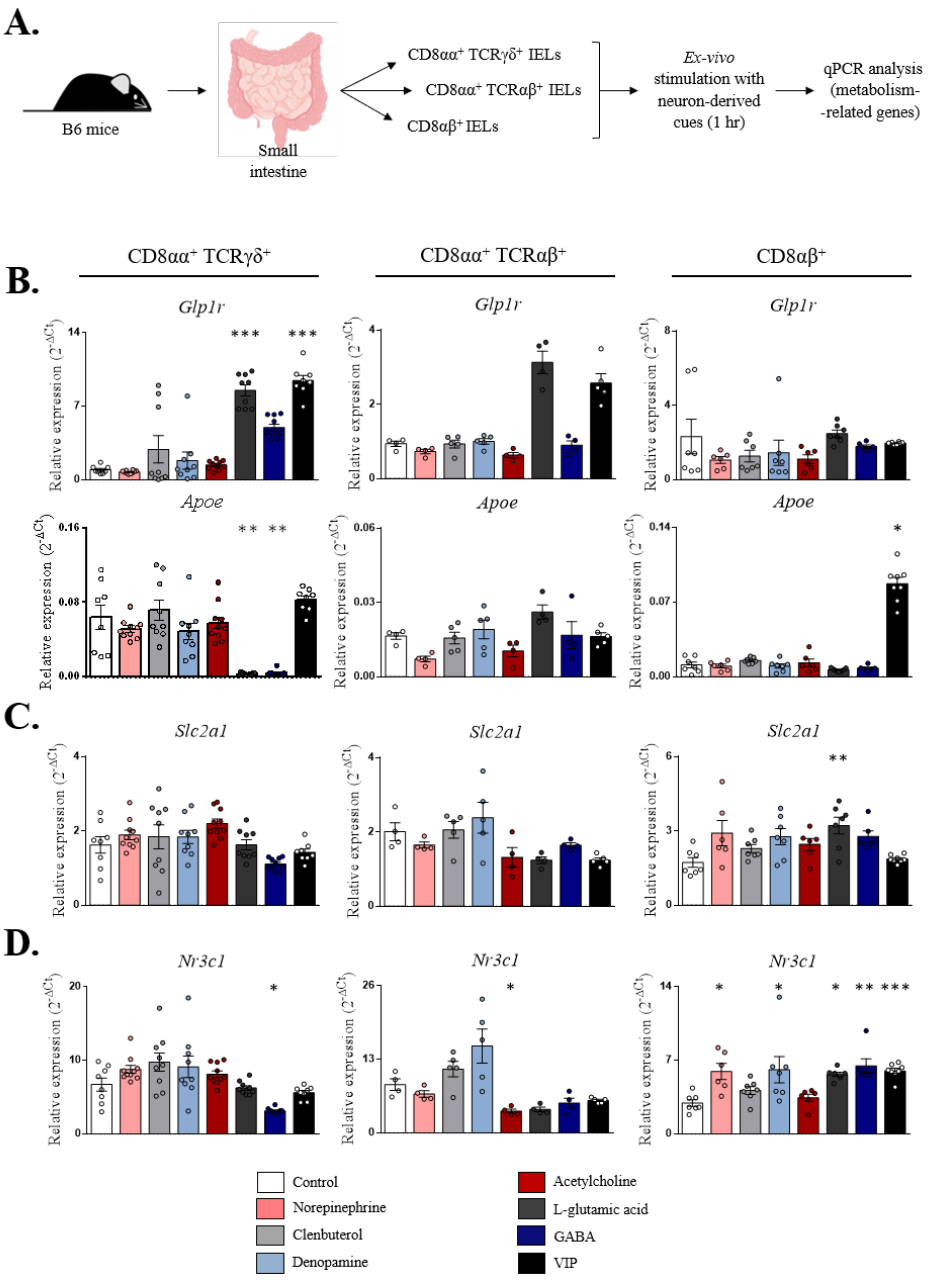

Figure 4.2: **CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$, CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ and CD8$\alpha\beta^+$ IELs' subpopulations directly respond to *ex-vivo* stimulation with neuron-derived cues by differentially changing the expression of genes related with metabolism.** **(A)** Representative scheme of the experimental strategy. CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$, CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ and CD8$\alpha\beta^+$ main subpopulations of intestinal IELs were purified by sorting and stimulated for 1 hr with the neurotransmitters, neuropeptide or their agonists. QPCR was performed for genes involved in lipid metabolism **(B)**, glucose metabolism **(C)** and corticosteroids-involving metabolism **(D)**. Results were normalized to *Hprt* expression. n = 7-9 for CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$, n = 4-5 for CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$, n = 6-7 for CD8$\alpha\beta^+$ for each experimental group. Data represented as mean ± SEM. One-way ANOVA was applied for normal distributed groups and Kruskal-Wallis Test for the others. *P < 0.05, **P < 0.01 and ***P < 0.001.

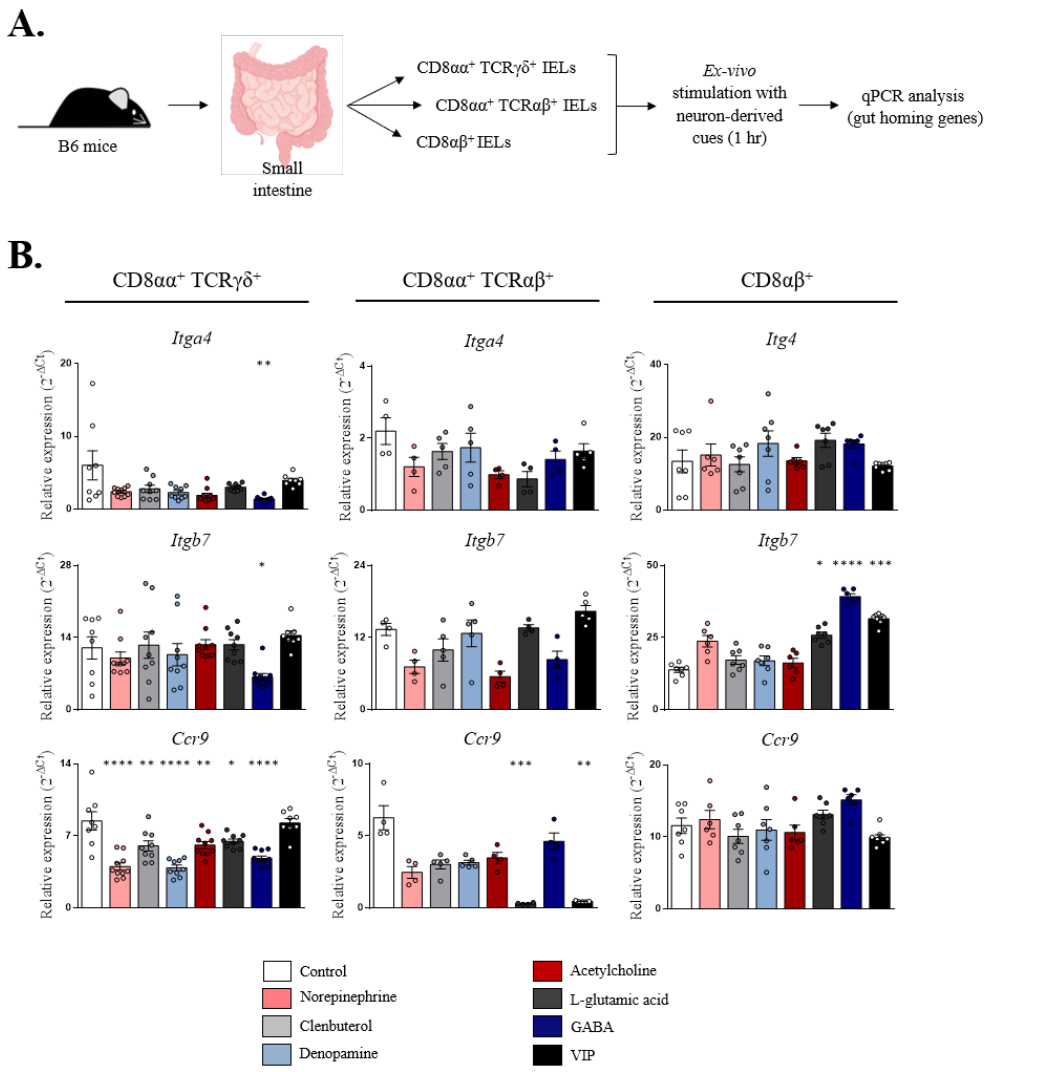

Figure 4.3: **CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$, CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ and CD8$\alpha\beta^+$ IELs' subpopulations directly respond to *ex-vivo* stimulation with neuron-derived cues by differentially changing the expression of gut-homing markers-coding genes. (A)** Representative scheme of the experimental strategy. CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$, CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ and CD8$\alpha\beta^+$ main subpopulations of intestinal IELs were purified by sorting and stimulated for 1 hr with the neurotransmitters, neuropeptide or their agonists. QPCR was performed for gut-homing markers-coding genes **(B)**. Results were normalized to *Hprt* expression. n = 7-9 for CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$, n = 4-5 for CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$, n = 6-7 for CD8$\alpha\beta^+$ for each experimental group. Data represented as mean ± SEM. One-way ANOVA was applied for normal distributed groups and Kruskal-Wallis Test for the others. *P<0.05, **P<0.01, ***P<0.001 and ****P <0.0001.

4.4 Co-culture of Natural IELs with Intestinal Organoids Does Not Induce Metabolic Responses in Epithelial Cells

It has been shown that IELs can control GLP-1 levels by directly capturing some of the available and released GLP-1 using their GLP-1 receptors [174]. However, whether IELs

can influence GLP-1 production by L-cells by changing their gene expression is not known. Our previous results showed that stimulation with VIP induced the expression of the *Glp1r* gene in natural IELs (Figure 4.2B). Moreover, we also found that these cells have a similar pattern of *Glp1r* and *Vipr2* basal expression levels along the small intestine (Figure 4.5 B and C). To gain insight into this, we further investigated whether the direct stimulation of intestinal epithelial cells with VIP or their co-culture with VIP-stimulated natural IELs, could be also a way to control GLP-1 levels by altering the expression of the GLP-1-coding gene expressed by L-cells: the proglucagon gene, *Gcg* [175, 176]. To address this question, we took advantage of intestinal epithelial organoids' cultures. These self-organized 3D clusters of cells closely replicate the structure and cellular composition of a functional native intestinal epithelium (*i.e.* mini-guts) [213]. We co-cultured intestinal epithelial organoids with the intestinal natural IELs' subpopulations CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ and CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$, respectively. Prior to co-culture, IELs were stimulated with either VIP (5 μM) or vehicle for one hour. We also tested a direct effect of VIP (5 μM) on intestinal epithelial organoids. The expression of *Gcg* was further determined by quantitative real-time PCR after 16 hours of co-culture or stimulation (Figure 4.6A). To investigate other metabolism- and inflammation-related genes expressed by epithelial cells, the expression of *Fabp2* – involved in the uptake, intracellular metabolism and transport of long-chain fatty acids by intestinal epithelial cells [214] -, *Il18* – Type I-inducing proinflammatory cytokine-coding gene constitutively expressed by intestinal epithelial cells with critical roles on intestinal homeostasis and inflammation [215, 216]- and *Il15* - pro-inflammatory cytokine-coding gene [217]- was also quantified.

For any of the genes tested, no significant differences were triggered upon the organoids' co-culture with both non-stimulated and VIP-stimulated CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ or TCR$\alpha\beta^+$ IELs' subpopulations (Figure 4.6B). In addition, the direct stimulation of intestinal organoids with VIP did not induce any difference in the expression of the genes tested (Figure 4.6B).

Altogether, these results show that neither the direct stimulation with VIP nor the addition of non-stimulated and VIP-stimulated natural IELs to intestinal epithelial organoids are capable of modulating the metabolic and inflammatory genetic profile of epithelial cells, for the genes tested.

4.5 A Diet Rich in Fat Downregulates *Glp1r* and *Vipr2* Genes' Expression and Modulates the Inflammatory Profile of Natural IELs

Dietary changes positively or negatively affect gut microbiota composition and intestinal immunity, which, in turn, modulates intestinal integrity, permeability and inflammation. It has been shown that HFDs, rich in short-chain saturated fatty acids, compromise the

barrier function of the intestine by diminishing epithelial integrity and increasing intestine's permeability, which can ultimately lead to systemic inflammation and metabolic dysfunctions [198, 201, 218]. To gain insights into the consequences of a diet rich in fat on intestinal IELs, we examined the effects of the HFD on IELs' distribution and gene expression patterns along the small intestine. To guarantee that we were inducing an obesity-like phenotype, both normal chow diet (ND)- and HFD-fed mice were weighted weekly and a GTT was performed one week prior to the analysis. All experiments were performed when mice displayed a 40% increase in their Body Weight (BW) and glucose intolerance comparing with the ND-fed mice (Figure 4.7A). After 16 weeks of HFD regimen, we analysed the total number of IELs and percentages of each IEL subpopulation in different intestinal segments (duodenum, jejunum and ileum). The same procedure was performed for control mice that were fed a ND for the same amount of time. We also purified natural and induced IELs from the three intestinal segments by FACS and compared their gene expression profiles by qPCR.

The natural IELs' subpopulations remained similar in number and frequency along the intestinal segments under ND and HFD-feeding (Figure 4.7B). However, a CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ CD4$^+$ IEL subpopulation almost totally disappeared along the small intestine in the mice fed with HFD. The numbers and frequency of this subpopulation were significantly decreased in all intestinal segments (Figure 4.7 C). The induced IEL subpopulation, CD8$\alpha\beta^+$, displayed higher frequency in the duodenum and jejunum of mice fed with an HFD (Figure 4.7 B).

To understand whether a diet rich in fat could induce changes in IEL's expression of genes related with metabolism and inflammation, we quantified the expression of *Glp1r*, *Vipr2*, *Ifng* and *Tnfα* genes in the natural IELs from different intestinal segments by qPCR.

Regarding the metabolic profile, both *Glp1r* and *Vipr2* genes showed to be downregulated in natural IELs in duodenum and jejunum from HFD-fed mice. In the ileum, this downregulation was only observed for the *Glp1r* gene but, again, in both CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ and TCR$\alpha\beta^+$ subpopulations (Figure 4.8 A and B). Regarding the inflammatory profile of the natural IELs tested, both showed a significant increase in the expression of the *Tnfa* gene upon the HFD-feeding (Figure 4.8 C and D).

Altogehter, these results indicate that a diet rich in fat triggers the completely loss of a CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ CD4$^+$ IEL subpopulation along the length of the small intestine while simultaneously modulates the metabolic and inflammatory profile of natural IELs .

4.6 *Ex-vivo* Stimulation with Dexamethasone Shifts the Inflammatory Profile of IELs' Subpopulations

Glucocorticoids are steroid hormones that interact with immune cells to exert potent immunesuppressive and anti-inflammatory effects while controlling intermediate metabolism through a multitude of ways [35, 75, 88, 177]. However, little is known about the effect of

glucocorticoids on IELs' subpopulations. Given the high basal levels of expression of the glucocorticoid receptor gene *Nr3c1* in all IELs' subpopulations tested (as shown in Figure 4.1 C), we investigated whether the activation or absence of the glucocorticoid receptor could induce changes in IELs' metabolic and inflammatory profiles.

We first performed activation of the glucocorticoid receptor by directly stimulating each IEL subpopulation with Dexamethasone (0.1 μM) - a corticosteroid drug belonging to the glucocorticoid family [84]. Natural IELs (CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ and CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$) were purified as described above and *ex-vivo* stimulation assays were performed for 1 hour. We further quantified the expression of genes involved in lipid and glucose metabolism (*Glp1r* and *Slc2a1*) and genes for cytolytic enzymes and pro-inflammatory cytokines (*Gzmb*, *Tnfa* and *Ifng*) by qPCR. We also quantified the expression of *Vipr2* to verify a possible effect of glucocorticoids on this neuropeptide receptor. Although no significant differences were found for the *Glp1r* or *Slc2a1* genes for any of the subpopulations tested, the expression of *Vipr2* showed to be significantly upregulated in the CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ IEL subpopulation upon stimulation with Dexamethasone (Figure 4.9 B). Besides this, we noticed a shift in the inflammatory profile of all IELs, as *Gzmb* showed to be downregulated in the CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ IELs, *Tnfα* in the CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ and CD8$\alpha\beta^+$ IELs and *Ifng* in all IELs' subpopulations tested.

For the glucocorticoid receptor loss-of-function approach (Figure 4.9 C), we isolated the intestinal IELs' subpopulations from IL7Rα-Cre.Nr3c1$^{flox/flox}$ mice - in which lymphocyte's ability to recognize glucocorticoids is lost [219, 220] – and their correspondent IL7Rα-Cre.Nr3c1$^{wt/wt}$ littermate controls. We then compared gene expression profiles of IELs from these two groups by qPCR. In the absence of the glucocorticoid receptor, the expression of both *Glp1r* and *Vipr2* remained unchanged. In addition, no changes in the inflammatory profile between these two groups were observed (Figure 4.9 D).

Altogether, these results demonstrate that the activation of the glucocorticoid receptor Nr3c1 is sufficient to promote a shift in the inflammatory profile of all IELs'subpopulations and modulate the expression of *Vipr2* in the CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ subpopulation. Furthermore, a loss of the Nr3c1 receptor in the lymphocyte population does not affect neither inflammatory nor metabolic profiles of natural IELs at steady state.

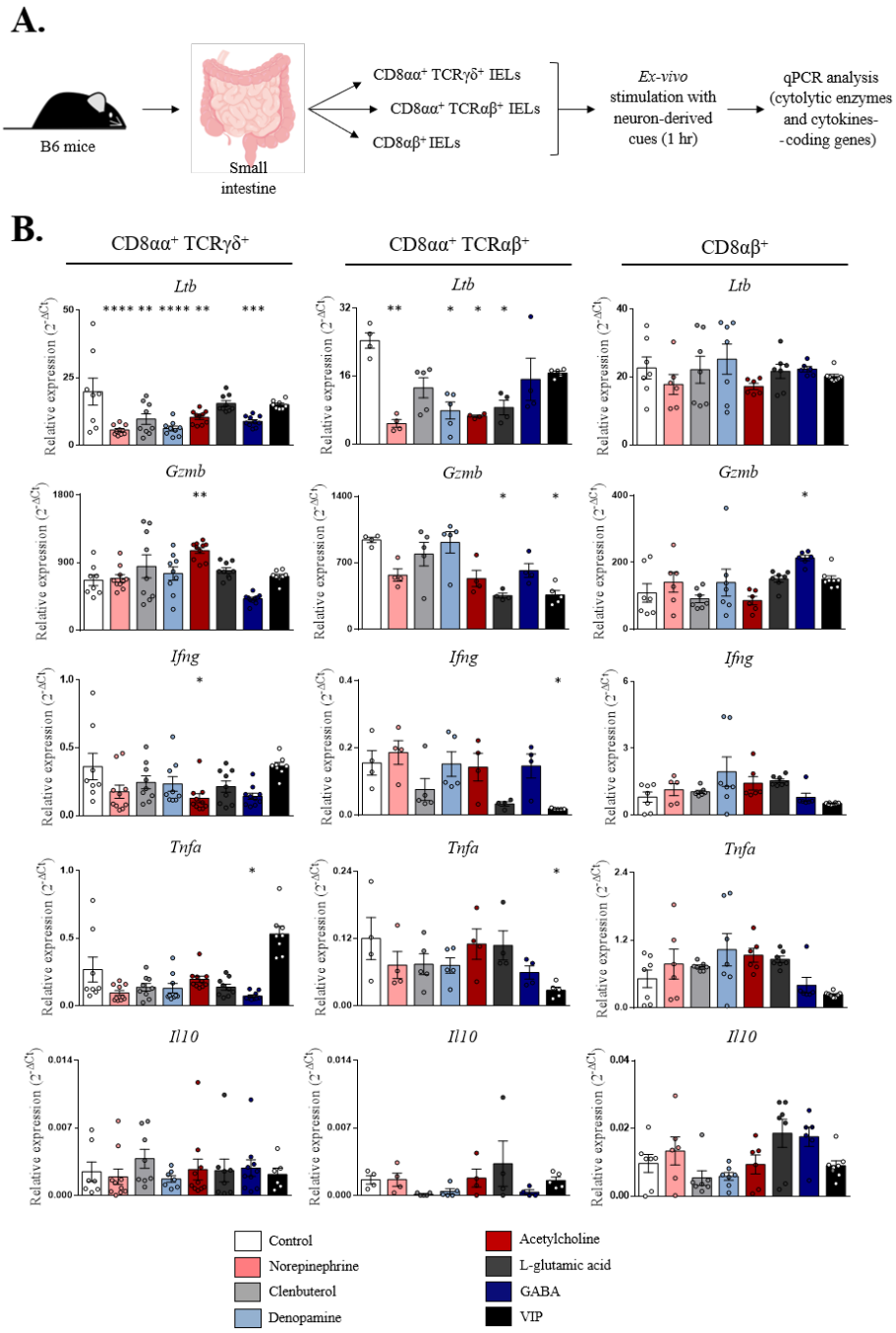

Figure 4.4: **CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$, CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ and CD8$\alpha\beta^+$ IELs' subpopulations directly respond to *ex-vivo* stimulation with neuron-derived cues by differentially changing the expression of cytolytic enzymes- and cytokines-coding genes. (A)** Representative scheme of the experimental strategy. CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$, CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ and CD8$\alpha\beta^+$ main subpopulations of intestinal IELs were purified by sorting and stimulated for 1 hr with the neurotransmitters, neuropeptide or their agonists. QPCR was performed for cytolytic enzymes- and cytokines-coding genes **(B)**. Results were normalized to *Hprt* expression. n = 7-9 for CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$, n = 4-5 for CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$, n = 6-7 for CD8$\alpha\beta^+$ for each experimental group. Data represented as mean ± SEM. One-way ANOVA was applied for normal distributed groups and Kruskal-Wallis Test for the others. *P<0.05, **P<0.01, ***P<0.001 and **** P<0.0001.

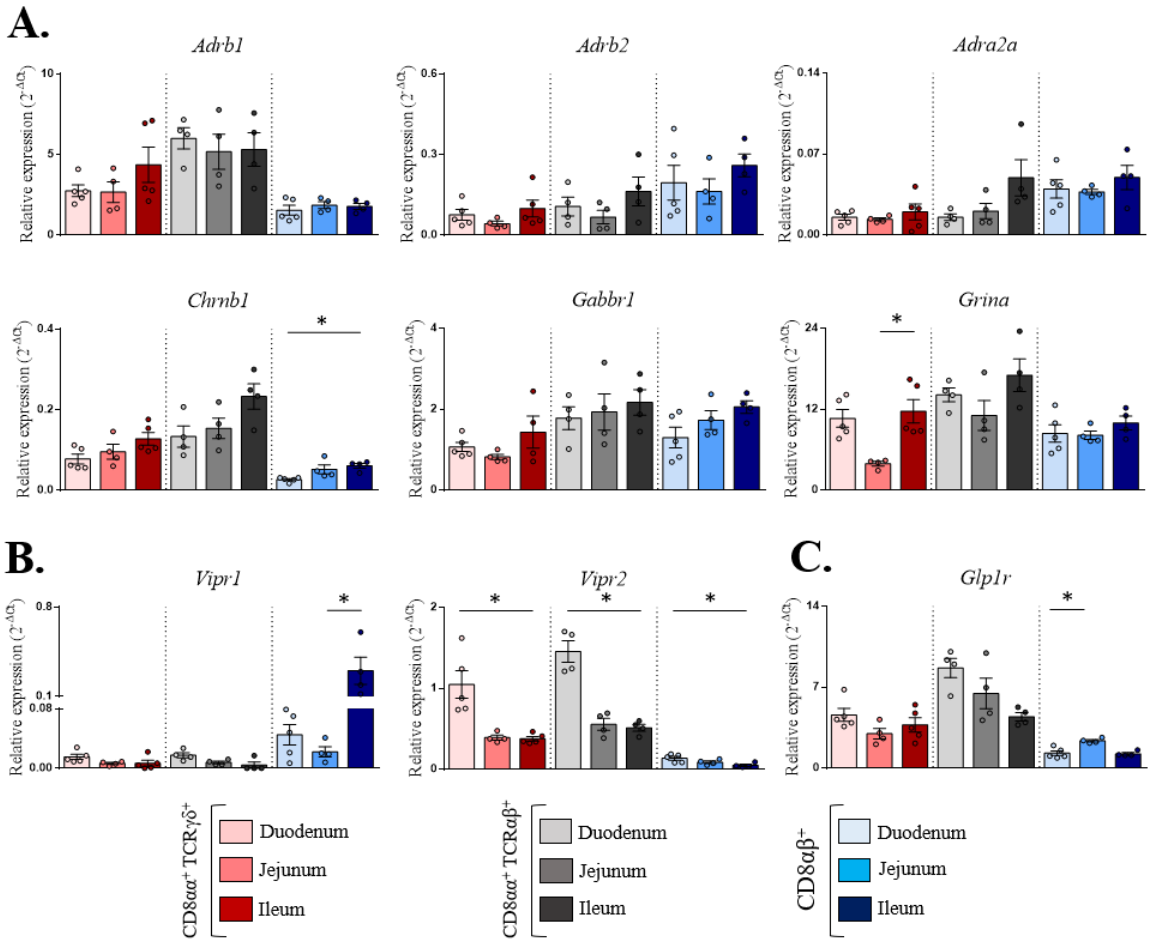

Figure 4.5: **CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$, CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ and CD8$\alpha\beta^+$ IELs' subpopulations display regional expression profile.** CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$, CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ and CD8$\alpha\beta^+$ main subpopulations of IELs were purified by sorting from duodenum, jejunum and ileum of 7 to 9 weeks old B6 male mice and qPCR was performed for **(A)** neurotransmitters-, **(B)** neuropeptides- and **(C)** GLP-1-receptors' genes. Results were normalized to *Hprt* expression. n = 4-5 for each experimental group. Data represented as mean ± SEM. Kruskal-Wallis Test was applied. *P<0.05, **P<0.01, ***P<0.001 and **** P<0.0001.

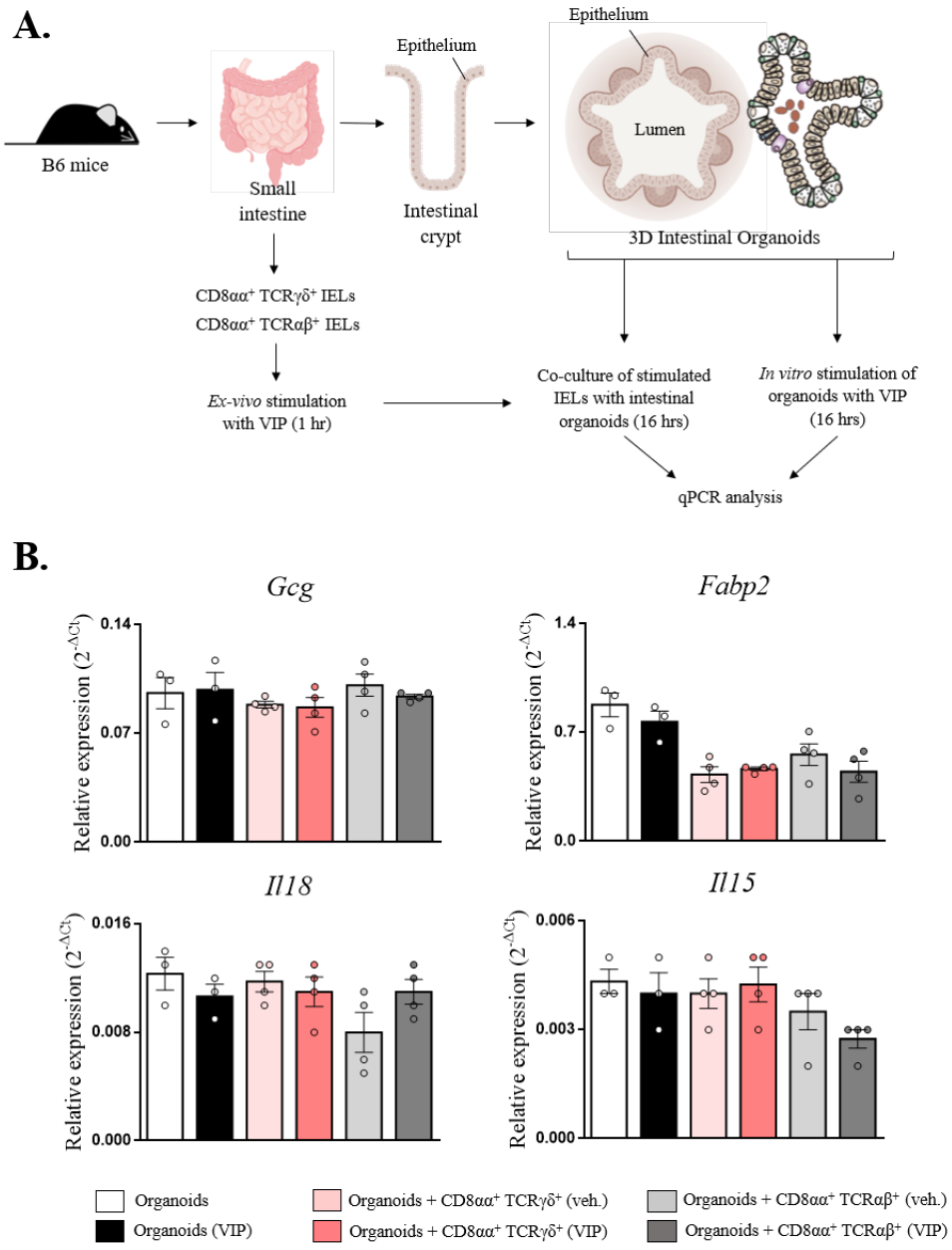

Figure 4.6: **Co-culture of CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ or CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ natural IELs with intestinal epithelial organoids does not induce metabolic nor inflammatory changes in intestinal epithelial cells. (A)** Representative scheme of the experimental strategy. Intestinal organoids were prepared from the small intestine of 7 to 9 weeks old B6 male mice. CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ and CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ IELs' subpopulations were purified by sorting from 7 to 9 weeks old B6 male mice, stimulated for 1 hr with VIP and co-cultured with the intestinal organoids. The direct effect of VIP in organoids was also tested. **(B)** QPCR was performed for metabolism related- and pro-inflammatory cytokines genes. Results were normalized to *Epcam* expression. n = 4 for each experimental group. Data represented as mean ± SEM. Kruskal-Wallis Test was applied.

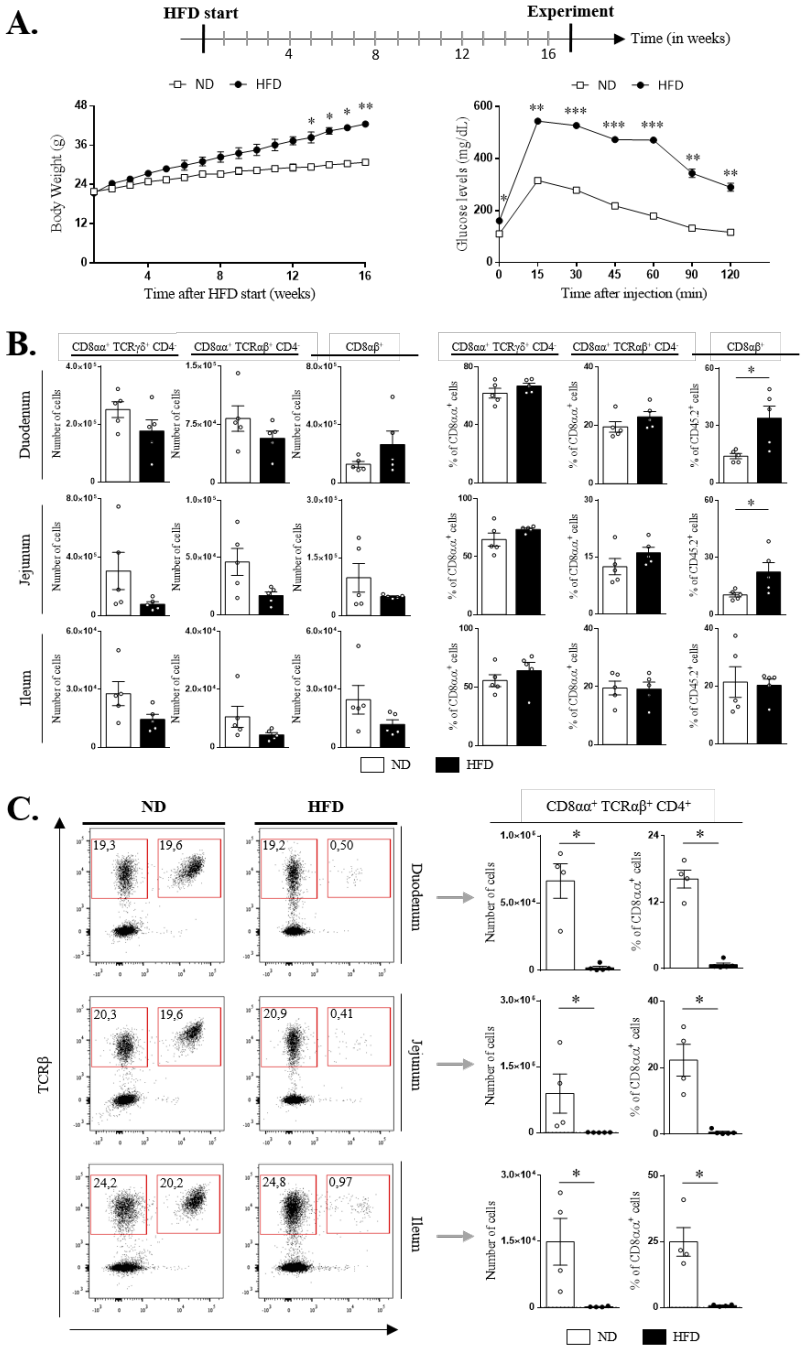

Figure 4.7: **A diet rich in fat leads to the complete loss of a CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ CD4$^+$ IEL subpopulation along the small intestine.** Duodenum, jejunum and ileum segments from 16 weeks old B6 mice fed with ND or HFD were collected and analysed. **(A)** Body weight evolution following ND (white colour)- or HFD (black colour)-feeding (left panel) and glucose tolerance test results (right panel). **(B)** Numbers and percentages of CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ CD4$^-$, CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ CD4$^-$ and CD8$\alpha\beta^+$ IELs subpopulations from ND- and HFD-fed mice. **(C)** Representative FACS plots of CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ CD4$^+$ IEL subpopulation from ND- and HFD-fed mice (left panel) and correspondent numbers and percentages (right panel). n = 5 for each experimental group. Data represented as mean ± SEM. Non-parametric Mann-Whitney-test (two-tailed) was applied. *P<0.05.

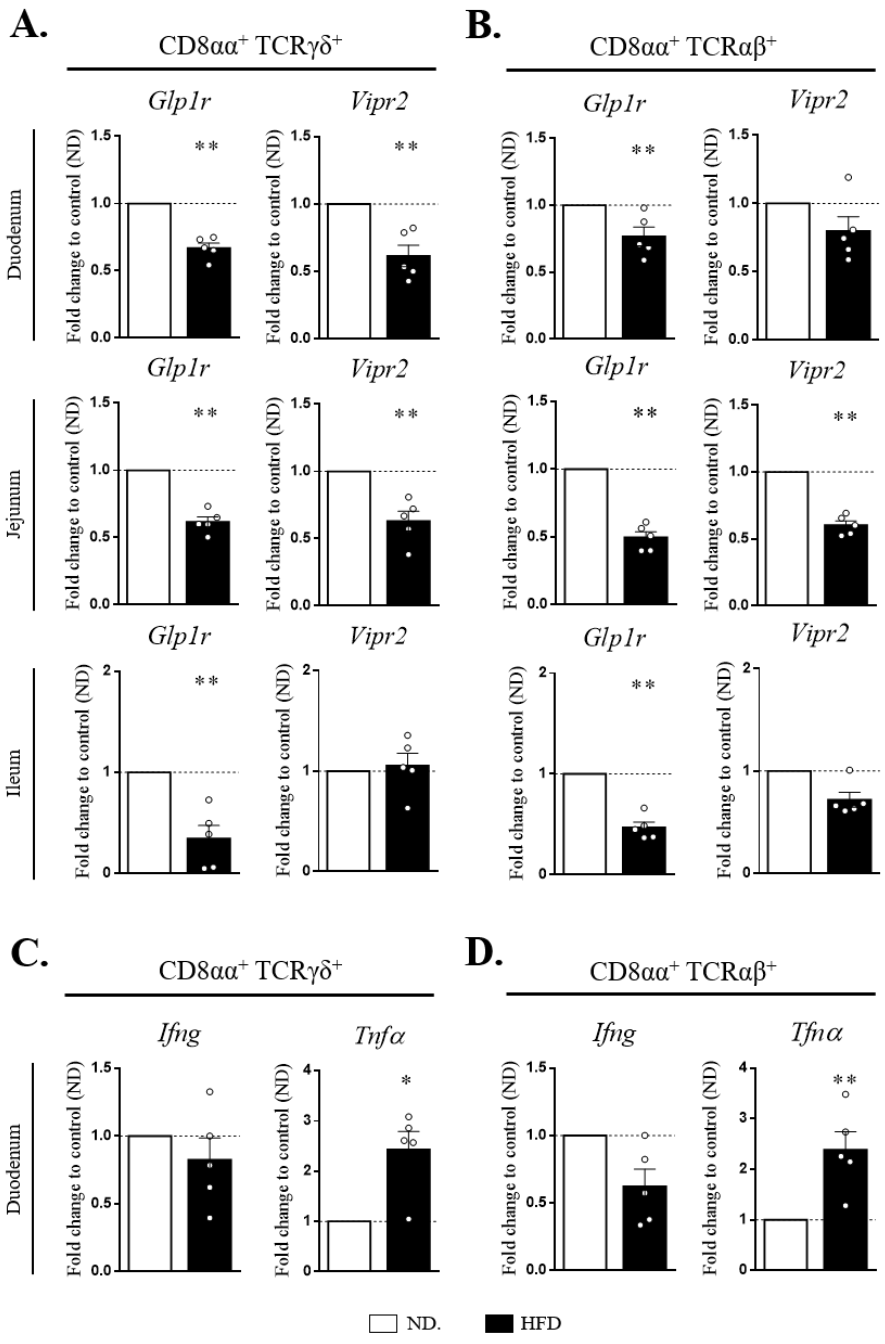

Figure 4.8: **A diet rich in fat modulates both metabolic and inflammatory profiles of CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ and TCR$\alpha\beta^+$ natural IELs.** CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$ CD4$^-$ and CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ CD4$^-$ natural IELs' subpopulations were isolated from the intestinal duodenum, jejunum and ileum from 16 weeks old B6 mice fed either a ND or HFD and qPCR was performed for **(A, B)** neuronal- and metabolism-related genes and **(C, D)** inflammation-related genes. Results were normalized to *Hprt*. n = 5 for each experimental group. Data represented as mean ± SEM. Non-parametric Mann-Whitney-test (two-tailed) was applied. *P<0.05, ** P<0.01.

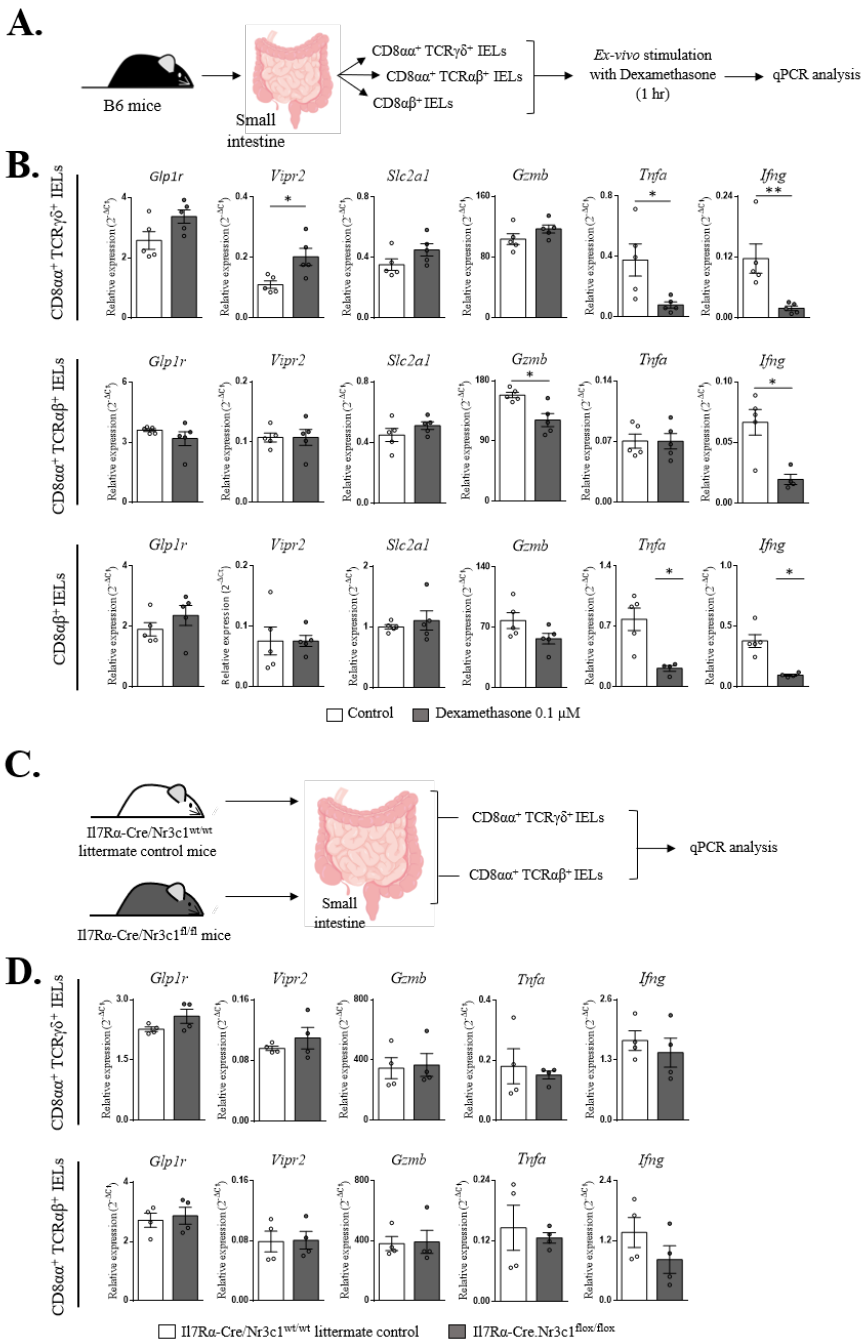

Figure 4.9: **The three major subpopulations of IELs respond to an *ex-vivo* stimulation with Dexamethasone by shifting their inflammatory profile. (A, B)** Representative scheme of the strategy used to perform *ex-vivo* stimulation with Dexamethasone in the three subpopulations of IELs and further quantitative PCR performed for metabolism related-, neuropeptide receptors-, cytolytic enzymes- and pro-inflammatory cytokines-coding genes. n = 5 for each experimental group. **(C, D)** Representative scheme of the strategy used for the glucocorticoid receptor loss-of-function approach and further quantitative PCR performed for metabolism related-, neuropeptide receptors-, cytolytic enzymes- and pro-inflammatory cytokines-coding genes; n = 4 for each experimental group. Results were normalized to *Hprt* expression. Data represented as mean ± SEM. Non-parametric Mann-Whitney-test (two-tailed) was applied. *P<0.05 and **P<0.01.

Chapter 5

Discussion

The intestinal IELs constitute one of the first lines of immune defence at the intestinal mucosa and recently have been shown to play a role in metabolism [136, 174]. Because they intervene in physiological and metabolic processes fundamental for the intestine and whole-body homeostasis, it is necessary that their responses are tightly controlled. The gut microenvironment, the microbiome and even the dietary antigens play a great part in regulating responses by these immune cells [7, 149, 162]. Here, we uncover a possible new layer of local and systemic tissue regulation through a neuro-immune interaction.

First, we observed that the three major subpopulations of IELs, CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$, CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ and CD8$\alpha\beta^+$, differentially express adrenergic, cholinergic, GABAergic, glutamatergic and VIP receptors' genes. Because no literature addressing the neuronal receptors in IELs is available, we therefore provide the first line of evidence that all the three subpopulations of intestinal IELs have the machinery to perceive neuronal cues.

Through an *ex-vivo* stimulation approach, we also demonstrate that the gene expression profile of intestinal IELs can be modulated by the abovementioned neuronal stimuli. Moreover, we unveil a so far unknown relationship between the neuropeptide VIP and the metabolism-related gene *Glp1r*, which may have strong implications in metabolism. GLP-1 is an incretin hormone released by enteroendocrine L-cells of the gut epithelium and it is responsible to induce postprandial pancreatic insulin secretion and exert glucose control [175–177]. It has been shown recently that IELs can control the bioavailability of GLP-1 by capturing it through their GLP-1 receptors [174] . In this study, we found that the *ex-vivo* stimulation of natural IELs with the neuronal transmitters L-glutamic acid and VIP leads to an increase of the *Glp1r* expression. These findings led us to speculate that the release of L-glutamic acid and VIP by, respectively, intestinal glutamatergic and VIPergic neurons could be part of the GLP-1 regulation pathway through intestinal

IELs. According to this, glutamatergic and VIPergic neurons would, respectively, release L-glutamic acid and VIP, which would be further perceived by their correspondent receptors in IELs: Grina and Vipr1 or Vipr2, respectively.

It is known that there is a regional specialization of the different IELs' subsets within the different parts of the intestine [90]. Here, we verified a zonal pattern of *Vipr2* expression in all IELs' subpopulations. Interestingly, a tendency for a very similar pattern was also observed for the *Glp1r* gene in the natural IELs. Based on our parallel results, we can reason that a possible communication between intestinal IELs and neurons to modulate *GLP1r*'s expression in natural IELs is most likely to happen through the neuropeptide VIP, via the Vipr2. Concordant with this, *Glp1r* and *Vipr2* genes' expression of duodenal and jejunal natural IELs changed in the exact same way for both genes upon a long-term challenge with a diet rich in fat (Figure 4.8 A and B). VIP is a pleiotropic neuropeptide that has been shown to play a role in metabolism [73, 77]. One of its metabolic functions is translated by an increase in the rate of digestion through an increase in gut motility and stimulation of proteins' breakdown in this organ [73, 207]. In addition to the abovementioned functions of GLP-1, it is also known that this hormone is responsible for slowing the rate of digestion [175–177]. Based on our results and on these opposite digestive functions displayed by these two molecules, VIP could potentially act as an inhibitor of the GLP-1's function by increasing the capture of GLP-1 by natural IELs. It is possible that, in response to high levels of released GLP-1, VIPergic neurons release VIP, which in turn would stimulate the expression of GLP-1r in IELs to maintain the right levels of GLP-1. On the other hand, it is also possible that VIP is released side-by-side with GLP-1 in response to certain nutritional cues as a mechanism to guarantee a balanced rate of digestion. Lastly, VIP release and sensing by *Vipr2* in natural IELs can also represent a secondary pathway responsible for accelerating the digestive process that is triggered in situations when a fast nutrient absorption is necessary. Therefore, a relationship between VIP and GLP-1r in natural IELs may have high impact on the circulating levels of GLP-1 and, therefore, on systemic metabolism.

In addition, we also demonstrate that VIP, at least in the context of GLP-1 production, does not act on intestinal epithelial cells. L-cells synthetize and secreted GLP-1 through the expression of their GLP-1 coding gene: the proglucagon gene, *Gcg* [175, 176]. Intestinal organoids cultures are often used in research as they closely replicate a functional native intestinal epithelium [208]. In this study, we found that an *in vitro* stimulation of intestinal organoids with VIP did not induce any change in the gene expression levels of *Gcg* by L-cells. Moreover, the co-culture of organoids with non-stimulated or VIP-stimulated IELs, triggered the exact same absence of response in the epithelial *Gcg* gene's expression. With this, we can therefore conclude that VIP modulates the expression of the GLP-1r in IELs but does not stimulate the production of GLP-1 by epithelial cells. In addition, we can also conclude that natural IELs itself do not influence GLP-1 production by L-cells, at least through the altering of their *Gcg* gene expression.

It is known that GLP-1 production is stimulated upon feeding [175–177]. Therefore,

we propose a model for what can be happening (also described in Annex I, Figure I.2) . According to this model, in a steady-state situation, where it is known that L-cells produce basal levels of GLP-1, the *Glp1r* is kept at low expression levels in IELs. After meal consumption, where it is known that the levels of GLP-1 are high, the sensing of dietary antigens by L-cells, by absorptive enterocytes or by both, can trigger an increase release of GLP-1. To avoid detrimental metabolic consequences of what high amounts of GLP-1 may bring, IELs can start to upregulate their *Glp1r* to also capture some of the GLP-1 available, therefore contributing to a balance level of this hormone. It can happen that the sensing of high GLP-1 levels is made by IELs itself but also by VIPergic neurons. The transport of GLP-1 from L-cells to the blood and lymph is rapid [176, 177]. Therefore, if needed, VIPergic neurons can release VIP and increase the expression of the GLP-1 receptor to increase the decrease of GLP-1 available. Thus, in this model, VIPergic neurons can act synergistically and through IELs as a secondary and quick regulatory mechanism of GLP-1 levels. Concordant with this model, is the fact that our co-culture experiments showing IELs-independent basal levels of *Gcg* resemble a steady-state condition, the fact that the expression of *Glp1r* was upregulated upon stimulation with VIP and the fact that there was a spacial and responsive correlation between *Glp1r* and *Vipr2* genes in duodenum and jejunum, the two intestinal regions where is it known to happen the majority of intestinal nutrient absorption.

Moreover, we also show that both *Glp1r* and *Vipr2* genes were downregulated in natural IELs from mice fed with an HFD. It was previously demonstrated that HFD reduces intestinal *Gcg* expression and GLP-1 production [221], therefore it would be naturally advantageous for these animals' health to increase the circulating levels of this hormone. This compensatory mechanism could be potentially achieved through the downregulation of the GLP-1 receptor and/or through blocking the neuropeptide VIP sensing.

In conclusion, throughout this study we have demonstrated that intestinal IELs can perceive and respond to specific neuronal signals, but also that these immune cells are involved in much more than just immune defence. Our results indicate that the metabolic role of intestinal IELs is modulated by the neuropeptide VIP and that strongly seems to be involved in the homeostasis and regulation of metabolism and the normal digestive process. However, several questions remain open: What would happen to the levels of GLP-1 if we blocked the *Vipr2* in natural IELs in ND- and HFD-fed mice? What would happen to the GLP-1 receptors in IELs in a situation of food starvation? What are the signals triggering GLP-1 release by L-cells? Are there other players in this GLP-1-mediated homeostatic pathway? To solve some of these questions, different experimental approaches need to be done. The first approach would be to specifically delete *Vipr2* in natural IELs from the intestinal tissue to understand what would happen to L-cells and GLP-1 levels. Usually, this is achieved by using specific Cre-lines that allow the loss of a specific receptor from a specific cell through the Cre-lox system [210, 211]. However, because no Cre-line is available for natural IELs, this process becomes more complex.

One option would be to use CD8α-Cre line as it marks peripheral lymphocytes, however it is not specific to IELs [222]. Other approach could be to perform an adoptive transfer of natural IELs from *Vipr2* KO and *Vipr2* WT mice to an immunodeficient host mice that do no express effective T, B, NK cells, the Rag2$^{-/-}\gamma c^{-/-}$ [223]. By comparing the metabolic profile of these mice, we could have an idea about the role of IEL-VIP signalling in metabolism.

It would also be interesting to understand the role of the CD8$\alpha\alpha^{+}$ TCR$\alpha\beta^{+}$ CD4^{+} IEL at the intestinal epithelium and why an HFD led to a complete loss of this population. Can this population have an impact in shaping metabolism?

In addition to all of this, our work also gives preliminary data showing how the inflammatory profile of intestinal IEL can be also modulated by neuronal and endocrine signals. Therefore, besides the relevance of our findings in the VIP-IEL-GLP-1 axis, this work also opens new doors for future studies involving the treatment of enteric inflammatory diseases.

Overall, this work provides evidence of a direct neuroimmune communication within the different subpopulations of intestinal IELs that can represent a powerful signalling axis for modulating their function. Such knowledge will pave the way for new clinical approaches and therapeutic strategies to treat enteric inflammatory and metabolic diseases that are of major public concern and where, either directly or indirectly, IELs can be playing a role.

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Annex I

Supplementary Material

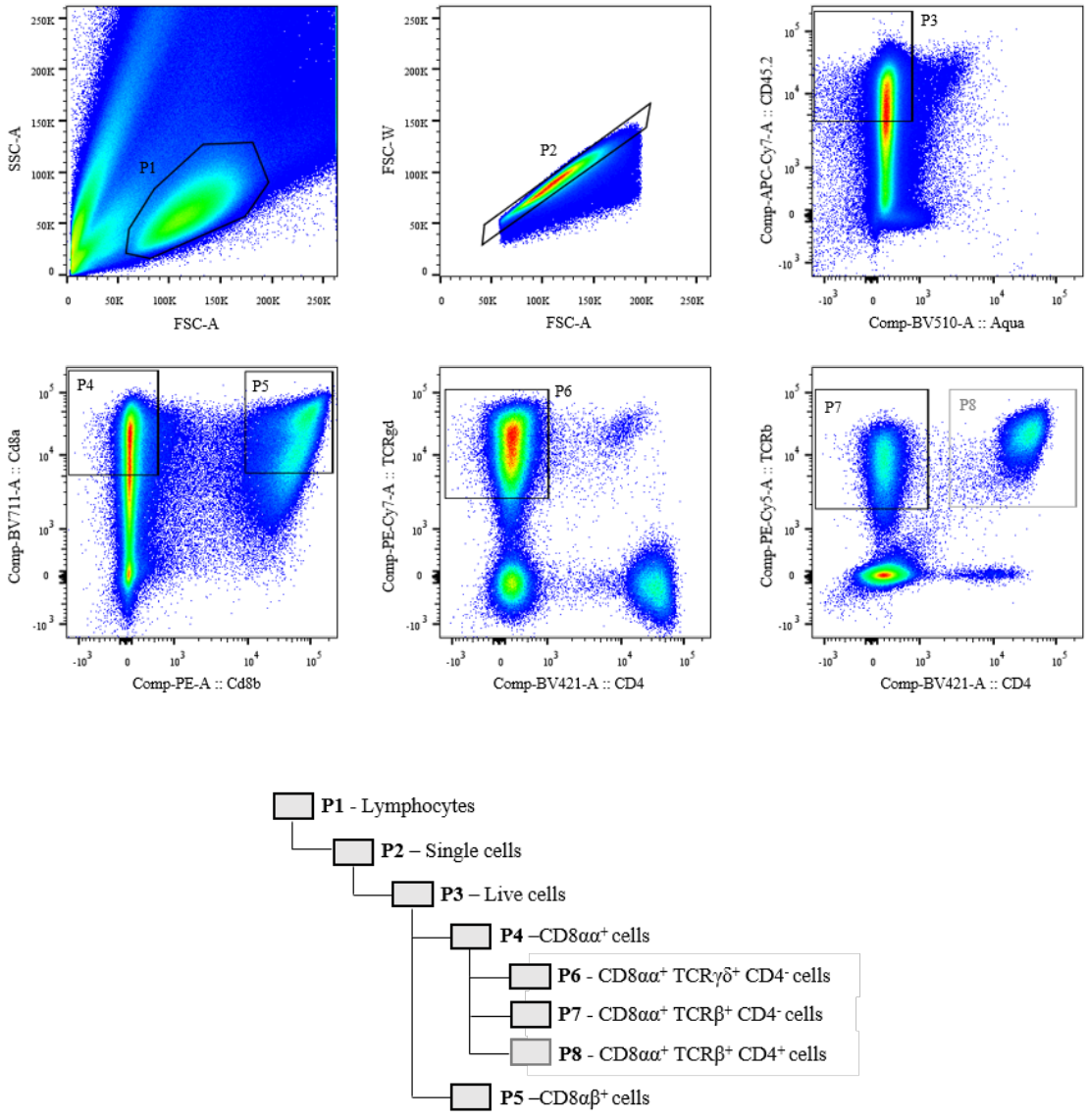

Figure I.1: **Representative FACS Plots and Gating Strategy Used to Purify CD8$\alpha\alpha^+$ TCR$\gamma\delta^+$, CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ and CD8$\alpha\beta^+$ IELs' Subpopulations by Sorting.** The conjugated primary antibodies used and their correspondent markers are shown in the axis.

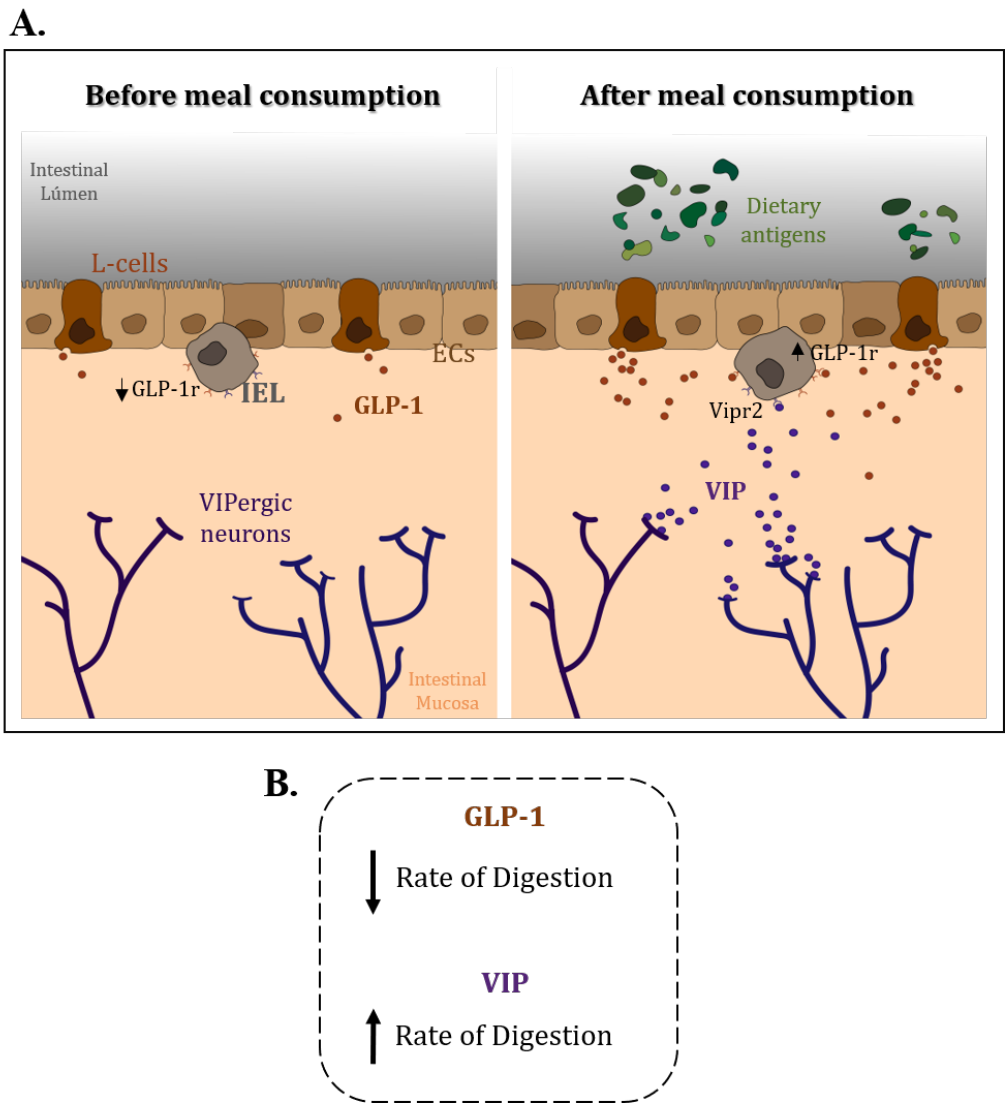

Figure I.2: **Representative Diagram of a Possible Model Proposed in this Work to Explain the VIP-IEL-GLP-1 Axis. (A)** Before meal consumption (left pannel), the *Glp1r* gene in intestinal IELs is likely expressed at low levels due to the low basal levels of GLP-1 secreted by the enteroendocrine L-cells of the gut epithelium. After meal consumption (right pannel), L-cells increased dramatically the synthesis and release of GLP-1. In response to high levels of GLP-1, the surrounding VIPergic neurons can release the neuropeptide VIP, which is further sensed by natural IELs - most likely through their Vipr2 - in order to increase the expression of their *Glp1r*. Because the neuropeptide VIP and the hormone GLP-1 display opposite digestive functions - translated by an increase or decrease of the rate of digestion, respectively (as represented in **(B)**) - and because GLP-1 is metabolized within minutes, this model propose that the VIPergic neurons can act synergistically and through IELs as a secondary and possible quick regulatory mechanism of the GLP-1 levels. This possible communication between the neuropeptide VIP, the natural IELs and the GLP-1 hormone (VIP-IEL-GLP-1 axis) offers a possible new layer for whole-body metabolism's regulation. Abbreviations: IEL, Intraepithelial Lymphocyte; ECs, Epithelial Cells; GLP-1, Glucagon-like Peptide 1; GLP-1r, Glucagon-like Peptide 1 Receptor; VIP, Vasoactive Intestinal Peptide; Vipr2, Vasoactive Intestinal Peptide Receptor 2.