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MODULATION OF ANTI-MICROBICAL DEFENSES IN THE GASROINTESTINAL TRACT BY ACETYLCHOLINESTERASE INHIBITORS.

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MODULATION OF ANTI-MICROBIAL DEFENSES IN THE GASTROINTESTINAL TRACT BY ACETYLCHOLINESTERASE INHIBITIORS

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Medical Sciences (Biochemistry and Molecular Biology)

Under the Supervision of Dr. Maria J. Fernandez-Cabezudo

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Declaration of Original Work

I, Alreem Abdulaziz Al-Dalali Al-Mansori, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Modulation of Anti-Microbial Defenses in the Gastrointestinal Tract by Acetylcholinesterase Inhibitors*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Maria J. Fernandez-Cabezudo, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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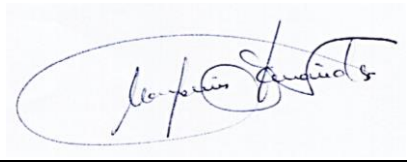
   Signature  Date 16 May 2020

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Abstract

Inflammation is a crucial defense mechanism that protects the body from the effect of invading pathogens. However, the inflammatory response needs to be controlled in order to avoid systemic manifestations with serious consequences to the host. Accumulating evidence indicates that the inflammatory response is tightly regulated through immunological and neural pathways. Previously, it has been demonstrated that cholinergic stimulation by paraoxon, a specific and irreversible inhibitor of acetylcholinesterase (AChE), improved survival in mice following an oral infection with virulent *Salmonella enterica* serovar *Typhimurium* (*S. typhimurium*). However, paraoxon is an organophosphorous compound unsuited for human use. In this study, the main objective is to investigate the efficiency of rivastigmine, an FDA-approved inhibitor of AChE, on murine mucosal defenses in the gastrointestinal (GI) tract against *Salmonella* infections. Results show that cholinergic stimulation with rivastigmine enhanced host survival following an oral-route infection, and this correlated with lower bacterial load in systemic organs, including liver and spleen. Interestingly, while bacterial loads in systemic organs were decreased, bacterial loads were higher in intestinal content and feces compared to saline control group, suggesting enhanced bacterial shedding in the GI tract. Morphological analysis of the small intestine (ileum) showed that rivastigmine induced the degranulation of goblet cells and Paneth cells, two specialized secretory cells involved in innate immunity, and demonstrated a significant increase in the thickness of mucin layer in these mice. Immunohistochemical study of the immune population present at the intestinal mucosa revealed that rivastigmine treatment resulted in minor changes in the lymphoid population in the epithelium (intraepithelial lymphocytes or IELs) and lamina propria (LP). A comparative flowcytometric analysis of the different leukocytic populations present in the two isolated compartments of the intestinal mucosa (IEL and LP) following either paraoxon or rivastigmine treatment, demonstrated that only paraoxon induced an increase in the $CD8^+$ population in the LP. Moreover, intestinal epithelium of rivastigmine-treated mice presented a decrease in $CD8^+\gamma\delta TCR^+$ cells. These findings indicate that rivastigmine-mediated cholinergic modulation increases the innate defense mechanisms at the level of the intestinal lumen, delaying the bacterial translocation from intestine to LP and systemic organs, ultimately leading to enhanced

initial protection against a lethal bacterial infection. However, complete protection in this model appears to require the recruitment of professional T cells to the LP, which was only observed after treatment with irreversible AChE inhibitors, like paraoxon. The data highlight the crucial interactions between neural and immune systems that act at the GI mucosal interface to protect the host against invading pathogens.

Keywords: Acetylcholinesterase Inhibition, *Salmonella enterica*, Neuro-immune, Intestine, Mucosal Barrier, Goblet Cells, Paneth Cells, Intestinal Epithelium, Lamina Propria.

Title and Abstract (in Arabic)

تحوير الدفاعات المضادة للميكروبات في الجهاز الهضمي بواسطة مثبطات الأستيل كولينيستراز

الملخص

يعد الالتهاب آلية دفاعية مهمة تحمي الجسم من تأثير غزو مسببات الأمراض. ومع ذلك، يجب السيطرة على الاستجابة الالتهابية من أجل تجنب المظاهر الجهازية مع عواقب وخيمة على المضيف. تشير الأدلة المتراكمة إلى أن الاستجابة الالتهابية يتم تنظيمها بإحكام من خلال المسارات المناعية والعصبية. في السابق، لقد أثبت أن رفع مستوى الناقل العصبي الاستيل كولين عن طريق الباروكسون، وهو مثبط محدد لا رجعة فيه لأسيتيل كولينيستراز(AChE)، أدى إلى حماية الفئران من عدوى السالمونيلا القاتلة مع ذلك، فإن الباروكسون مركب فوسفور عضوي غير مناسب للاستخدام البشري. في هذه الدراسة، استهدفنا دراسة كفاءة عقار ريفاستغمين، وهو مثبط معتمد من إدارة الأغذية والعقاقير لـ AChE، على دفاعات الغشاء المخاطي للفأر في الجهاز الهضمي ضد عدوى السالمونيلا. أثبتنا أن رفع مستوى الناقل العصبي الاستيل كولين عن طريق ريفاستغمين يعزز بقاء الفئران بعد الإصابة البكتيرية عن طريق الفم، وهذا يرتبط مع انخفاض التعداد الجرثومي في الأعضاء الرئيسية، بما في ذلك الكبد والطحال. ومن المثير للاهتمام، أنه بينما انخفض التعداد الجرثومي في الكبد والطحال، كانت البكتيريا أعلى في محتوى الأمعاء والبراز مقارنة بمجموعة الفئران التي لم تأخذ العقار، مما يشير إلى تخلص الجسم من البكتيريا المعززة في الجهاز الهضمي. وأظهر التحليل المورفولوجي للأمعاء الدقيقة أن ريفاستغمين تسبب في تحلل خلايا الكأس وخلايا البانث، وهما خليتان إفرازيتان متخصصتان في المناعة الأولية، وأظهروا زيادة كبيرة في سمك الغشاء المخاطي في هذه الفئران. وكشفت دراسة كيميائية مناعية في الأمعاء أن علاج ريفاستغمين أدى إلى تغييرات طفيفة في الخلايا المناعية الموجودة في (IEL) and (LP). أظهر تحليل مقاييس التدفق المقارن لمختلف كرات الدم البيضاء الموجودة في الجزءين المعزولين من الغشاء المخاطي في الأمعاء (الظهارة و بروبريا الصفيحة) بعد العلاج بالباروكسون أو ريفاستغمين، أن الباروكسون هو الذي تسبب فقط في زيادة عدد سكان + CD8 في بروبريا لامينا. وعلاوة على ذلك، قدمت ظهارة معوية من الفئران المعالجة ريفاستغمين انخفاض في خلايا +CD8+γδTCR. تشير هذه النتائج إلى أن التحوير الكوليني الذي يتوسطه ريفاستغمين يزيد من آليات الدفاع الفطرية على مستوى التجويف المعوي، مما يؤخر الانتقال

البكتيري من الأمعاء إلى بروبريا الصفيحة والأعضاء الجهازية، مما يؤدي في النهاية إلى تعزيز الحماية الأولية ضد العدوى البكتيرية الفتاكة. ومع ذلك، لتحقيق الحماية الكاملة في هذا النموذج، يبدو أنه من الضروري توظيف خلايا T احترافية في بروبريا الصفيحة التي يتم تحقيقها فقط بعد العلاج بمثبطات ACHE التي لا رجعة فيها مثل الباروكسون. تسلط البيانات الضوء على التفاعلات الحاسمة بين الجهاز العصبي والجهاز المناعي التي تعمل عند واجهة الجهاز الهضمي المخاطية لحماية المضيف من مسببات الأمراض.

مفاهيم البحث الرئيسية: مثبطات الأستيل كولين استيراز، السالمونيلا انتريكا، المناعة العصبية، الأمعاء، حاجز الغشاء المخاطي، خلايا الكأس، خلايا البانث، ظهارة الأمعاء، بروبريا لامينا.

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Dedication

To my beloved parents and family

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List of Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's Disease
AMPs	Anti-Microbial Peptides
BSA	Bovine Serum Albumin
BuChE	Butyryl-Cholinesterase
CFUs	Colony Forming Units
ChAT	Choline Acetyltransferase
DCs	Dendritic Cells
Defa	Defensin
DTNB/TNB^-	5,5-Dithio-Bis-(2-Nitrobenzoic Acid)/2-Nitro-5-Thiobenzoate
EDTA	Ethylenediaminetetraacetic Acid
ENS	Enteric Nervous System
ER	Endoplasmic Reticulum
FAE	Follicle-Associated Epithelium
FBS	Fetal Bovine Serum
GALT	Gut-Associated Lymphoid Tissue
GI	Gastrointestinal
GIT	Gastrointestinal Tract
Hb	Hemoglobin
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
IBD	Inflammatory Bowel Disease
IEL	Intraepithelial Lymphocytes

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILCs	Innate Lymphoid Cells
LPS	Lipopolysaccharide
M cells	Microfold Cells
mAChR	Muscarinic Acetylcholine Receptor
MHC II	Major Histocompatibility Complex Class Ii
MLN	Mesenteric Lymph Node
nAChR	Nicotinic Acetylcholine Receptor
NNCS	Non-Neuronal Cholinergic System
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate-Buffered Saline
PNS	Parasympathetic Nervous System
Pox	Paraoxon
PPs	Peyer's Patches
RA	Rheumatoid Arthritis
RBCs	Red Blood Cells
S. Typhymurium	*Salmonella Typhymurium*
SAO	Splanchnic Artery Occlusion
SNS	Sympathetic Nervous System
TNF	Tumor Necrotic Factor
UC	Ulcerative Colitis
VAChT	Vesicular Acetylcholine Transporter

Chapter 1: Introduction

1.1 Neuronal nervous system

The nervous system is a complex network of nerves and cells that enables an organism to interact with its surroundings. The nervous system has two components: the central nervous system and the peripheral nervous system. The central nervous system includes the brain and the spinal cord. The peripheral nervous system is divided into:

- Somatic nervous system.
- Autonomic nervous system.

The somatic nervous system is in charge of the voluntary movements. It consists of the (a) afferent branch that transmits sensory information from the periphery to the brain, and the (b) efferent branch that carries the information from the brain to the muscles causing their movements.

The autonomic nervous system controls and regulates involuntary functions of internal organs. It contains three different divisions:

- The sympathetic nervous system (SNS).
- The parasympathetic nervous system (PNS).
- The enteric nervous system (ENS).

The sympathetic nervous system innervates almost every tissue in the body and its activation leads to an overall increase activity and attention, what is called the “fight or flight” response (Koopman et al., 2011). The parasympathetic nervous system is smaller than the SNS and innervates only the head, viscera and external genitals. Opposite to the SNS, the PNS promotes “rest and digest” processes (Kenney & Ganta,

2014; Koopman et al., 2011) through the vagus nerve, its major nerve. The enteric nervous system is the most complex division of the peripheral nervous system (Gershon & Erde, 1981). It is composed of a network of neurons embedded into the wall of the gut forming two plexuses: the myenteric, located between the longitudinal and circular smooth muscle of the GI tract, and the submucosal, present in the GI submucosa (Waxenbaum & Varacallo, 2019).

The neurotransmitters used for the SNS are norepinephrine (presynaptic neurons) and acetylcholine (postsynaptic neurons). The PNS uses exclusively acetylcholine (ACh) and the ENS is known to use several neurotransmitter like ACh, nitrous oxide and serotonin (Koopman et al., 2011; McConalogue & Furness, 1994).

ACh was the first neurotransmitter identified in 1926 by Otto Loewi who received, together with Sir Henry Dale (who first isolated ACh from an animal organ), the Nobel prize in Medicine in 1936 (Fujii et al., 2017a). In the following years, the neuronal cholinergic system was explored and ACh was recognized as the neurotransmitter in the postganglionic parasympathetic system, the preganglionic neurons of the sympathetic system, the human axilla sweat glands and several brain regions. Acetylcholine is the essential neurotransmitter of the parasympathetic nervous systems (Lott & Jones, 2019). It is synthesized in the presynaptic neurons by the enzyme choline acetyltransferase (ChAT) from acetyl CoA and choline. Then, ACh is stored in secretory vesicles, where it enters with the help of the vesicular acetylcholine transporters (VAChT). Upon neuronal stimulation and membrane depolarization, ACh is released to the synaptic cleft where it binds to its receptors expressed in the postsynaptic membrane. In the cleft, and soon after its secretion, acetylcholine is hydrolyzed and converted into the inactive metabolites, choline and acetic acid, by the

enzyme acetylcholinesterase (AChE). Choline is then reabsorbed through a high-affinity choline transporter into the pre-synaptic neuron to be reused for ACh synthesis (Kawashima & Fujii, 2008). Figure 1 demonstrates the life cycle of ACh.

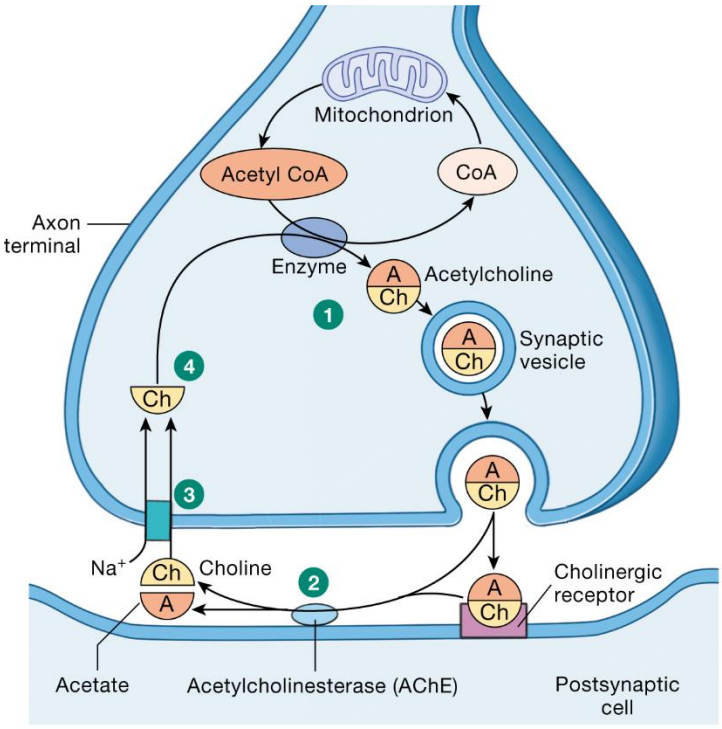

Figure 1: Acetylcholine life cycle. Acetylcholine is made from choline and acetyl CoA in the cytoplasm of the cell and stored inside secretory vesicles. Upon stimulation, ACh is released to the cleft where it binds to its receptors and soon hydrolyzed by the acetylcholinesterase enzyme into acetate and choline. Choline is transported back into the axon terminal to be used again for the synthesis of ACh (Blog, 2014).

Some drugs act as acetylcholinesterase inhibitors, causing the accumulation of acetylcholine in the cleft and, therefore, a hyperstimulation of the postsynaptic cell.

There are two types of ACh receptors: nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (mAChR). nAChRs are ligand-gated ion channels composed of five protein subunits that are arranged symmetrically like staves around a barrel. The subunit composition is totally different between different tissues. The nAChR subunits in neurons may be either α (ranging from α2 to α10) or β (ranging

from β2 to β4) and can be arranged as homomeric receptors such as α7 nAChR or heteromeric receptors such as (α4)2(β2)3. nAChR in muscles cells is an heteromeric structure composed of 2 α1 subunits and one of each of β1, γ (or ε) and δ (Abreu-Villaça, Filgueiras, & Manhães, 2011; Kawashima & Fujii, 2008). mAChRs are G-protein-coupled receptors categorized into five subtypes (M1, M2, M3, M4 and M5) that differ in their distribution in the central and peripheral nervous systems and in various organs and cells (Abreu-Villaça et al., 2011).

1.2 Non-neuronal cholinergic system

The word cholinergic refers to a system that not only uses the neurotransmitter acetylcholine (ACh) to communicate, but also is able to synthesize ACh, ACh transporters, ACh receptors and the enzymes for its degradation. In perspective of phylogenesis, before the nervous system was established, the non-neuronal cholinergic system (NNCS) already existed in non-neuronal cells such as bacteria, algae and protozoa (Wessler, Kirkpatrick, & Racke, 1999). In multicellular organisms, the skin is one of the best-known cholinergic organs. But apart from the skin, the NNCS has been described in many other organs such trachea, kidney, small and large intestines, and its role in disorders has already been described (Beckmann & Lips, 2013). Almost all types of cells seem to express cholinergic receptors, however, receptor expression alone is not an indication of NNCS, rather than its capacity to synthesize and releasing ACh. Cells that synthesize exclusively cholinergic receptors may serve as effector cells for non-neuronally as well as neuronally synthesized and released ACh. Non-neuronal cholinergic cells have the same molecular elements for synthesis and release of ACh than neurons (Wessler, Kilbinger, Bittinger, Unger, & Kirkpatrick, 2003). Thus, the

presence of an NNCS has to be classified before considering its involvement in organ-specific disorders.

1.2.1 Integumentary system

The integument consists of the skin and its derivatives, such as glands, hair and nails. In the skin, keratinocytes synthesize ACh in large quantities; a single keratinocyte is able to synthesize and release an average of 2×10^{-17} mol ACh and 7×10^{-19} mol ACh per minute, respectively (Grando, Kist, Qi, & Dahl, 1993). Moreover, keratinocytes express muscarinic and nicotinic ACh receptors, AChE and the transporters needed for an efficient auto-/paracrine cholinergic loop. NNCS in the skin regulates the keratinocyte connection, proliferation, differentiation, apoptosis, adhesion and migration (Grando, Pittelkow, & Schallreuter, 2006) which confers the NNCS an important role on the maintenance of healthy skin. In fact, NNSC seems to play a role in several dermatological diseases such Atopic dermatitis (Grando et al., 2006).

1.2.2 The digestive system

The digestive system comprises of pharynx, oesophagus, stomach, small and large intestines, and glandular organs such as liver, pancreas and salivary glands. Many cholinergic neurons innervate the digestive tract (Harrington, Hutson, & Southwell, 2010) and, therefore, there are many impacts that can contribute to neuronal rather than non-neuronal ACh release. M2 and M3 mAChR are responsible for mediating smooth muscle cell contraction in gastrointestinal tract, although they are less abundant but, functionally, more relevant than M1,M4 and M5 mAChR (Ruggieri & Braverman, 2013). Thuong Nguyen et al. (2000) showed that ChAT, AChE and various nAChR subunits can be found in the oesophageal epithelium and reduced

levels of ACh and signaling may result in reduced function of the oesophageal sphincter and eventually gastro-oesophageal reflux disease (Saegusa et al., 2011). Other studies reporter the involvement of the NNCS on cell-cell contact modulation and the alteration of elements needed for the synthesis and release of ACh (CHT1, ChAT, VAChT and OCT1) in the oesophageal mucosa under pathological conditions (Wolf-Johnston et al., 2012).

Inflammatory bowel diseases like ulcerative colitis and Crohn's disease have showed significantly reduced AChE activity and higher levels of miRNA-132, a post-transcriptional inhibitor of AChE (Maharshak et al., 2013). Interestingly, the response to nicotine varies between patients with Crohn's disease or ulcerative colitis as smoking clearly aggravates the symptoms for Crohn's disease (Hilsden, Hodgins, Timmer, & Sutherland, 2000), but it appears to have protective role in ulcerative colitis and may even decrease mucosal inflammation in this patients (De Jonge & Ulloa, 2007).

In patients with ulcerative colitis has been observed an increased expression of M2 mAChR in the colon epithelium and a downregulation of ChAT and α3-nAChR (Richardson et al., 2003). Using a mouse model of experimental colitis, α7-nAChR-deficient mice experimented an increased in severity of intestinal inflammation which confers to the α7-nAChRs a major role in inflammation (Ghia et al., 2009). The anti-inflammatory response to α7-nAChR stimulation, is most likely mediated in the intestine by resident immune cells expressing this receptor (Lakhan & Kirchgessner, 2011). In addition, the nAChR subunit α5 also appears to play a major role in colitis as nAChR subunit α5-deficient mice presented an increased susceptibility to experimental colitis (Orr-Urtreger, Kedmi, Rosner, Karmeli, & Rachmilewitz, 2005).

1.3 Cholinergic system in the immune cells

Many studies have demonstrated that most immune cells, including T and B cells, macrophages and dendritic cells (DCs), express the components required for the effective and independent cholinergic system: muscarinic and nicotinic ACh receptors, ChAT and AChE. Moreover, it has been suggested that the ACh produced by immune cells may modulate the immune function by regulating cytokine and antibody production (Fujii et al., 2017b).

1.3.1 ACh

ACh produced by immune cells was first described in human peripheral blood mononuclear leukocyte (MNL) fraction, where the majority are lymphocytes with a small fraction of monocyte (Kawashima, Kajiyama, Fujimoto, Oohata, & Suzuki, 1993; Kawashima, Oohata, Fujimoto, & Suzuki, 1989; Yamada, Fujii, & Kawashima, 1997). The detection of ACh in multiple human leukemic cell lines (Fujii et al., 1999; Fujii et al., 1996) as well as rat T and B lymphocytes verified the existence of ACh in immune cells (Rinner, Kawashima, & Schauenstein, 1998) which explained the presence of ACh in the spleen, despite of the absence of a cholinergic innervation (Fujii et al., 2017a). Moreover, the content of ACh in human leukemic T cell lines is greater than those of B cell lines, pre-lymphoma cell lines, or monocytic cell lines (Fujii et al., 1999; Kawashima & Fujii, 2000). Among rat lymphocytes, T cells had a considerably greater ACh content than B cells; and $CD4^+$ T cells produce more ACh than $CD8^+$ T cells (Rinner et al., 1998) and, as expected, higher content of ACh was correlated with greater ChAT activity (Rinner & Schauenstein, 1993).

1.3.2 ChAT

In 1993, Rinner and Schauenstein reported an ACh- synthesizing activity and the presence of ChAT in rat T and B cells present in thymus, spleen and blood (Rinner & Schauenstein, 1993). Later studies confirmed the presence of ChAT in T and B cells, macrophages, and DCs (Kawashima, Yoshikawa, Fujii, Moriwaki, & Misawa, 2007; Koarai et al., 2012) and their upregulation occurs in response to TLR agonists that elicit MyD88-dependent signal transduction in a cell-intrinsic manner (Reardon et al., 2013), indicating that immunological activation of T cells induces the upregulation of ACh synthesis that modulates the immune function (Fujii et al., 2017a).

1.3.3 ACh receptors

1.3.3.1 mAChR

Five different mAChR have been identified (M1, M2, M3,M4 and M5) (Bonner, Buckley, Young, & Brann, 1987; Bonner, 1989; Bonner, Young, Bran, & Buckley, 1988) and classified into two different groups based on their functional coupling (Fujii et al., 2017a). M1, M3 and M5 are coupled to the Gq/11 protein mediating the activation of PLC and, through second messengers, the release of Ca^{2+} from intracellular stores (Eglen & Nahorski, 2000). The other 2 subtypes, M2 and M4, are coupled to Gi/0 protein that mediates the inhibition of the adenylate cyclase and, therefore reducing the cAMP formation (Felder, 1995; Migeon, Thomas, & Nathanson, 1995). All subtypes of muscarinic AChR (M1, M2, M3,M4 and M5) have been found in T and B lymphocytes, DC, and macrophages of human, mice and rats (Fujii et al., 2017a; Kawashima, Fujii, Moriwaki, & Misawa, 2012; Kawashima et al., 2007). Their pattern of expression and intensities on the immune cells is variable and

dependent of their immunological status (Qian, Galitovskiy, Chernyavsky, Marchenko, & Grando, 2011). Moreover, it has been shown that M5 mAChRs are upregulated upon stimulation which suggest their involvement in the regulation of the immune function (Fujii et al., 2017a).

1.3.3.2 Nicotinic

Nicotinic receptors are pentamers composed of 1-5 different subunits. They have been described as 10 α (α1-10), 4 β (β1-4), γ, δ, and ε nAChR subunits. In all nAChRs, at least two copies of the α subunit are always present among the five subunits (Fujii et al., 2017a). Upon activation of the nAChRs there is an increase in the permeability of the membrane to Na^+, K^+ and Ca^{2+} which leads to depolarization and excitation of the membrane. In immune cells, Ca^{2+} influx specially through the α7nAChRs results in a strong anti-inflammatory effect on macrophages (Báez-Pagán, Delgado-Vélez, & Lasalde-Dominicci, 2015; Corradi & Bouzat, 2016; De Jonge & Ulloa, 2007; Egea et al., 2015). Most immune cells, including T cells, MNLs, DCs and macrophages express nAChR subunits. In these cells, mRNAs encoding for nAChR α2, α5, α6, α7, α9, α10 and β2 subunits were found to be expressed, while α3 and β3 were not encoded in any immune cell (Fujii et al., 2017b; Grando, Kawashima, Kirkpatrick, Kummer, & Wessler, 2015; Kawashima et al., 2012). Variable results have been reported for the presence of mRNAs for nAChR with α4, α9 and β4 subunits, (Kawashima et al., 2007).

1.3.4 AChE

To terminate the activity of the ACh, the enzyme AChE hydrolyzes ACh molecules into choline and acetate. This enzymatic breakdown takes place within few milliseconds which does not give time to the ACh to diffuse to the surrounding tissues.

Therefore, ACh must be released within the spaces where direct contact between cells occurs. Studies on C57BL/6J mice showed expression of AChE mRNAs in lymphocytes, bone marrow-derived DCs and macrophages (Kawashima et al., 2007). However, the physiological relevance of AChE in immune cells is not fully understood. Table 1 summarizes the present of cholinergic system in immune cells.

Table 1: Cholinergic system components in immune cells.

Immune cells	ACh	ChAT	AChR	AChE
T cells	√	√	√	√
B cells	√	√	√	-
Macrophages	-	√	√	√
DCs	-	√	√	√

1.4 Cholinergic modulation of the immune cells

In the body's response to infection, innate immune cells have an important protective role. Macrophages and neutrophils are the first immune cells recruited to the sites of infection where they have an effective function in the elimination of pathogens (Hurst et al., 2001; Zhang & Wang, 2014). It has been shown that nicotinic agonists can block the synthesis and release of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6 and HMGB1 in a dose-dependent manner from activated macrophages and macrophage cell lines. However, anti-inflammatory cytokines such as IL-10 are not usually affected (Borovikova et al., 2000; Sun et al., 2013; Wang et al., 2004; Wang et al., 2003). These studies involved pretreatment with non-selective and α7 selective AChR agonist followed by a challenge with LPS or another TLR-ligand to induce a cytokine response, (Khan et al., 2012; Wang et al., 2004; Wang et al., 2003).

Previous experiments suggested that nicotinic and muscarinic ACh receptors are present in immune cells to regulate their immune function. In immune cells, specific agonist stimulation of nAChR and mAChR induced an enhanced cytotoxicity, cGMP formation and intracellular Ca^{2+} signaling (Kawashima & Fujii, 2000). Culture of human leukemic T cell (CCRF-CEM) and B cell lines (Daudi) with ACh or mAChR agonists, like bethanechol and oxotremorine-M, induced a rapid increase of Ca^{2+} and up-regulation of C-fos expression, an intranuclear transcription regulator (Fujii & Kawashima, 2000). Moreover, these effects were found to be blocked by atropine, an antagonist for mAChRs (Kawashima et al., 2012). These findings suggested that ACh produced by T cells binds to the mAChRs expressed on their own plasma membrane or on other cells located nearby to activate nuclear C-fos signaling, acting as an autocrine and/or paracrine factor.

Other studies showed cholinergic activity during antigen presentation to naïve $CD4^+$ T cells. Naïve CD4 T cell are circulating until they recognize antigens presented on MHC class II molecules in secondary lymphoid organs by mature DCs. Recognition of antigen through specific receptors (TCRs) and interaction of CD80/CD86 (B7) co-stimulatory molecules via CD28 causes their activation. Following antigen presentation, an upregulation of ChAT mRNA expression, ChAT activity, ACh, AChE and M5 mAChRs was observed which indicates an active cholinergic activity upon stimulation (Fujii, Watanabe, Fujimoto, & Kawashima, 2003; Fujii, Watanabe, Inoue, & Kawashima, 2003; Kawashima & Fujii, 2003, 2004).

SLURP-1, a receptor protein that potentiates the α7nAChR (positive allosteric ligand) was demonstrated to be involved in the control of immune function in thymus and spleen, as well as peripheral blood MNLs, DCs and macrophages. Furthermore, $CD205^+$ SLURP-1^+ DCs are surrounded by $CD4^+$ T cells and other immune cells

expressing markers for monocyte/macrophages, DCs and B cells indicating strong association between SLURP-1$^+$ DCs and CD4$^+$ T cells, specific subtypes of DCs, macrophages and B cells during antigen presentation and other immunological reactions (Fukaya et al., 2012).

In vivo studies with α7nAChR-KO mice demonstrated an involvement of α7nAChR in the negative regulation of the immune function. Activation of α7nAChR inhibited the synthesis and release of pro-inflammatory cytokines (TNFα, IFNγ and IL-6) in spleen which, in turn, suppressed the production of IgG immunoglobulin (Fujii et al., 2007). These supported the existence of a non-neuronal cholinergic system in the immune cells that regulates the immune function.

Studies with in vivo disease models have shown that nicotine-based cholinergic stimulation prevents the development of rheumatoid arthritis (RA) by inhibiting the differentiation of CD4$^+$ T cells into Th17 cells, reducing the expression of the RORc transcription factor, the percentage of IL-17A$^+$ T cells percent, and the concentration of IL–17A; however it has no influence on the IL-17A$^+$ T cell proliferation.. Furthermore, stimulation of the cholinergic anti-inflammatory pathway could alter the distribution of Th-17 cells within the collagen-induced arthritis through the inhibitory Th-17 cell differentiation (Wu et al., 2018). Moreover, it has been demonstrated that pretreatment with AChE inhibitors prevented the development of hyperglycemia in STZ-treated C57BL/6 mice. The increased peripheral cholinergic activity was associated with a reduction in proinflammatory cytokines (IL-1β, IL-6, and IL-17) and to a shift in STZ-induced immune response from a predominantly disease-causing IL-17-expressing Th17 cells to IFNγ-positive Th1 cells. (George et al., 2016).

1.5 The inflammatory reflex

The fact that ACh is the major neurotransmitter in the cholinergic nervous system, and it also participates in the communication between cells in the immune system, suggested that ACh may participate in the interactions between the nervous and immune systems. Furthermore, immune cells, as discussed, express muscarinic and nicotinic receptors and the vagus nerve (a major parasympathetic nerve) innervates many organs including lymphoid tissues. It was in 2000 when K. Tracy's group demonstrated that the interaction between the immune and nervous systems is essential for the modulation of the inflammatory response (Borovikova et al., 2000). Initially, it was assumed that cholinergic stimulation of macrophages in the spleen was direct via the vagus nerve and mediated by the α7 AChR for which this pathway was named "cholinergic anti-inflammatory pathway" (Borovikova et al., 2000; Wang et al., 2003). Further studies showed that the vagus nerve does not innervate directly the spleen, rather it innervates the celiac ganglion and stimulates the adrenergic splenic pathway, which relays stimuli through splenic T cells to the splenic macrophages (Rosas-Ballina et al., 2008). The connection between the nervous system and the immune system was then well stablished. Therefore, both branches (cholinergic and adrenergic) of the autonomous nervous system regulate immune responses by what is called the "inflammatory reflex". The inflammatory reflex as shown in Figure 2 is able to boost the ability of afferent action in the periphery by the existence of endogenous and exogenous molecular products, which transmit the signal to the brain in the central nervous system (Pavlov, Wang, Czura, Friedman, & Tracey, 2003). The vagus nerve then transmit an efferent action potential and release ACh in the celiac ganglion. ACh binds to the α7 nAChR on the adrenergic splenic nerve that releases the neurotransmitter noradrenaline in the spleen, which binds to β2-adrenergic receptors

expressed in $CD3^{+}CD4^{+}CD25^{-}$ T cells (Vida et al., 2011) but only a particular subset of these cells ($CD4^{+}CD44^{hi}CD62L^{lo}$) will produce ACh in response to adrenergic stimulation (Peña et al., 2011; Rosas-Ballina et al., 2011). Afterwards, ACh will interact with α7 nAChR expressed on macrophages resulting in the suppression of proinflammatory cytokines (like IL-1 and TNF-α) production thus reducing the inflammatory responses (Borovikova et al., 2000). A shock model of the splanchnic artery occlusion (SAO) is characterized by systemic inflammatory reactions caused by increased TNF-α production and the accumulation of neutrophils in different organs, in particular lungs and liver, and ultimately organ failure. In an animal model of SAO, vagus nerve stimulation reduced NF-κb activation and decreased the mRNA TNF-α expression leading to a reduction of TNF-α levels in the circulation. Vagus nerve stimulation also reduced the accumulation of leukocytes in ileum and lungs probably due to the decrease in TNF-α which reduced the inflammatory cascade. These modulations by vagus nerve stimulation enhanced the time and rate of mice survival that led to a protective impact (Altavilla et al., 2006). In animal models of inflammatory diseases such acute kidney injury, obesity and collagen-induced arthritis, cholinergic stimulation using nAChR agonists lead to a decrease in the inflammatory reactions (Chatterjee et al., 2012; Van Maanen et al., 2009).

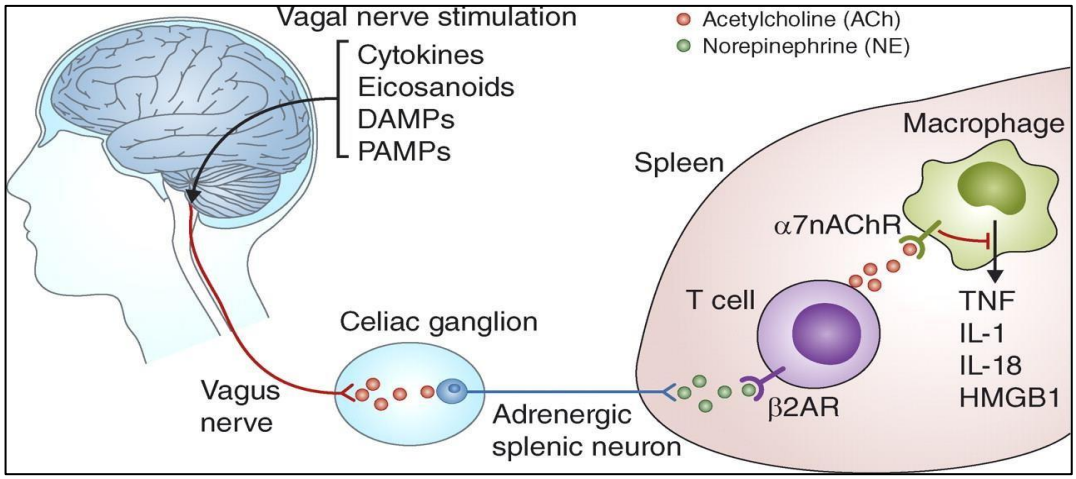

Figure 2: The inflammatory reflex. Prototypical immune regulation circuit reflex consists of afferent, efferent vagus nerve signals that react to diseases and wounds in molecular substances, such as cytokines, eicosanoides, DAMPs and PAMPs. The induction of neurons in the spleen results in norepinephrine produced in the vicinity of T cells which can secrete acetylcholine. Acetylcholine passes the marginal area and reaches into the red pulp, where it interacts with α7 nAChR expressed on cytokine producing macrophages. The production and release of TNF, IL-1, IL-18, HMGB1, and other cytokines are inhibited with α7 nAChR signal transduction (Andersson & Tracey, 2012).

Using live infectious models, some studies have shown that the decreased in production of pro-inflammatory cytokines and cell recruitment to the site of infection induced by cholinergic stimulation, affected the bacteria clearance and, therefore the animal survival rate which was lower than in the control non-stimulated group (Van Westerloo et al., 2005). This impact was overturned in α7 nAChR deficient mice where the recruitment of neutrophils was faster (Giebelen et al., 2008). On the other hand, using a murine model of endotoxemia and sepsis, cholinergic stimulation and activation of the inflammatory reflex through selective α7 nAChR agonist, vagus nerve stimulation and other ACh imitating agents was able to improve the survival of infected animals (Pavlov, 2008). Moreover, cholinergic stimulation induced a reduction in pro-inflammatory cytokines such TNF-α, protecting the vital organs from TNFa-induced tissue injury during sepsis, and preventing multiple organ failure

(Borovikova et al., 2000; Van Westerloo et al., 2005). Furthermore, pretreatment with an specific AChE inhibitor, paraoxon, enhanced the survival of mice infected with lethal *S. Typhimurium* (Al-Barazie et al., 2018; Fernandez-Cabezudo et al., 2010). Furthermore, the activation of the M3 mAChR enhanced Th1 and Th2 adaptive immune reactions in *Salmonella* infection by enhancing IFN-γ and IL-13 production respectively, which result in protection (Darby et al., 2015).

1.6 Immuno-physiology of the gastrointestinal tract

Digestion and absorption of distinct nutrients required for survival are the primary roles of the gastrointestinal system (GIT). GIT is also considered the primary defense against microorganisms and food antigens that are orally ingested. The defense mechanisms include saliva, gastric acidity, intestinal mucosa, peristaltic movement, epithelial cell membrane and intracellular junctional complexes that inhibit antigens translocation and insure their elimination (Singh et al., 2009).

The small intestine is human body's longest organ, and the most significant component of digestion. The adult small intestine is about 16 feet and has 1-inch diameter, but due to the presence of villi and microvilli its surface of absorption is 500 times higher (Santaolalla, Fukata, & Abreu, 2011). There are three separate components in the small intestine: duodenum, jejunum and ileum where nutrient intake takes place.

The gut is continually exposed to a variety of dietary and environmental pathogenic and non-pathogenic antigens. To maintain gut homeostasis specifically in intestine, the body has developed various mechanisms including the mucosal and epithelial barrier, intestinal innate and adaptive system and pro-inflammatory signaling pathways (Feng & Elson, 2011). These mechanisms control the gut flora and the pathogenic population

that drives inflammation (Geremia, Biancheri, Allan, Corazza, & Di Sabatino, 2014; Konrad, Cong, Duck, Borlaza, & Elson, 2006).

In the intestinal mucosa, the first layer of protection against pathogens is the epithelium facing the intestinal tract's luminous surface. It consists of several types of cells that originate from progenitor stem cells: absorptive enterocytes, goblet cells which produce mucus, enteroendocrine hormone-producing cells, Paneth cells that produce anti-microbial peptides, and M cells, specialized in antigen testing (Figure 3). Below the epithelium is the lamina propria, which contains immunocompetent cells such T, B cells, macrophages and DC (Niess et al., 2005; Rescigno, 2006).

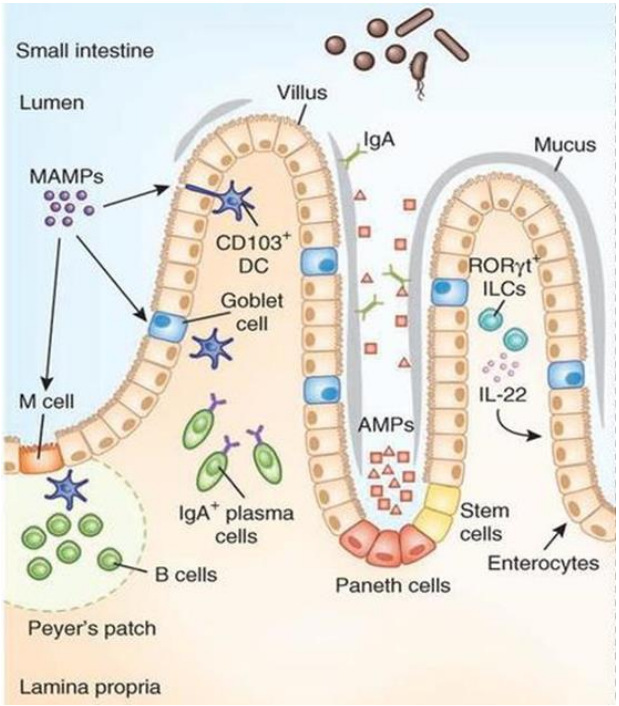

Figure 3: Components of the intestinal epithelial barrier in small intestine. The intestinal epithelium in the small intestine consists of a single layer of enterocytes interspersed by specialized cells. Goblet cells between the epithelial cells responsible to produce mucus. Paneth cells in the crypt release antimicrobial peptides (AMP). Antigen testing across the intestinal barrier is mediated by microfold (M) cells. Below the epithelium is the lamina propria, which contains immunocompetent cells such T and B cells, and dendritic cells (DC) (Bossche, 2017).

The innate immune response in the mucosa is mainly driven by macrophages and dendritic cells. Dendritic cells are located below the intestinal epithelium but the cytoplasmic extensions of these cells are interdigitated between the epithelial cells in order to face the luminal contents and present foreign components to T cells in the lamina propria and underlying lymphoid follicles (Niess et al., 2005; Rescigno, 2006). T and B cells are the components of the adaptive immune system in the LP and Peyer's patches. However, in the intestinal environment it is not worth separating adaptive and innate immune responses, since there is an intimate relationship to the microbiota present in gut (Santaolalla et al., 2011).

An important component of the mucosal barrier is the mucous layer produced by goblet cells. This mucous layer constitutes a perfect niche for microbiota to live protected from being washed out and, at the same time serves as a nutrient for some members of the microbiota like saccharolytic bacteria (Garrett, Gordon, & Glimcher, 2010). The intestinal mucins produced by the goblet cells are arranged in a bilayer composed of a firm inner layer free from bacteria and a looser external layer (Johansson et al., 2008). The most abundant type of intestinal mucin is MUC2 and, in fact, mice with missense MUC2 mutations had been shown to develop intestinal chronic inflammation similar to ulcerative colitis (UC) in humans. Moreover, defective mutant MUC2 multimers caused stress conditions in the endoplasmic reticulum (ER) and enhanced the unfolded protein response (Heazlewood et al., 2008). Finally, in human UC, the intestine frequently shows a loss of goblet cells and therefore, mucus depletion (Gersemann et al., 2009).

Paneth cells present in the intestinal crypts, produce different antimicrobial molecules such as zinc, lysozyme, and defensins that play a major role in a host defense against antigens. The release of antimicrobial molecules from Paneth cells is induced by

microbiota through cell-autonomous MyD88-dependent activation of toll-like receptors (TLRs), and it helps in protecting the epithelial barrier from breaking by commensal and pathogenic bacteria (Vaishnava, Behrendt, Ismail, Eckmann, & Hooper, 2008). Several studies have shown the significance of Paneth cells for the intestinal homeostasis (Kaser & Blumberg, 2009).

1.6.1 Gut-associated lymphoid tissue (GALT)

Gut-associated lymphoid tissue (GALT) is a component of the mucosa-associated lymphoid tissue (MALT) that protect the body from pathogen invasion in the gut. GALT includes Peyer's patches, mesenteric lymph nodes and different scattered lymphocytes present in the intestinal lamina propria and intestinal epithelium (Forchielli & Walker, 2005).

1.6.1.1 Peyer's patches

Peyer's patches are aggregated lymphoid nodules, are small organized lymphatic tissue found in the small intestine, specially in the ileum region. About 100 of them are found in humans and 8-10 in mice. They play an important role in the intestine where they direct the intestinal bacteria populations and inhibit the growth of pathogenic bacteria. In general, structurally PPs have three different compartments: the follicle-associated epithelium (FAE), subepithelial dome (SED) and follicular region as shown in Figure 4.

- Few characteristics make FAE different from typical small intestinal villus epithelium. FAE has a smaller number of goblet cells, and consequently thinner mucus layer (Ermund, Gustafsson, Hansson, & Keita, 2013), and contains antigen-sampling microfold cells (M cells), specialized in the absorption and

transport of lumen antigens (Owen & Jones, 1974). In fact, the cytoplasm of M cells contains tiny transcytosis endocytic vesicles that allow these cells to sample the contents of lumen (particular and pathogenic matter) and transport them to the basolateral membrane throughout the epithelium. FAE is less permeable to ions and macromolecules due to the high expression of tight junction proteins (Markov, Falchuk, Kruglova, Radloff, & Amasheh, 2016). Finally, as opposed to intestinal villus, the basal lamina of follicle-associated epithelium is more porous compared to intestinal villus (Takeuchi & Gonda, 2004).

- In the SED area dendritic cells (DCs) are very abundant making up ~10% of the total PPs population. DCs are able to uptake and process the antigens transported by M cells and present them to mucosal T and B cells in order to produce antigen-specific responses.
- In the follicular area, the most abundant cells are B cells, representing approximately 60% of the population. T cells are mostly located in the interfollicular region (IFR) of the PPs and represent around 25% of the population. 45% of the T cells are $CD4^+$, 35% are $CD8^+$ and 20% are $CD4^-/CD8^-$ T cells (Iwasaki & Kelsall, 2000). Among the T cells, $CD4^+/CD25^+$ (10%) cells and $CD8^+/CD25^+$ (5%) cells are richer in Peyer's patches than in the peripheral blood (Jung, Hugot, & Barreau, 2010).

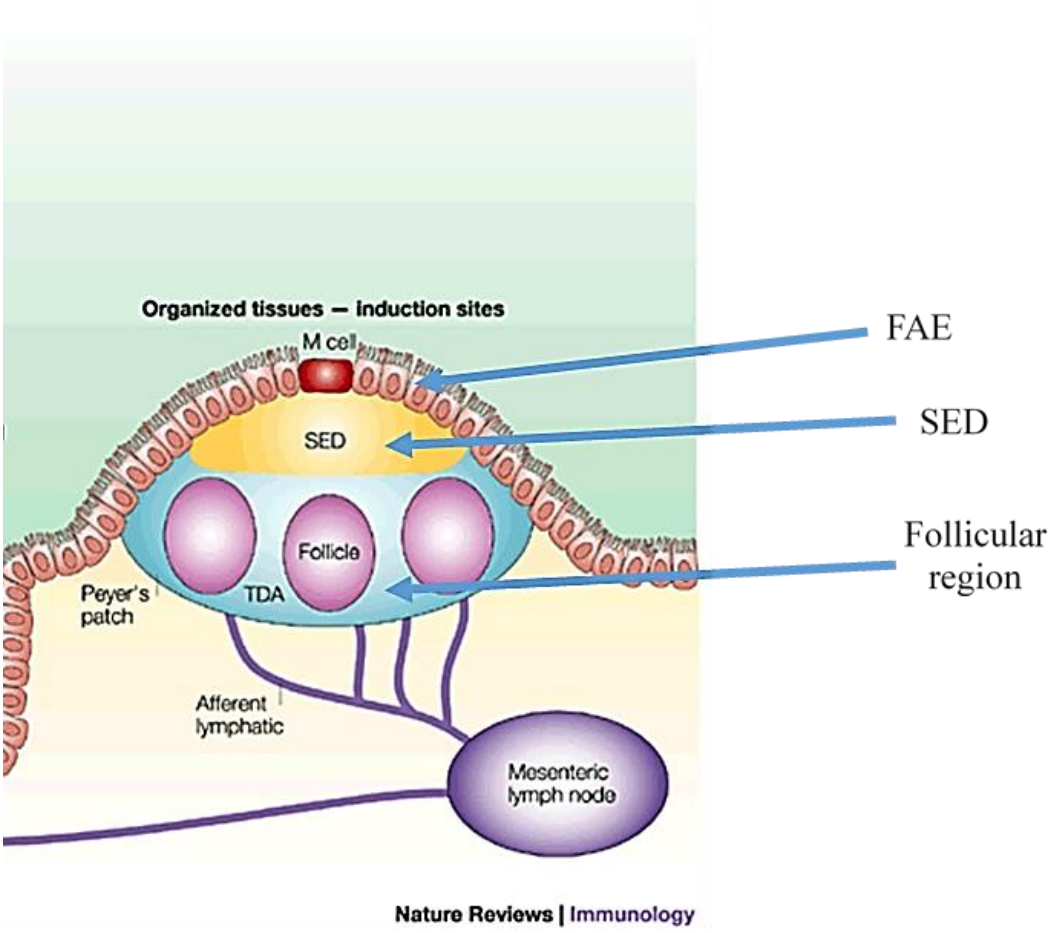

Figure 4: PPs three different compartments. The follicle-associated epithelium (FAE), subepithelial dome (SED) and follicular region (Mowat, 2003).

1.6.1.2 The mesenteric lymph nodes (MLNs)

They present in the intestinal mesentery are the primary sites where nutrients and microbial agents are taken by lymph fluid from the intestinal lamina propria. They serve as central sites where tolerance to food particles is induced at the same time that avoid the systemic spread of microorganisms. In each MLN, three areas can be observed: (a) the cortex with follicles consisting of B cells and dendritic cells, (b) paracortex that contains large proportion of T-cells and dendritic cells and finally, (c) the medulla, composed of B cells and T cells (Ramiro-Puig, Perez-Cano, Castellote, Franch, & Castell, 2008). Antigen presenting cells carry the antigen and transport it

from the intestinal epithelium to the MLNs to be presented to the naïve T cells in the paracortex where they initiate the adaptive immune response. Subsequently, the activated T cells leave the MLNs and migrate to other organs like liver and spleen (Kobayashi et al., 2004; Ramiro-Puig et al., 2008). B cells in MLN contribute to the release of secretory IgA in the gut lumen (Yamamoto et al., 2000).

1.6.1.3 The intestinal mucosa

Constituted by the intestinal epithelium, the basement membrane and the lamina propria (LP). LP is a thin layer of loose connective tissue lying below the epithelium, that contains a population of very different cells like fibroblasts, lymphocytes, plasma cells, macrophages, DCs, eosinophilic leukocytes, and mast cells. The epithelium contains IELs (intraepithelial lymphocytes) in the interepithelial space between the tight junctions and the basal membrane of the gut epithelium (Lefrançois & Lycke, 1996).

The lymphocytes in this intestinal compartment are T cells, both $CD8^+$ and $CD4^+$, which do not need antigen presentation, instead they interact directly with the antigen and produce cytokines causing the killing of infected target cells (Lefrançois & Lycke, 1996), (Hopper et al., 2006). Intraepithelial $CD8^+$ T lymphocytes can be divided into two major groups, based on expression of either an αβ T-cell receptor (TCR) or a γδ TCR. The percentage of both groups are almost equal in mice, while in humans the majority of IELs express αβ TCR with only 15% γδ TCR^+ (Turvey & Broide, 2010). Many important immune functions have been related to γδTCR^+ T cells, including rapid production of cytokines that may regulate pathogen clearance, inflammation, and intestinal homeostasis. γδTCR^+ T cells stimulate inflammation by the production of IL-17 or IFN-γ that induce the lysis of infected cells through lytic granules or death

receptors (Hamada et al., 2008; Martin, Hirota, Cua, Stockinger, & Veldhoen, 2009), at the same time, they inhibit inflammation by the production of IL-10 (Rhodes, Andrew, Newton, Tramonti, & Carding, 2008) and produce keratinocyte growth factor (KGF) that regulates epithelial cell integrity and healing. It has been shown that absence of γδ TCR^+ T cells causes defects in the tight junction formation resulting in increased intestinal permeability, and translocation of pathogens across the epithelial barrier in infection models (Dalton et al., 2006).

Finally, the intestinal mucosa contains the innate lymphoid cells (ILCs), a recently described family of lymphocytes with phenotypes and functions that are similar to T cells. However, ILCs are present mostly in non-lymphoid tissue and lack the expression of lineage markers and antigen receptors and do not undergo clonal selection and expansion upon stimulation. Instead, they respond directly to various stimuli and produce cytokines that aid in the initiation of immune responses. ILCs develop from a common lymphoid progenitor and their differentiation depends on the expression of specific transcription factors that will suppress the generation of B and T cells to direct the production of the different types of ILCs. ILCs have been classified into three groups (ILC1, ILC2 and ILC3) depending on the transcription factor expressed and cytokines produced as shown in Figure 5 (Eberl, Colonna, Di Santo, & McKenzie, 2015). ILC1s, ILC2s, and ILC3s are the "innate" equivalents of Th1, Th2, and Th17 $CD4^+$ effector cells, respectively, based on the type of cytokine produced (Eberl et al., 2015): ILC1 and Th1 cells can produce IFNγ and TNFα; ILC2, like Th2 cells, can produce IL-4 and IL-13; ILC3, like Th17 cells, can produce IL-17 and IL-22. Moreover, similar transcription factors are required for the differentiation and development of Th cells and ILCs: T-bet for Th1 and ILC1, GATA-3 for Th2 and

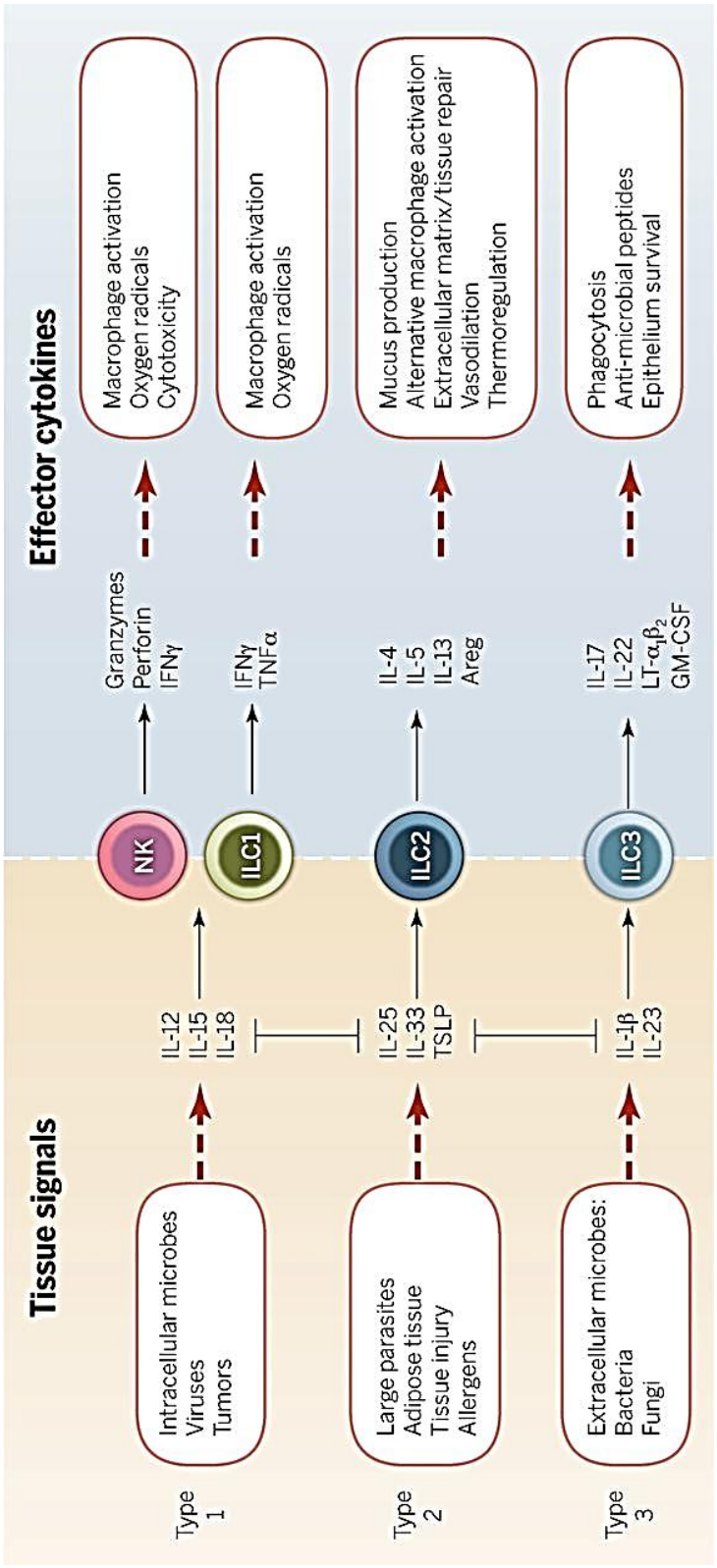

Figure 5: Activation and functions of ILCs. The tissue signals expand and activate ILC1s, ILC2s, and ILC3s depending on specific transcription factor. ILC directly response to the stimuli and produce the cytokines that develop the immune response (Eberl et al., 2015).

ILC2 and RORrt for Th17 and ILC3 (Artis & Spits, 2015; Zhong & Zhu, 2015; Zook & Kee, 2016).

1.7 Effect of the cholinergic system on the GI tract

Several studies showed the effect of cholinergic system and its components on various intestinal activities like motility, secretions and material absorption. Moreover, the cholinergic system is able to modulate the innate immune response in different intestinal diseases like inflammatory bowel disease (IBD). During surgical manipulation of the intestine, macrophages are activated, inducing the production of inflammatory cytokines, upregulation of adhesion molecules, and invasion of neutrophils and other leukocytes. To avoid these consequences, Mattioli and his colleagues showed that electrical vagus nerve stimulation (VNS) for 5 minutes before intestinal manipulation avoided macrophages activation and preserved GI motor function. However, using the intestinal muscularis inflammation model, splenectomy did not have any effect on the anti-inflammatory response, indicating that VNS exerts a direct effect over the GI immune system preventing postoperative ileus (Matteoli et al., 2014)

In other studies where colitis was induced in rats by TNBS (2,4,6-trinitrobenzene sulfonic acid) administration, pretreatment with AChE inhibitors (neostigmine and physostigmine) decreased colitis severity (Meregnani et al., 2011; Miceli & Jacobson, 2003). Moreover, using the same TNBS model in mice, subdiaphragmatic bilateral vagotomy or removal of the splenic nerve caused an increase in disease severity of colitis (Munyaka et al., 2014; O'Mahony, van der Kleij, Bienenstock, Shanahan, & O'Mahony, 2009). Other studies have shown that defects in nAChRs on immune cells could lead to the development of IBD. However, many other factors such as smoking

could affect the development and progression of IBD (Cosnes, 2008; Lunney & Leong, 2012) . Moreover, AChE microRNA-132 (miR-132) which function is to regulate the expression levels of other genes, has been proposed to have some potential in decreasing AChE in immune cells and, therefore, to attenuate inflammation (Shaked et al., 2009).

Using an infectious model, it has been demonstrated that AChE inhibition and, therefore, activation of the cholinergic pathway is able to induce a protection against virulent oral enteric pathogens. This protection was correlated with massive degranulation of two types of intestinal secretory cells, goblet and Paneth cells, which enhanced the gastrointestinal barrier defense mechanisms (Al-Barazie et al., 2018). All these studies highlight the anti-inflammatory effect that cholinergic activation exerts on the intestine.

1.8 *Salmonella* infection

Salmonella spp. are Gram-negative flagellated bacteria that can cause food and water-borne gastroenteritis and typhoid fever in humans (Mathur et al., 2012). *Salmonella enterica* serovar *Typhimurium* causes a systemic infection in mice that resembles typhoid fever caused by *S. enterica* serovar Typhi in humans. All *Salmonella* species are facultative anaerobic intracellular pathogens (Jantsch, Chikkaballi, & Hensel, 2011) that can invade several types of cells like epithelial cells, M cells, macrophages and dendritic cells (LaRock, Chaudhary, & Miller, 2015).

In order to infect the host, *Salmonella* display a variety of mechanism to cross the gastrointestinal epithelial barrier. First, *Salmonella* induce membrane ruffling in M cells to gain access and infect M cells present in the intestinal epithelium located above the Peyer patches. After infection, M cells die and *Salmonella* is translocated to the

Peyer's Patches (PP) (Jones, Ghori, & Falkow, 1994). The subepithelial DCs in the PPs take up *Salmonella* following M cell invasion (Hopkins, Niedergang, Corthesy-Theulaz, & Kraehenbuhl, 2000). After exposure to antigen (*Salmonella*), immature DCs become mature DC and present the microbial peptide to naïve B and T-cells in the mesenteric lymph nodes (Murphy, Travers, & Walport, 2012). *Salmonella* can also directly interact with IECs to get internalized (Finlay & Falkow, 1990) and translocate to the intestinal lamina propria (Chieppa, Rescigno, Huang, & Germain, 2006; Niess et al., 2005).

Intestinal epithelial cells can also directly recognize invading pathogens through the interaction between pathogen-associated molecular patterns (PAMPs) present in bacteria and Toll-like receptors in the epithelial cells. Each type of TLRs recognizes a unique PAMP. It is TLR4 that recognizes the lipopolysaccharide (LPS) from Gram-negative bacterial cell walls (Hallstrom & McCormick, 2011). Upon PAMP recognition the immune response is initiated by activation of the NFκB that leads to the production of proinflammatory cytokines and chemokines. Secretion of IL-8 induces recruitment of neutrophils while CCL20 chemokine production attracts immature dendritic cells (Patel & McCormick, 2014). *Salmonella* can also interact with TLRs in macrophage and induce the production of different cytokines such as IL-1, IL-6, and IL-23 which are important for Th17 cell differentiation and recruitment of neutrophils (Godinez et al., 2009; Murphy et al., 2012). Activated macrophages can also produce IL-18 and IL-12 that are involved in the production of IFNγ cytokine (Murphy et al., 2012).

1.9 Acetyl cholinesterase inhibitors

Several diseases that result from cognitive shortages in elderly people are the reasons behind the discovery of cholinesterase inhibitors. Since ACh has been demonstrated to be involved in cognitive processes, the idea of increasing acetylcholine levels by inhibiting the cholinesterase enzyme to restore cognitive loss "cholinergic hypothesis" has got much attention and interest (Kandiah et al., 2017; Martorana, Esposito, & Koch, 2010).

Degeneration of cholinergic neurons in the brain causes a reduction in the levels of acetylcholine and cholinergic function, which lead to diseases such as Alzheimer's disease (AD). Most AChE inhibitors bind to the active site of the AChE enzyme and inhibit the binding of ACh, reducing the degradation of synaptic acetylcholine, improving brain acetylcholine levels in a dose-dependent manner and enhancing cholinergic transmission in patients with Alzheimer's and other dementias. AChE inhibitors have some side effects such as, constriction of the pupils in the eyes, vasodilation, increased secretion of sweat, saliva and tears, slow heart rate, mucus secretion in the respiratory tract and constriction of the airways (Kandiah et al., 2017). AChE inhibitors can be divided into two groups: reversible and irreversible. The reversible inhibitors, competitive or non-competitive, are used as therapeutic agents. Reversible acetylcholinesterase inhibitors have temporary effects on the enzyme maintaining the functionality of the active site. Once the reversible inhibitors are removed, the acetylcholinesterase enzyme recover its activity. On the other hand, irreversible AChE inhibitors used at high dose have toxic effect through non-reversible phosphorylation of esterases in the central nervous system. The acute toxic effects are related to irreversible inactivation of AChE (Colovic, Krstic, Lazarevic-Pasti, Bondzic, & Vasic, 2013). The typical symptoms of acute poisoning are agitation,

muscle weakness, muscle fasciculations, miosis, hypersalivation and sweating. Severe poisonings may cause respiratory failure, unconsciousness, confusion, convulsions and/or death (Costa, 2006; World-Health-Organization, 1986). Most of the organophosphorus used as pesticides are irreversible acetylcholinesterase inhibitors.

Rivastigmine ((S)-3-[1-(dimethylamino)ethyl]phenyl N-ethyl-N-methylcarbamate) is a pseudo-irreversible, carbamate-type, brain-selective, dual acetyl- and butyryl-cholinesterase (BuChE) inhibitor (Kandiah et al., 2017). Rivastigmine has been approved in 60 countries including all members of the European Union and the USA (Birks, Chong, & Grimley Evans, 2015) for the treatment of Alzheimer's and other dementias as it reduces the degradation of synaptic acetylcholine and, therefore, increases the levels of ACh available for synaptic transmission which causes symptomatic therapeutic effects. The pharmacokinetic profile showed that compared with the oral formulation, rivastigmine transdermal patch provides smoother and continuous as well as controlled drug delivery over 24 h, thereby resulting in fewer side effects (Kandiah et al., 2017).

Previous studies showed that chronic administration of a specific and irreversible inhibitor of AChE, paraoxon, improved the survival of mice following an oral infection with virulent *Salmonella* bacteria. This was mediated by cholinergic pathway-dependent enhancement of anti-microbial immune defenses acting at the level of the gastrointestinal mucosal surfaces (Al-Barazie et al., 2018). However, paraoxon is an organophosphorus compound unsuited for human use, therefore, the study aimed to test the efficacy of rivastigmine, an FDA-approved inhibitor of AChE, on mucosal defenses against *Salmonella* infection. Results show that rivastigmine treatment, previous to an oral infection with a virulent strain of *Salmonella* increased the survival of mice orally infected and delayed the translocation of pathogenic bacteria to the

systemic sites. Results showed that rivastigmine induced the degranulation of two specialized intestinal secretory cells involved in innate immunity, goblet cells and Paneth cells, and increased the thickness of the mucin layer in colon tissue. Immunohistochemical study of the ileum revealed that rivastigmine treatment resulted in few changes in the lymphoid population in the epithelium and lamina propria (LP). Further, evidence provided by flowcytometric phenotypic analysis that rivastigmine does not induce recruitment of T cells to the intestinal mucosa as effectively as paraoxon, suggesting the essential role that these cells play on the protection against an oral infection. The findings demonstrate an effect of cholinergic stimulation on immune cells that is correlated with the type of AChE inhibitor and, therefore, with the availability of ACh to bind their receptors.

Chapter 2: Hypothesis

Cholinergic stimulation using paraoxon, an irreversible AChE inhibitor, enhanced innate immunity at the level of intestinal epithelium by increasing goblet and Paneth degranulation and, therefore, inducing mucus and antimicrobial peptides secretion, respectively. Paraoxon treatment was also able to enhance protection against oral infection with virulent *Salmonella*. Depending on that, the first hypothesis of this study is cholinergic stimulation by rivastigmine, a more physiologically relevant AChE inhibitor, will also be able to exert the same effect as paraoxon. Also given the fact that immune cells express ACh receptors, so the second study hypothesis is cholinergic stimulation could also modulate the immune population present in the intestinal mucosa and contribute to the enhanced defense against bacterial infection as shown in Figure 6.

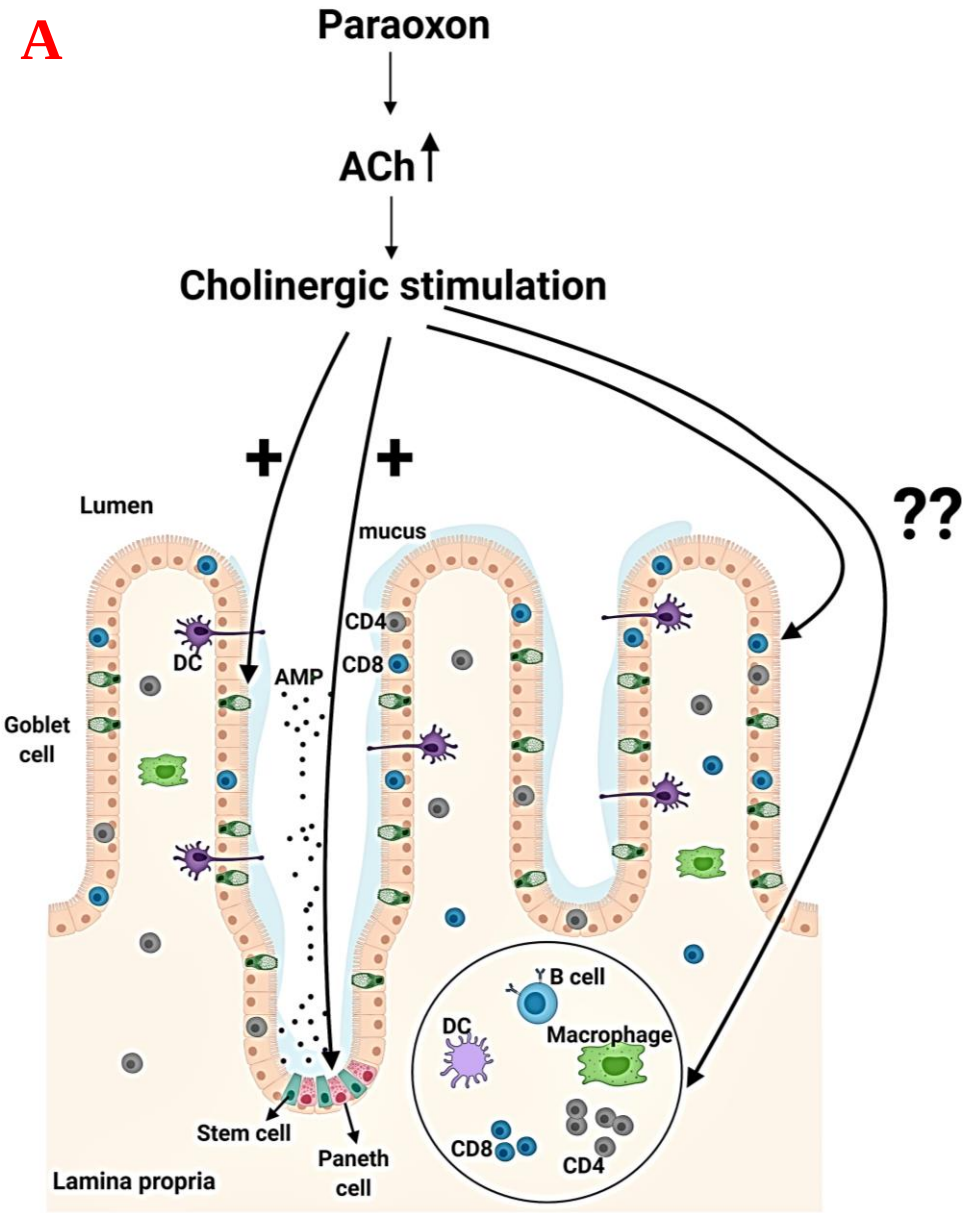

Figure 6: Project hypothesis. Schematic diagram representing the effect of AChE inhibition using paraoxon (A) or rivastigmine (B) on goblet and Paneth cells in the intestinal epithelium, which enhances protection against *Salmonella*. The hypothesis is that AChE inhibition also exerts an effect on professional immune cells in the intestinal mucosa.

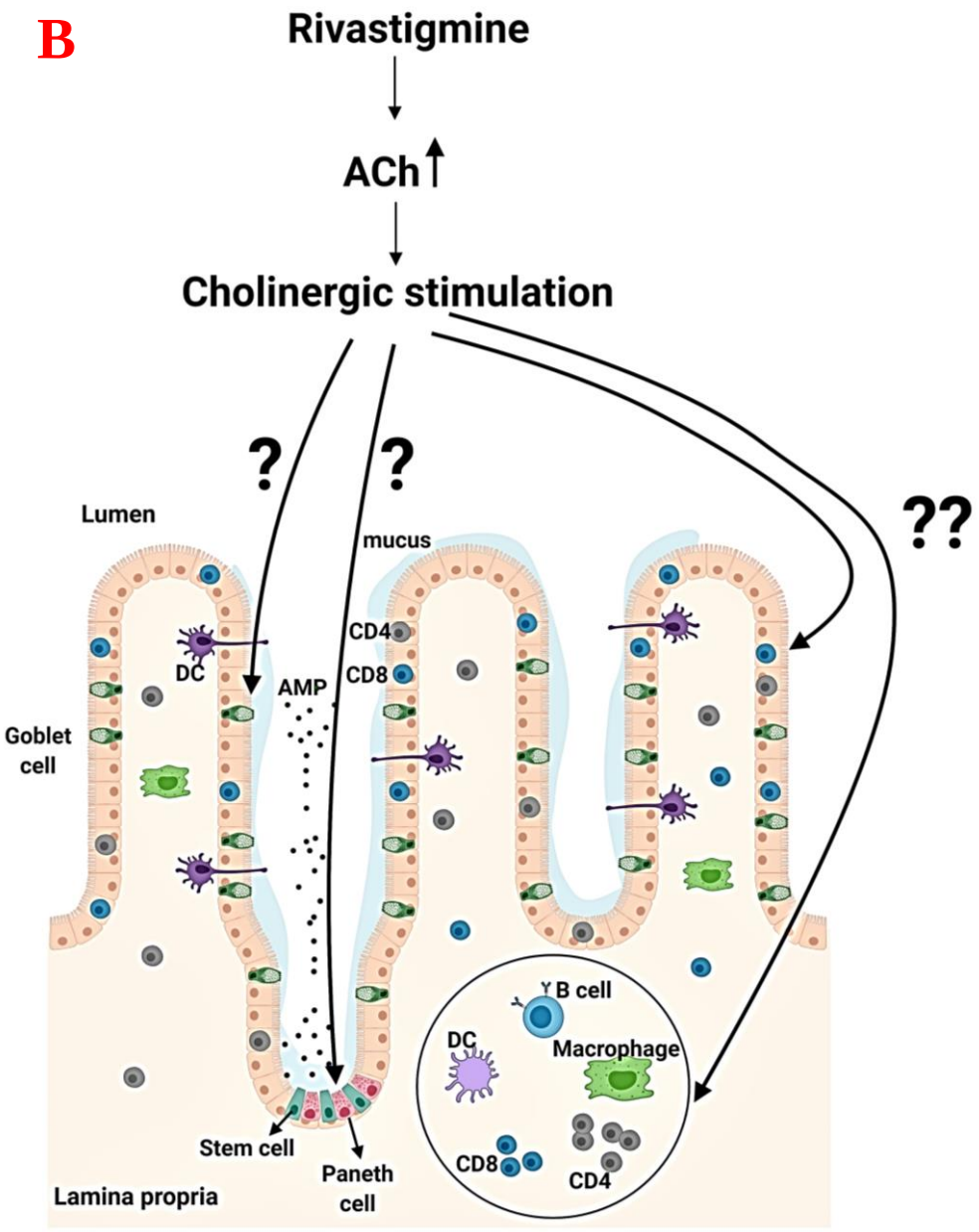

Figure 6: Project hypothesis. Schematic diagram representing the effect of AChE inhibition using paraoxon (A) or rivastigmine (B) on goblet and Paneth cells in the intestinal epithelium, which enhances protection against Salmonella. The hypothesis is that AChE inhibition also exerts an effect on professional immune cells in the intestinal mucosa (Continued).

Chapter 3: Aim and Objectives of the Study

Previously, it has been demonstrated that cholinergic stimulation using paraoxon, a specific and irreversible acetylcholinesterase (AChE) inhibitor, enhanced survival in mice following an oral infection with virulent *Salmonella enterica* serovar *Typhimurium* (*S. Typhimurium*). However, paraoxon is an organophosphorus compound unsuited for human use. This study aimed to investigate the effectiveness of rivastigmine, an FDA-approved inhibitor of AChE, on mucosal defenses against *Salmonella* infections.

The specific objectives were to:

- Characterize the effect of AChE inhibition by rivastigmine on mouse survival following oral infection with virulent *S. typhimurium.*
- Determine the effect of rivastigmine treatment on the bacterial load in the feces, intestinal content and organ homogenates at an early time point after infection.
- Investigate by electron microscopy the ultrastructural changes in intestinal epithelial cells, specially Paneth cells and goblet cells, following rivastigmine treatment.
- Investigate by light microscopy the effect of rivastigmine treatment on the secretion of mucin and lysozyme by goblet and Paneth cells, respectively.
- Investigate by immunohistochemistry the effect of rivastigmine on the distribution of the different immune cell populations in the intestinal mucosa. Where possible, quantification of labeled cells will be performed.
- Evaluate by flow cytometry the effect of AChE inhibition by paraoxon or rivastigmine on the cellular phenotype and activation status of lymphoid and myeloid cell populations present in the intestinal epithelium and lamina propria.

Chapter 4: Materials and Methods

4.1 Materials

All materials, standard solutions or antibodies used to conduct the experiment are mentioned below.

4.1.1 Standard solutions

Different solutions used for the different techniques. Table 2 shows the preparation of each solution.

Table 2: Standard solutions

Solution	Preparation
0.1 mol/ml phos phate buffer	• Solution 1: 17.8 g of $Na_2HPO_4.2H_2O$ in 1L distilled water • Solution 2: 2.27 g of KH_2PO_4 in 200 ml distilled water To adjust the pH, solution 2 was added, gradually, to solution 1 until pH became 7.4, then, it was filtered and stored at 4°C.
10 mM DTNB	396.3 mg DTNB in 100 ml phosphate buffer. Stored at -20°C.
6 mM ethoprorazine	20.94 mg in 10 ml of 12 mmol/L HCl. Stored at -20°C.
Transformation buffer	200 mg potassium ferricyanide, 50 mg potassium cyanide, 1g sodium bicarbonate and 500 µl 100% Triton X-100 in 1L distilled water
10x PBS	87.66 g NaCl, 2.56 g $NaH_2PO_4.H_2O$ and 11.94 g Na_2HPO_4 dissolved in 1 L of distilled water.
Staining buffer for flow cytometric analysis	1 ml fetal calf serum and 0.1 g NaN_3 was dissolved in 100 ml PBS.
HBSS	0.4 g KCl, 0.09 g $Na_2HPO_4.7H_2O$, 0.06 g KH_2PO_4, 0.35 g $NaHCO_3$, 0.14 g $CaCl_2$, 0.1 g $MgCl_2.6H_2O$, 0.1 g $MgSO_4.7H_2O$, 8 g NaCl and 1 g D-glucose in 1 L distilled water, pH adjusted to 7.4 and stored at 4°C.
Retrieval solution for immunocytochemistry	1.21 g Tris base, 0.37 g EDTA with 1000 ml water, pH adjusted to 9.
Blocking buffer (BSA) for immunocytochemistry	1 g albumin from bovine serum with 100 ml PBS with tween.
Pre-digestion solution for intestinal lymphocyte isolation	15 mM EDTA, 5% FBS, 100 mM DTT and HBSS (w/o)
Enzyme mix mixture for gentleMACS	100 µL of Enzyme D + 50 µL of Enzyme R + 12.5 µL of Enzyme A into a gentleMACS C Tube containing 2.35 ml of pre-heated digestion solution.
Carnoy's fixative	60 ml of methanol, 30 ml of chloroform and 10 ml of acetic acid.
10% Formalin for fixation	100 ml 37% formaldehyde in 900 ml PBS.
HBSS without calcium/magnesium (HBSS w/o)	0.4 g KCl, 0.09 g $Na_2HPO_4.7H_2O$, 0.06 g KH_2PO_4, 0.35 g $NaHCO_3$, 8 g NaCl and 1 g D-glucose, 10 ml of 1 M HEPES in 1 L distilled water, pH adjusted to 7.4 and stored at 4°C.

4.1.2 List of materials used and suppliers

All chemicals used in different techniques are shown in Table 3.

Table 3: List of materials used and suppliers

Chemicals	Company	Catalog #
Paraoxon ethyl	Sigma	36186
Rivastigmine	Sigma	4617
$Na_2HPO_4.2H_2O$	Panreac	131679.1210
NaCl	Sigma	102H3660
NaN_3	BDH	UN No /687
D-glucose	Sigma	G-5767
$NaHCO_3$	Gibco-BRL	11810-074
Lead citrate	Agar scientific	R1210
uranyl Acetate	Agar scientific	R1260
Glutaraldehyde	Agar scientific	R1011
Agar 100 epoxy	Agar scientific	R1043
Dodecenyl Succinic Anhydride	Agar scientific	R1051
Methyl Nadic Anhydride	Agar scientific	R1081
2,4,6 Tri (dimethylaminoethyl)	Agar scientific	R1065
Probylene Oxide	Agar scientific	00236
EM stain 336	Agar scientific	AGG1260D
Tetramethylbenzidine TMB	BD	51-2607 KC
Toloudine blue	Agar scientific	R1727
Osmium tetroxide	Agar scientific	R1024
Mesh cover grid	Agar scientific	G2100C
Agar 100 Resim	Agar scientific	R1043

4.1.3 List of antibodies used for FACS staining

All antibodies used for flowcytometry experiments are shown in Table 4.

Table 4: List of antibodies used for FACS staining

Antibody	Host	Conjugate	Catalog #	Company	Dilution
CD45	Rat	APC	103112	Biolegend	2.5 μg/ml
CD45	Rat	Alexa Fluor 700 (APC-R700)	103127	Biolegend	2.5 μg/ml
CD11b	Rat	APC-Cy7	101226	Biolegend	2.5 μg/ml
CD11b	Rat	Alexa Fluor 488	101217	Biolegend	2.5 μg/ml
CD16/CD32	Rat	-	101302	Biolegend	10 μg/ml
F4/80	Rat	PE	123109	Biolegend	0.75 μg/ml
CD11c	Armenian Hamster IgG	FITC	117306	Biolegend	2.5 μg/ml
Ly6G	Rat	BV-605	127639	Biolegend	2.5 μg/ml
Ly6c	Rat	PE	128008	Biolegend	0.75 μg/ml
CD8β	Rat	FITC	140404	Biolegend	1:200
CD4	Rat	PerCP	100432	Biolegend	2.5 μg/ml
CD4	Rat	APC	100412	Biolegend	2.5 μg/ml
7-AAD	-	PerCP	420404	Biolegend	1:100
CD3	Armenian Hamster IgG	FITC	100306	Biolegend	5 μg/ml
CD3	Rat	BV-785	100232	Biolegend	2.5 μg/ml
CD19	Rat	BV-650	115541	Biolegend	2.5 μg/ml
Zombie Aqua Fixable Viability Kit	-	BV-510	423101	Biolegend	1:200
I-A/I-E(MHCII)	Rat	BV-785	107645	Biolegend	2.5 μg/ml
I-A/I-E(MHCII)	Rat	APC-Cy7	107628	Biolegend	2.5 μg/ml
CD8α	Rat	APC-Cy7	100714	Biolegend	5 μg/ml

4.1.4 List of antibodies used for immunocytochemistry

Table 5 shows the list of antibodies used for immunocytochemistry to stain section of intestine.

Table 5: List of antibodies used for immunocytochemistry

Antibody	Host	Reactivity	Catalog #	Company	Dilution
CD8	rabbit	Anti-mouse	Ab183685	Abcam	1:2000
CD4	rabbit	Anti-mouse	Ab203035	Abcam	1:1000
F4/80	rat	Anti-mouse	T-2006	BMA Biomedicals	1-100
IFN-γ	rabbit	Anti-mouse	Ab231036	Abcam	1:200
Ki67	rat	Anti-mouse	ab21700	Abcam	Pre diluted
Lysozyme	rabbit	Anti-mouse	A0099	Dako	1:300
Mucin	rabbit	Anti-mouse	Sc 15334	Santa Cruz	1:100
Secondary antibody (FITC)	goat	Anti-rabbit	111-095-003	Jackson Immune Research	1:100
Signa lstain boost IHC		Anti-rabbit	8114S	Cell signaling	Pre diluted
Streptavidin FITC			11-4317	e-bioscience	1:100
Biotin	Goat	Anti-rat	3030-08	SouthernBiotech	1:200

4.1.5 Mice

BALB/c mice purchased from Harlan Olac (Bicester, UK), were bred in the animal facility at the College of Medicine and Health Science, United Arab Emirates University (UAEU). Mice were housed in plastic cages with a controlled light and dark cycle of 12 hours each at 24-26°C and received rodent chow and water ad libitum. All animals for this study were male and used at 8-12 weeks of age, weighing 20-25 g. All studies involving animals were carried out in accordance with, and after approval of the animal research ethics committee of the College of Medicine and Health Science, UAEU (Protocol no.AE/06/81).

4.2 Methods

All methodology used to conduct experiments will be listed below.

4.2.1 Paraoxon preparation

Paraoxon ethyl (Sigma Chemicals Co., St Louis, MO) solution was prepared at a concentration of 10 mmol/L in anhydrous acetone, and further diluted in 1x PBS to a stock solution of 1 mmol/L. Working solution with a final concentration of 80 nmol/ml was also prepared in 1x PBS. Each mouse received daily injection of 40 nmol/0.5 ml of paraoxon (equivalent to 0.44 mg/kg of body weight) by intraperitoneal (i.p.) injection. Control animals received an equivalent volume of 1x PBS (0.5 ml). Injections were given daily for 5 consecutive days, followed by a 2-day break, and this cycle was repeated for a total of 3 weeks.

4.2.2 Rivastigmine preparation

50 mg/ml of Rivastigmine (Sigma Chemicals Co., St Louis, MO) stock solution was prepared in DMSO**.** Working solution was prepared in 1x PBS to a final concentration of 17.7 µg/µl. Each mouse received daily subcutaneous injection of 200 µl of Rivastigmine (equivalent to 2 mg/kg). Control animals received an equivalent volume of 1x PBS (200 µl). All injections were given subcutaneous daily for 5 days, followed by a 2-day rest, and this cycle was repeated for a total of 3 weeks.

4.2.3 Experimental protocol

Male BALB/c mice with matched age and weight were randomly divided into two groups. First group received daily injection of PBS and served as control. Second group received daily injection of 40 nmol of paraoxon or 2 mg/kg of rivastigmine.

Mice were weighed weekly and blood collected and analyzed for acetyl cholinesterase (AChE) activity (from only paraoxon-treated mice). At the end of the third week of treatments, animals were either sacrificed or infected with a virulent strain of *S. Typhimurium* (SL1344) and sacrificed at indicated time points. In other experiments, infected animals were followed for survival.

4.2.4 Red blood cells AChE activity

20 µl of venous blood was collected from the tail, diluted in 2 ml of 0.1 M phosphate buffer, and stored at -20°C until analyzed. After thawing, 1 ml of the sample was incubated with 2 ml of phosphate buffer, 100 µl of 10 mM DTNB (5,5'-dithiobis-2-nitrobenzoic acid) and 10 µl of 6 mM ethoprorazine for 20 minutes in a water bath set at 37ºC. Then, 50 µl of the substrate, 28.4 mmol/L acetylthiocholine, was added immediately before measuring the absorbance. The change in the absorbance of the DTNB was measured at 436 nm using Du-70 spectrophotometer (Beckman coulter Inc. Pasadena, CA, USA). To measure the total hemoglobin (Hb) content, 1 ml of the blood sample was incubated with 1 ml of transformation buffer for 10 minutes at room temperature. Then absorption at 546 nm was read against water blank. AChE activity was calculated using the absorbance coefficient of TNB- at 436 nm (ε =10.6 mM^{-1} cm^{-1}). Values were normalized to the Hb content (determined as cyanmethemoglobin) and expressed as mU/µM/HB (116). All enzyme activity was expressed as percentage of the baseline activity (100%).

4.2.5 Bacterial strain and preparation

In this study, wild-type virulent strain of *Salmonella enterica* serovar *Typhimurium* (*S. Typhimurium*) SL1344 was used. To grow the bacteria, Tryptic Soy (TS) agar plates

used without ampicillin (100 µg/ml). Aliquots from frozen bacteria stock were plated on TS agar plates. To prepare required dosage, three to five colony-forming units (CFU) were cultured overnight in 10 ml of Tryptic soy broth (TSB) without ampicillin (1 µg/ml) and then diluted 1:5 in fresh TSB without ampicillin (1 µg/ml) and incubated for two hours shaking at 200 rpm and 37ºC. On a spectrophotometer at λ=600, 1 ml of the culture was read to determine bacterial concentration against TSB as blank. Suitable dilutions of the log- phase bacterial suspension was made in pyrogen-free 1x PBS to prepare the indicated doses and their verification. To confirmed bacterial dose, the number of CFUs counted in the plates. For oral inoculation, sodium bicarbonate (200 µl of 7.5%) was given 4 minutes before oral administration of SL1344 (200 µl) in order to neutralize the stomach acid. Gavage needles (CADENCE science, Staunton, VA, USA) were used for the administration of sodium bicarbonate and SL1344.

4.2.6 Determination of bacterial load in target organs and fecal pellets

Fecal pellets were freshly collected from infected animals at day three post infection before sacrificed, weighed, and homogenized in 0.5-1 ml cold 1x PBS. Then, 100 µl from the homogenate or appropriate dilution were plated on SS agar plates with Streptomycin (200 µl/ml). CFUs counts were determined after overnight incubation at 37°C.

Mice were sacrificed at day three post oral infection. Spleen, liver, intestine and intestinal content were aseptically removed and homogenized in 1 ml cold 1x PBS in an Ultra-turrax T-25 tissue homogenizer (Janke & Kunkle, Staufenim Breisgau, Germany). 100 µl aliquot of the homogenate or appropriate dilution were plated on SS agar with Streptomycin. CFUs counts were determined after overnight incubation at 37°C.

4.2.7 Flow cytometry

$0.5x10^6$ cells/well from isolated cells were incubated in U shaped bottom 96-well plate (BD) for 30 minutes at 4°C in 50 µl of staining buffer containing anti-CD16/CD32 monoclonal antibody (clone 2.4G2) to block the FcII/III receptors and, therefore, prevent any non-specific binding of the antibodies. Then, the plate was spun at 900 rpm for 5 minutes at 4°C, decanted, and cells incubated with zombie antibody for exclusion of non-viable cells. Afterwards, the plate was spun at 900 rpm for 5 minutes at 4°C, decanted and cells incubated at 4°C for 30 minutes with different combinations of conjugated monoclonal antibodies (Biolegend or Novus as detailed in above Table 5) in a total volume of 100 µl/well. Then, plates were spun, decanted and cells washed twice with staining buffer, 200 µl/well. Finally, cells were re-suspended in 200 µl of staining buffer and either immediately read or fixed overnight by adding 100 µl of 4% paraformaldehyde. Data were collected on 30,000 cells using BD FACS Celesta (BD biosciences, Mountain View, CA, USA) and analyzed using BD FACS Diva software (BD).

4.2.8 Isolation of intestinal epithelial cells and lamina propria

Mice were treated with paraoxon or saline for three weeks. At day three from the last injection, mice were sacrificed, and whole small intestine excised. Intestinal fecal content was removed by flushing cold HBSS w/o. Residual fat tissue and Peyer's patches were carefully removed. Intestine was, then, weighed (only 1 g of tissue was used), opened longitudinally and cut into small pieces (0.5-1 cm) to which 20 ml of pre-digestion solution was added (each sample in a different 50 ml tube) and incubated in a shaking bath at 37°C for 20 minutes. Afterwards, intestinal tissue was vortexed for 10 seconds, and passed through a 100 µm-pore strainer. Supernatant was collected

in 50 ml tube and discarded. Intestine pieces were resuspended again in 20 ml of pre-digestion solution, incubated, vortexed and passed them again through the strainer. This time supernatant was collected and kept. The tissue incubation was repeated one more time and supernatants pulled, centrifuge at 300× g for 10 minutes at room temperature. Supernatant was then, discarded and pellet containing IELs resuspended in 5 ml PBS.

Intestinal tissue was further processed to obtain leukocytes from the lamina propria (LP). Intestine tissue was transferred into gentleMACS C tubes containing the enzyme mixture and incubated at 37°C under continuous shaking for 30 minutes. Then, C tube was placed into the gentleMACS dissociator, to homogenate the intestinal tissue for 30 seconds (m_intestine_01 program). Then, tube was spun, and pellet collected. Afterwards, 5 ml of PBS buffer was added, cell suspension passed through a 100 µm strainer and placed on a 50 ml tube. Strainer was washed with 10 mL of PBS buffer and LP cell suspension spun at 300× g for 10 minutes at room temperature, supernatant discarded, and cells resuspended in 5 ml PBS.

4.2.9 Light microscopy analysis of the ileum

Ileum (the distal part of the intestine) was removed and immediately fixed in 10% Formalin or in Carnoy's fixative. After overnight fixation, tissues were placed in histological cassettes, dehydrated with a series of alcohols, 70% to 95% to 100%, cleared with xylene and then infiltrated and embedded in paraffin (Sherwood Medical, St. Louis, Mo, USA). Using Shandon Finesse 325 manual microtome (Thermo Scientific, Pittsburg, PA, USA), paraffin blocks were trimmed, 5 µm sections prepared and placed on gelatin coated slides. The process of dehydration was done at room temperature by immersing the sections in xylene I solution for 5 minutes, xylene II for

5 minutes, absolute ethanol I for 3 minutes, absolute ethanol II for 3 minutes, 90% ethanol for 3 minutes, 80% ethanol for 3 minutes and finally 70% ethanol for 3 minutes. Antigen retrieval was performed by placing the tissue slides at 200°C for 10 minutes, and then cooled down for 30 mins on ice. Endogenous peroxidase activity was blocked using 1.5% H_2O_2 in PBS. After 3 wash cycles with PBS-T (5 minutes each), the slides were further blocked with 1% BSA in PBS for 45 minutes followed by incubation with the required concentration of primary antibodies diluted in BSA. After overnight incubation, slides were kept for one hour at room temperature, washed and then, incubated with the appropriate secondary antibodies for 30 minutes. 3 wash cycles with PBS-T (5 minutes each).

For DAB staining: the peroxidase activity was determined using DAB chromogen (Dako) for 5-10 minutes followed by wash with distilled water. Sections were then, stained for 1 minute with hematoxyline and rehydrated. Rehydration was in the following order: 70% ethanol for 1 minute, 80% ethanol for 1 minute, 90% ethanol for 1 minute, absolute ethanol I for 1 minute, absolute ethanol II for 1 minute, xylene I for 2 minutes and xylene II for 2 minutes. Finally, the sections were mounted with DPX (Panreac, Spain) and images captured with an Olympus BX51 microscope model V-LH100HG (Olympus Corporation, Japan).

For Florescent staining: slides were countered stained with propidium iodide for 30 minutes at 37°C. Then, they were washed with PBS and finally mounted with florescence mounting medium (Dako).

4.2.10 Electron microscopy analysis of the ileum

Ileum (the distal part of the intestine) was removed and immediately fixed in a mixture of 2.5% glutaraldehyde and 2% formaldehyde solution (pH= 7.2 in phosphate buffer)

overnight at 4°C. Samples were rinsed with 0.1 M phosphate buffer, post-fixed in 1% aqueous osmium tetroxide for 1 hour and then rinsed in distilled water. Then, samples were dehydrated in ascending series of graded ethanol, infiltrated with Agar100 epoxy, and finally embedded in the same resin where they polymerized at 65°C for 24 hours. Blocks were trimmed, semithin (0.5 mm) sections cut in an ultramicrotome Leica EM UC7 (Leica, Welzlar, Germany) and stained with 1% aqueous toluidine blue on glass slides. Ultrathin (95-100 nm) sections were prepared on 200 mesh copper grids and contrasted with 2% uranyl acetate for 40 minutes, and lead citrate for an additional 25 minutes. Grids were then air dried on filter paper and the sections examined and photographed with TENAI G2 Spirit Transmission Electron Microscope (FEI, Hillsboro, Oregon, USA).

4.2.11 Statistical analysis

Statistical significance between control and treated groups was analyzed by unpaired two-tailed Student's t-test or the log rank (Mantel-Cox) test for Kaplan-Meier functions (for survival analysis) using GraphPad Prism software (San Diego, CA, USA). Differences between experimental groups were considered significant when p values were < 0.05.

Chapter 5: Results

5.1 Rivastigmine exposure retarded body weight gain

In previous studies where paraoxon used, as irreversible AChE inhibitor, the effectiveness of the treatment monitored by measuring the activity of the enzyme AChE in peripheral blood after 24 hours of paraoxon injection. However, in this study rivastigmine used, a reversible AChE inhibitor and, it is not possible to detect any changes in the RBC AChE activity after 24 hours of rivastigmine injection. Then, in order to monitor the efficiency of the rivastigmine treatment, body weight was followed in all animals during the treatment period. Body weights were recorded just before starting the treatment with either saline (control) or rivastigmine and considered these weights as baseline. Once the treatment started, mice were weighed weekly and the percentage change in body weight calculated. Results showed a gradual increase of body weights in saline treated animals of 4% after the first week, 7% after the second week and 10% at the endo of the third week. On the rivastigmine-treated group, mice showed an increase of only 0.7% in their body weights after the first week of treatment. The following week (week 2), mice experimented an increase of 2% of their initial weights. Their body weight after the three weeks treatment increased by only 4% (Figure 7). The differences in body weight growth between the control and experimental group at each time point and during the experimental period was statistically significant confirming the effectiveness of the rivastigmine treatment.

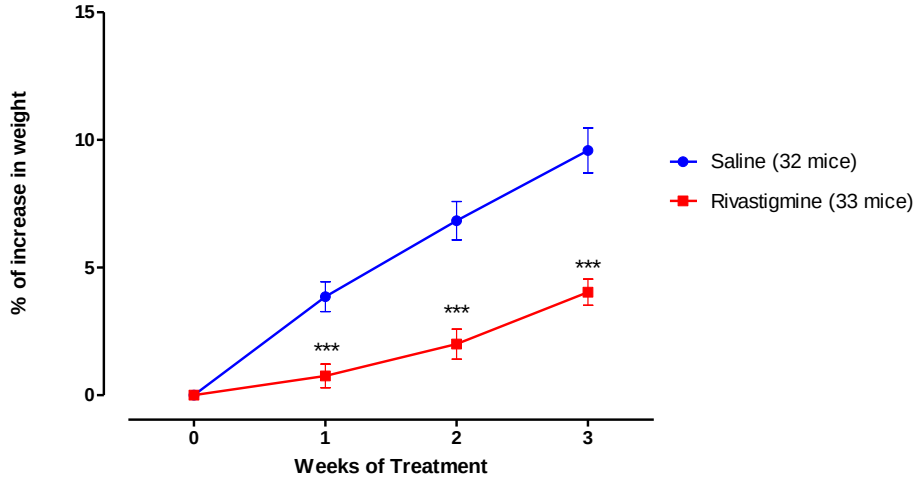

Figure 7: Retarded growth in rivastigmine-treated mice. Mice were treated with rivastigmine or saline for three weeks. Weekly change in body weight was calculated as percentage of the initial weight. Depicted are the mean values ± SD of data pooled from 3 independent experiments. Asterisks denote statistically significant differences between the control and experimental groups ***$p<0.001$.

5.2 Rivastigmine treatment enhanced host survival after a lethal infection with SL1344

Next, study aimed to test if rivastigmine treatment could also protect mice from a lethal oral infection, as demonstrated paraoxon treatment did (Al-Barazie et al., 2018). After three weeks treatment with rivastigmine or saline, mice were inoculated orally with SL1344, a virulent strain *of S. Typhimurium* at a lethal dose of $2x10^4$ CFUs/mouse. Mice were then followed for survival for up to day 30 post-infection as shown in Figure 8. Results show that rivastigmine treatment increased significantly the time of survival to 23 days (14 days medial survival) after infection compared to the 11 days survival (medial survival of 9.5) in saline-treated group. However, eventually all animals in both groups succumbed to the infection.

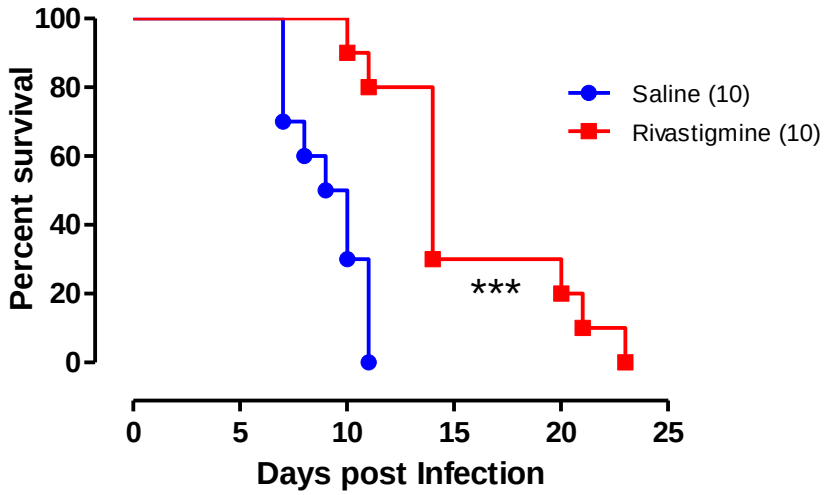

Figure 8: Rivastigmine treatment enhanced host survival after a lethal oral infection. Mice were treated with rivastigmine or saline for three weeks. At the end of the treatment mice were infected orally with a dose of $2x10^4$ CFUs/mouse of SL1344 and followed for survival up to 30 days post infection. Depicted are the mean values ± SD of data pooled from two independent experiments. Asterisks denote statistically significant differences between the control and experimental groups (***$p<0.001$). Chi squared (Mantel-Cox) statistical test was used for this analysis.

5.3 Rivastigmine reduces the initial bacterial translocation and systemic dissemination of oral *Salmonella*

After three weeks treatment with saline or rivastigmine, mice were orally infected with a high dose of virulent *Salmonella* strain SL1344 ($1x10^5$ CFUs/mouse). Mice were, then, sacrificed at day 3 post-infection, and spleen, liver and intestine aseptically removed. Fecal pellets were also collected just before sacrifice.

Bacterial load estimation showed higher number of CFUs in the fecal pellet and intestinal content of rivastigmine-treated mice when compare with saline-treated control group (Figure 9). However, the opposite was observed in target organs like spleen and liver where the number of bacteria were higher in the control group than in the rivastigmine-treated group (Figure 9). These results suggest that rivastigmine

treatment prevented the translocation and, therefore, the dissemination of bacteria. Interestingly, no differences between the control and rivastigmine groups were found in the number of CFUs of the whole intestine (tissue + content). This is probably due to the fact that in the control group bacteria is not in the content anymore but it is already in the intestinal tissue after translocation, while in the rivastigmine-treated group is in the intestinal content but not in the tissue.

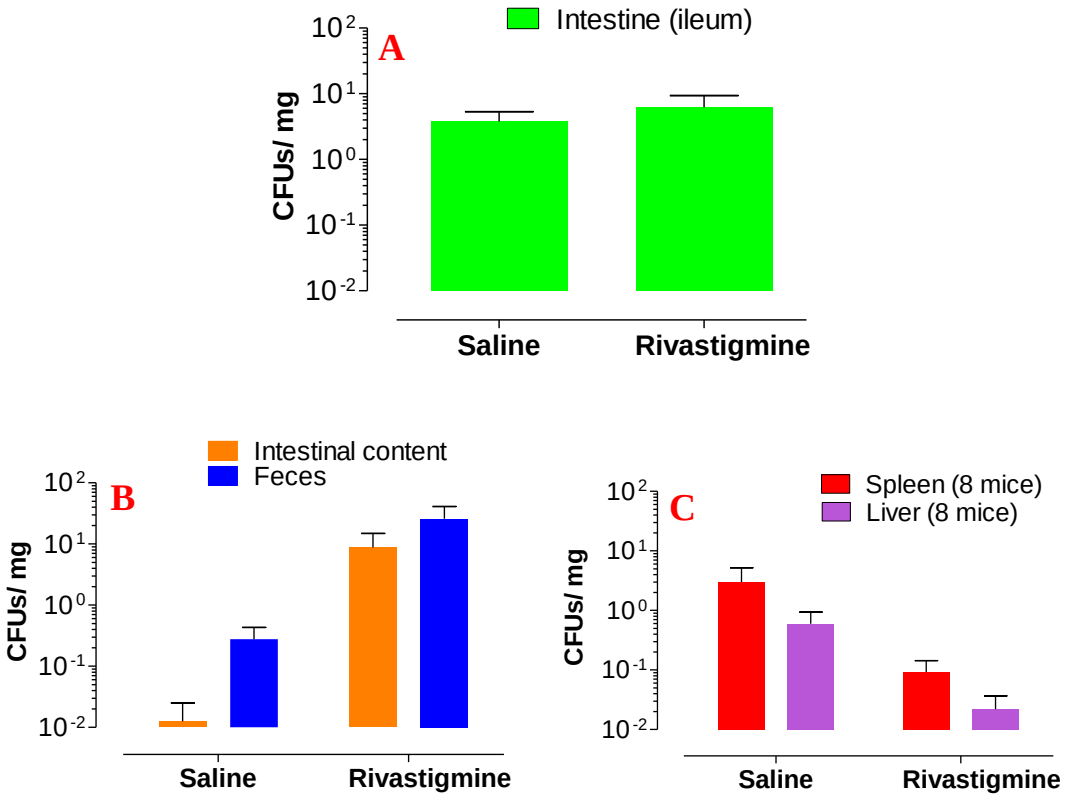

Figure 9: Reduction in the bacterial load in systemic organs of rivastigmine pre-treated mice after 3 days of oral infection. Mice were treated with saline or rivastigmine for 3 weeks and then infected orally with SL1344. Mice were sacrificed and organs homogenates aliquots were plated to determine bacterial load. Bacterial loads were determined as CFUs/mg in intestine, feces, intestinal content, spleen and liver at day 3 post infection as shown in A-B and C. Graph shows mean ± SEM of data from 2 independent experiments.

Spleens obtained, from saline- and rivastigmine-treated mice, at day 3 post-infection with high dose of SL1344 were weighted and plotted in Figure 10. Although no statistically significant difference was found between the spleen size of both groups, the saline pre-treated group showed an average of 76 mg compared to the 65 mg in the rivastigmine-treated group. This small splenomegaly correlates with the higher bacterial load found in the control group.

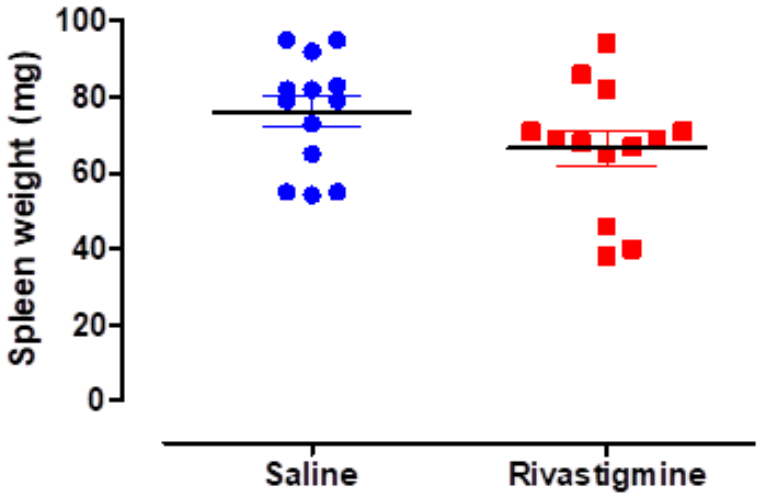

Figure 10: Spleen weights after 3 days of oral infection. Mice were treated with saline or rivastigmine for 3 weeks and then infected orally with SL1344. At day 3 post-infection, mice were sacrificed and spleen weight measured. Graph depicts mean ±SD of data from 3 independent experiments.

5.4 Morphological analysis of the intestinal mucosa revealed an induction of mucus and lysozyme secretion following rivastigmine administration

Next, study aimed to investigate if rivastigmine treatment induces any changes on the intestinal mucosa as this is the site where orally administered bacteria will encounter the first host defense mechanism against infection. More specifically, looked at morphological changes at the level of the intestinal epithelium that could affect the translocation of bacteria from the intestinal lumen to the submucosa. Following three

weeks of treatment with rivastigmine or saline (control), mice were sacrificed and small intestine (ileum) collected and prepared for morphological analysis. Within the intestinal epithelium the focusing were on characterizing, by electron- and light microscopy, the effect of rivastigmine treatment on the main cells involved in the mucosal defense against pathogens: goblet cells, Paneth cells and immune cells.

5.4.1 Goblet cells: mucin production

5.4.1.1 Rivastigmine treatment induced degranulation of Paneth cells

Goblet cells are located among the columnar epithelial cells of the intestinal epithelium. Intestinal goblet cells in saline-treated mice are large and rounded and showed the presence of an extensive endoplasmic reticulum as well as densely packed granules located on the apical side of the nucleus (Figures 11A-B). However, intestinal goblet cells observed in the rivastigmine-treated group appeared depleted granules with the cellular apical side complete open to the intestinal lumen where granules are being released (Figures 11C-D). This massive secretion seemed to disrupt the cell membrane and, often to induce the release of cytoplasmic fragments and cell death.

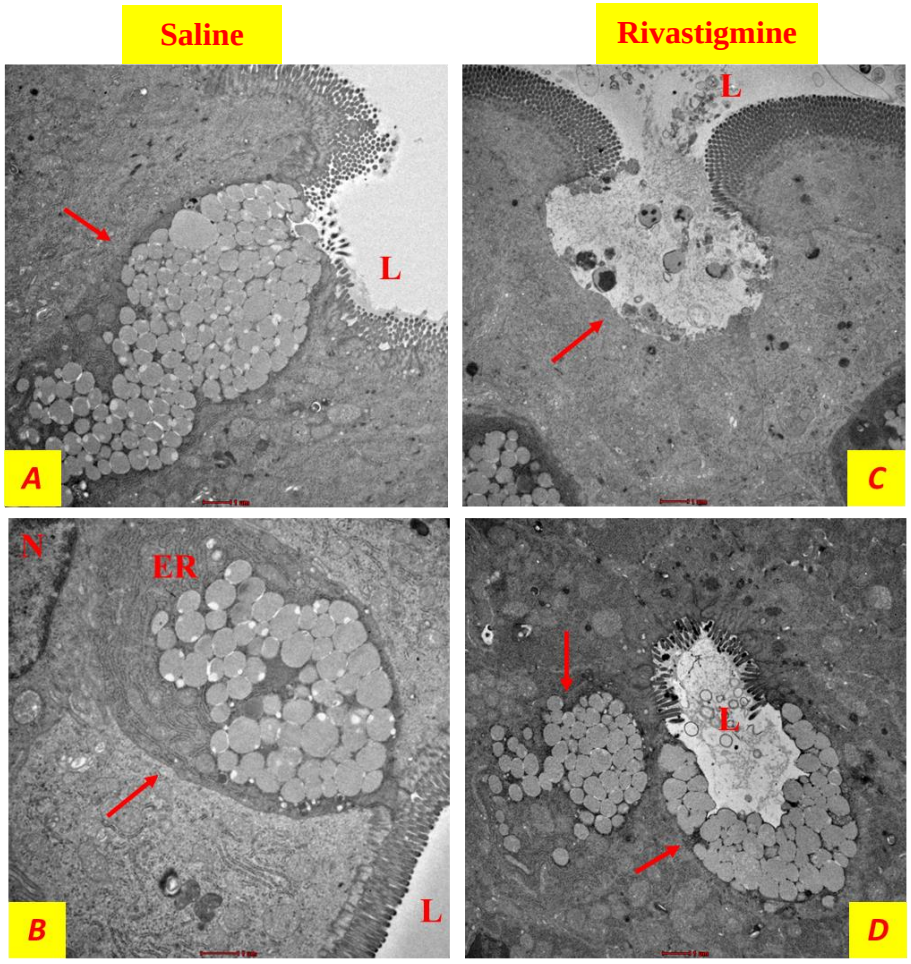

Figure 11: Rivastigmine treatment induced degranulation of Paneth cells. Goblet cells in ileum sections from saline- (A-B) and rivastigmine-treated mice (C-D). Goblet cells (arrow) in saline treated animals showed large and rounded mucous globules. In contrast, goblet cells from rivastigmine treated animals presented an active degranulation of the granules to the intestinal lumen, or complete degranulated cell. Figure bars indicate 1 μm. L: intestinal lumen; N: nucleus; ER: endoplasmic reticulum.

5.4.1.2 Administration of rivastigmine induced the release of mucin to the intestinal lumen

Goblet cells synthesize several bioactive molecules that are secreted to the intestinal lumen forming the intestinal mucus layer. The most abundant of these molecules is mucin. So, also analyzed goblet cells degranulation immunohistochemically using a specific anti-mucin antibody. As shown in Figures 12A-B, the distal region of the small intestine (ileum) in saline-treated mice showed rounded positive cells with dense

staining in their cytoplasm along the intestinal epithelium. On the other hand, equivalent sections from ileum of rivastigmine-treated mice presented less intense staining and an opened apical region releasing the mucin to the intestinal lumen (Figures 12C-D).

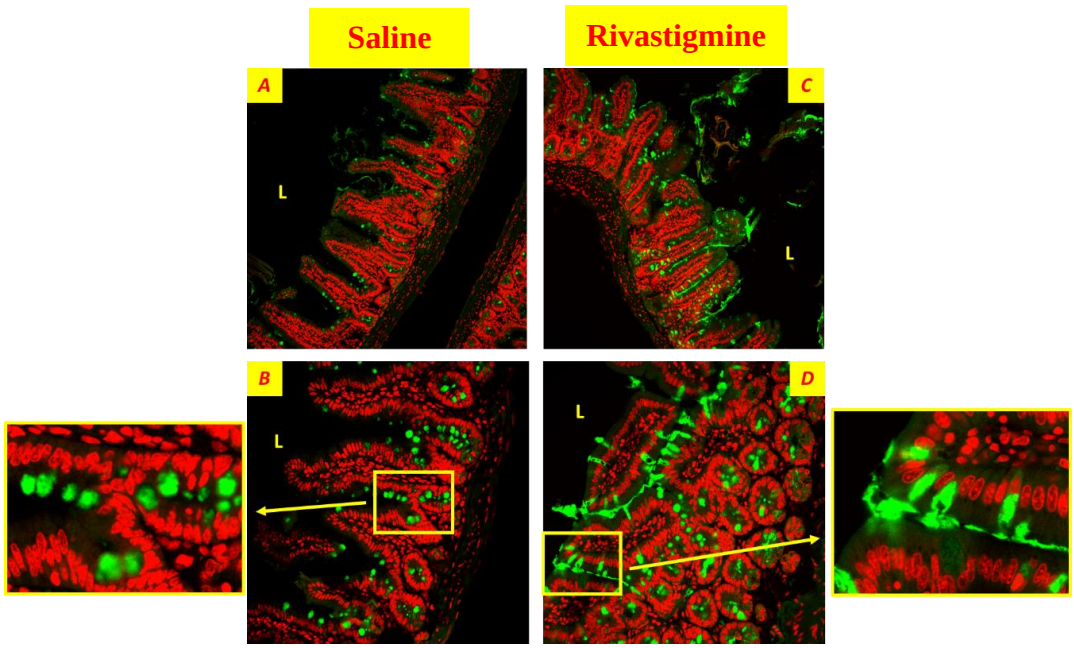

Figure 12: Administration of rivastigmine induced the release of mucin to the intestinal lumen. Light confocal micrographs of ileum sections showing immunofluorescence of mucin-containing (green) goblet cells in (A-B) saline and (C, D) rivastigmine-treated mice. Original magnification (A-C) 200x and (B-D) 400x. All photos are representative of 3-4 mice/group. L, lumen; mm, muscularis mucosa; M, mucin.

5.4.1.3 Rivastigmine treatment increases the thickness of the intestinal mucin layer.

Next, the study investigates if the level of degranulation and, therefore, mucin accumulation in the intestinal lumen differed between treatments with rivastigmine or paraoxon. Colon tissue was collected after treatment with saline or rivastigmine or paraoxon and processed for histological analysis. Tissue sections were stained with Alcian blue that labels the sulphated (high acidity) complex mucins. Colon tissue

obtained from control group showed a thin outer loose and inner adherent mucus layers (Figure 13A) compared to the much thicker layers found in the rivastigmine- (Figure 13B) and paraoxon- (Figure 13C) treated groups. Measurements of the dense inner layer further demonstrated a ~ 2-fold increase in its thickness following treatment with AChE inhibitors (rivastigmine and paraoxon) compared to the control group (Figure 13D). Therefore, rivastigmine and paraoxon seem to induce the same level of degranulation of the intestinal goblet cells.

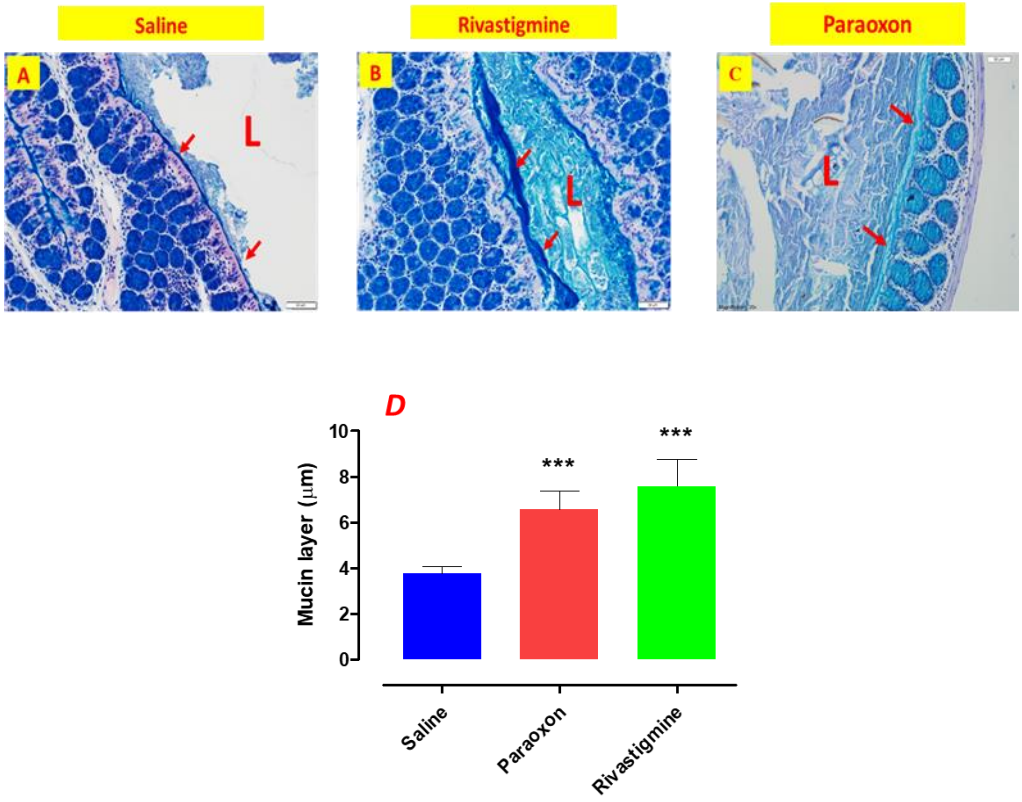

Figure 13: Rivastigmine treatment increases the thickness of the intestinal mucin layer. Colon from saline and rivastigmine-treated mice was collected and fixed in Carnoy's solution. After processing and cutting, sections were stained with Alcian Blue to visualize mucin content. (A) Thin inner mucin layer (arrow) in the intestinal lumen of saline-treated mice compared with a thicker inner mucin layer (arrow) present in the lumen of (B) rivastigmine and (C) paraoxon mice. (D) Graph depicts measurements of the thickness of the inner mucus layer in the lumen of the colon in all groups. Data are shown as the mean ± SEM of the measurements at high power field of three non-consecutive sections per mouse. Asterisks denote significance between control and experimental groups (***$p < 0.001$). Pictures are representative of 3 slides/ mouse, 4 mice per group. Figure bars indicate 50 mm. L, lumen.

5.4.2 Paneth cells

5.4.2.1 Rivastigmine induced degranulation of intestinal Paneth cells

Paneth cells are secretory cells unique to the small intestine and located at the base of the intestinal Lieberkühn crypts. As goblet cells, Paneth cells contain multiple secretory granules where different antimicrobial peptides are stored before being release to the intestinal lumen. These cells analyzed morphologically in order to determine if rivastigmine treatment induced any changes on them. Ultrathin sections of ileum from saline-treated group, showed intact Paneth cells with prominent basal nucleus, layered endoplasmic reticulum and large spherical electron-dense granules that most of the time appear surrounded with clear halos (Figures 14A-B). When ileum of rivastigmine-treated mice was analyzed, Paneth cells presented an abnormal morphology containing a stressed endoplasmic reticulum, large cytoplasmic vacuoles and fused secretory vesicles (Figure 14C). Moreover, occasionally, some Paneth cells seemed to be completely degenerated with almost empty cytoplasm that opens to the intestinal lumen (Figure 14D).

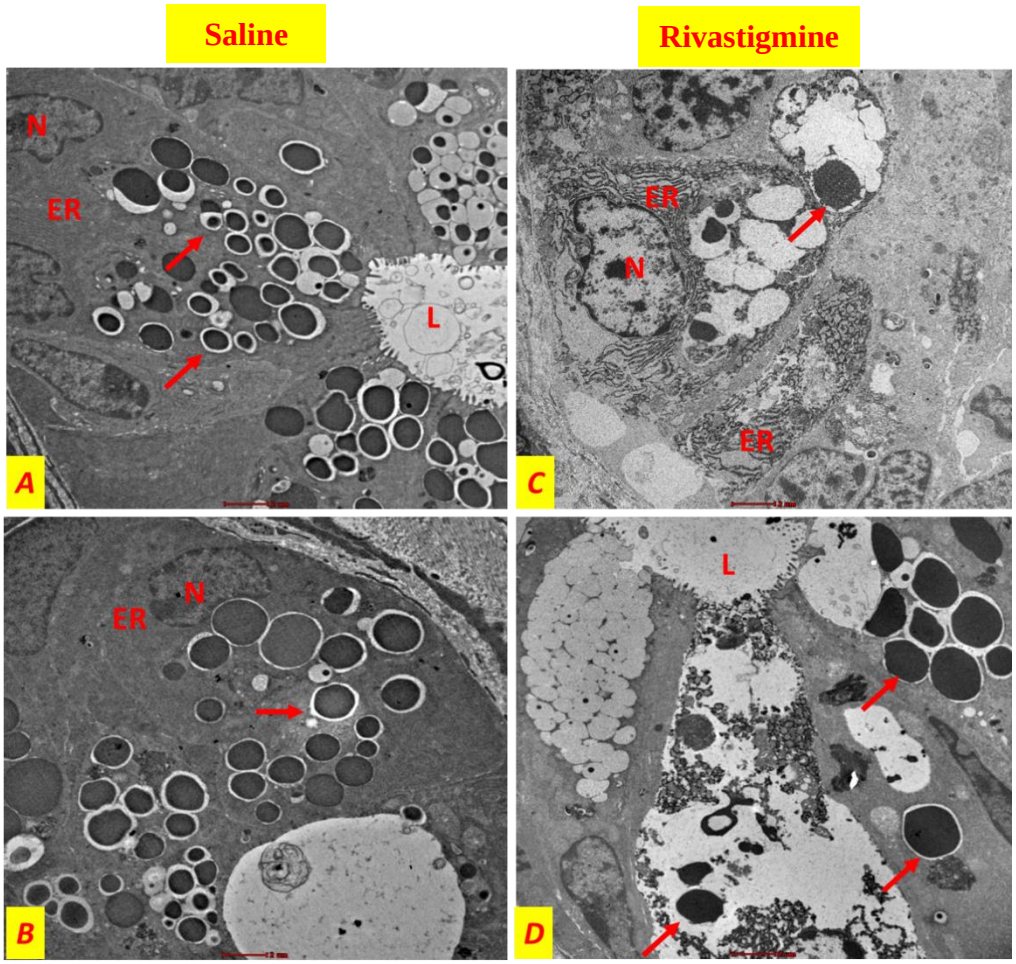

Figure 14: Rivastigmine induced degranulation of intestinal Paneth cells. Transmission electron micrograph of small intestine Paneth cells in the crypts of the small intestine of (A-B) saline- and (C-D) rivastigmine-treated mice. (A-B) In control saline group the cytoplasm of Paneth cells is filled with large spherical electron-dense secretory granules (arrow) and present a well-developed endoplasmic reticulum. (C-D) In rivastigmine-treated mice, Paneth cells contain fused secretory granules and have altered morphology. ER: endoplasmic reticulum; L: intestinal lumen; N: nucleus; V: vesicles. Bar =2 μm

5.4.2.2 Reduced lysozyme content in Paneth cells following rivastigmine treatment

Next, study investigated if the described ultrastructural changes in Paneth cells induced by rivastigmine correlated with the content of lysozyme, an antimicrobial protein produced by Paneth cell and secreted upon microbial stimulation. Paraffin-embedded

ileum sections where stained with anti-lysozyme antibody and observed by light confocal microscopy. Results showed an intense staining in Paneth cells granules of saline-treated mice whereas in mice treated with rivastigmine the lysozyme staining was substantially reduced (Figure 15).

This morphological analysis of the intestinal epithelium demonstrates that both AChE inhibitors induce degranulation of goblet and Paneth cells which could provide a similar grade of protection against a bacterial infection.

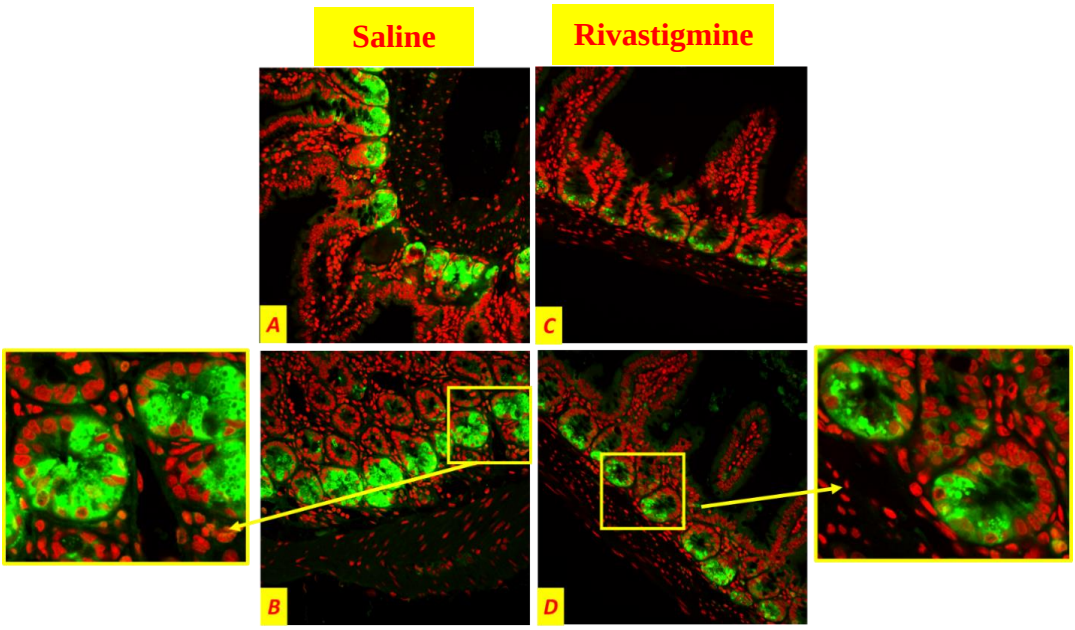

Figure 15: Rivastigmine reduces lysozyme levels in Paneth cells. Immunostaining with anti-lysozyme antibody of paraffin-embeded ileum sections showed a decrease in positively labeled (green) Paneth cells in (C-D) rivastigmine-treated mice compared to (A-B) saline-treated control mice. In pictures B and D, the demarcated area has been amplified and shown on the side squares. Propidium iodine (red) was used as a counterstain to visualize cellular nucleus. L: lumen. Original magnification 400x. Photos are representative of 3-5 mice/group/experiment.

5.5 Increased intestinal cell proliferation following rivastigmine administration

Next, it has been investigated if the increase on cell degranulation induced in Paneth and goblet cells by rivastigmine treatment was correlated with an increase in cell

proliferation. To study the mitotic rate of the intestinal epithelial cells, small intestine sections were stained with an anti-Ki67 antibody (Figure 16). In both groups (saline and rivastigmine) $Ki67^{+}$ cells were found in the bottom of the crypts among the Paneth cells, immediately above the Paneth cells and then, scatter in the lamina propria and some in the epithelium. Although, $Ki67^{+}$ cells were not quantified, intestinal tissue from rivastigmine-treated groups seem to present a higher number of proliferating cells at the bottom of the crypts, and in the lamina propria (Figures 16C-D) than the one from control saline group (Figures 16A-B). These results could indicate an increase in intestinal stem cell proliferation that will differentiate into new goblet and Paneth cells in the intestinal epithelium replacing the ones lost after forceful degranulation.

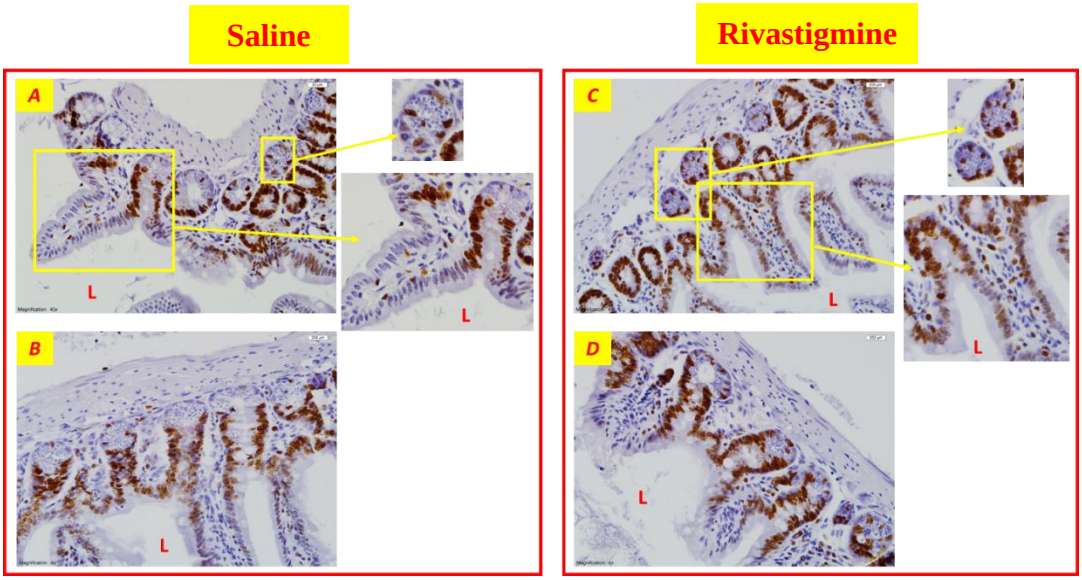

Figure 16: Increased intestinal cell proliferation following rivastigmine administration. Small intestine sections of saline (A-B) and rivastigmine-treated (C-D) mice stained with anti-Ki67 antibody to locate active proliferating cells. Small size pictures show magnification of the demarcated areas. Hematoxylin was used as a counterstain to visualize cellular nucleus. L: lumen. Original magnification 400x. Photos are representative of 3-5 mice/group/experiment.

5.6 Analysis of the immune cells present in the ileal mucosa

5.6.1 Immunohistochemical analysis

Immunohistological analysis of the intestinal mucosa was performed in order to investigate the possible changes induced by rivastigmine treatment on the main immune populations present in the intestinal epithelium and lamina propria. The distal part of the small intestine (ileum) was excised, fixed and embedded in paraffin. Sections were stained with specific antibodies against different immune populations.

5.6.1.1 $CD4^+$ cells were more abundant in the lamina propria of the ileal mucosa

Intestine of saline (Figures 17A-B) and rivastigmine-treated (Figures 17C-D) groups showed the presence of $CD4^+$ T cells mostly in the lamina propria and only few of them in the intestinal epithelium. Numerical quantification of $CD4^+$ cells (Figures 18A-B) reveled no statistical-significant differences between the two groups, although rivastigmine treatment reduced the number of these cells in the lamina propria and slightly increased in the epithelium.

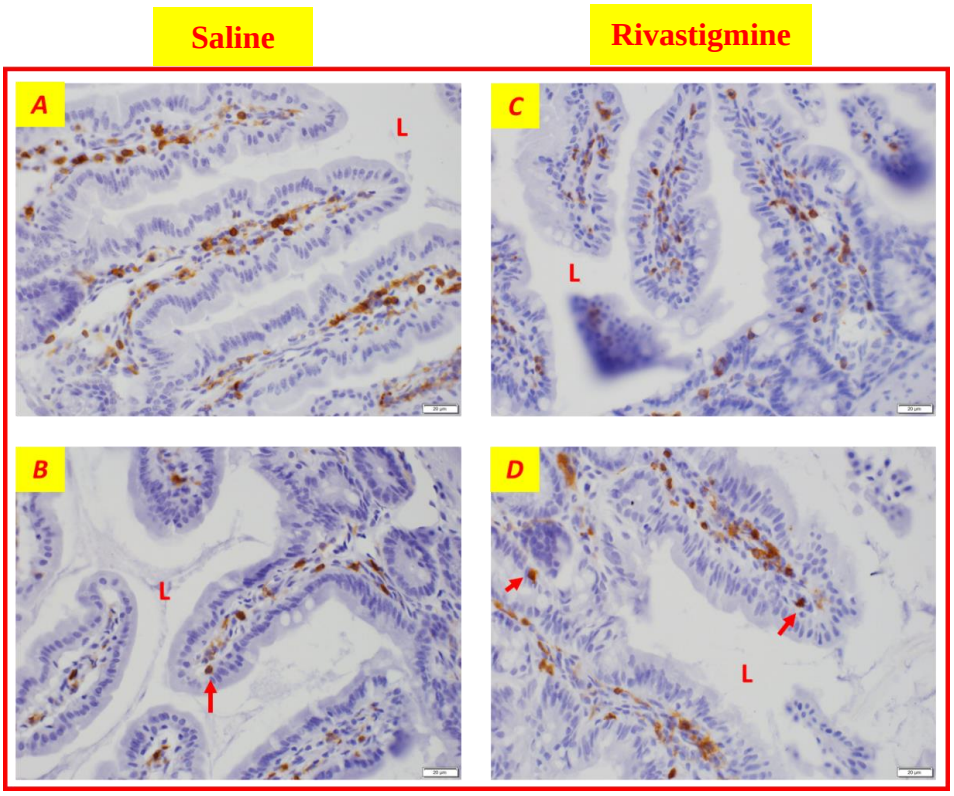

Figure 17: Immunohistochemical analysis of $CD4^+$ cells in the small intestine. Ileum sections obtained from saline (A-B) and rivastigmine (C-D) treated mice were stained with anti-CD4 antibody. Representative pictures are shown where positively stained cells are brown. Arrows point to IELs. L: lumen. Scale bars= 20 µm. Photos are representative of 3-5 mice/group/experiment.

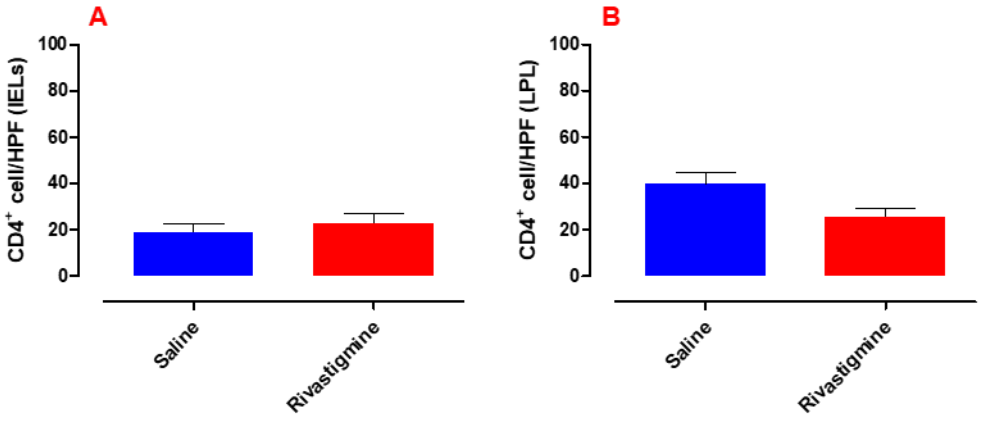

Figure 18: Quantitative analysis of the $CD4^+$ cells in the ileal mucosa. Brown labeled cells present in the intestinal epithelium (A) and in the lamina propria (B) of intestinal sections were counted in 10 HPF/section and 2-3 sections/mouse, then, averaged. The values represent the mean ± SEM of 4-5 mice/group and the results are pooled from 3 independent experiments.

5.6.1.2 CD8$^+$ cells preferentially located in the epithelial layer

Paraffin sections were also stained with an anti-CD8 α-chain (expressed by all CD8$^+$ cells). As shown in Figure 19, the majority of CD8$^+$ cells are located in the intraepithelial compartment. No differences in location were found between saline (Figures 19A-B) and rivastigmine (Figures 19C-D) groups. Quantification of CD8$^+$ cells (Figure 20) reveals that in saline-treated mice there were 2.75 folds more CD8$^+$ cells in the intraepithelial compartment than in the lamina propria (Figures 20A-B). Similarly, the intestinal epithelium of the rivastigmine-treated group contained 3.8 folds more CD8$^+$ cells than the lamina propria (Figures 20A-B). Nevertheless, there was no statistically significant difference in the number of CD8$^+$ T cells in the LP or IEL compartment between control vs. rivastigmine-treated mice.

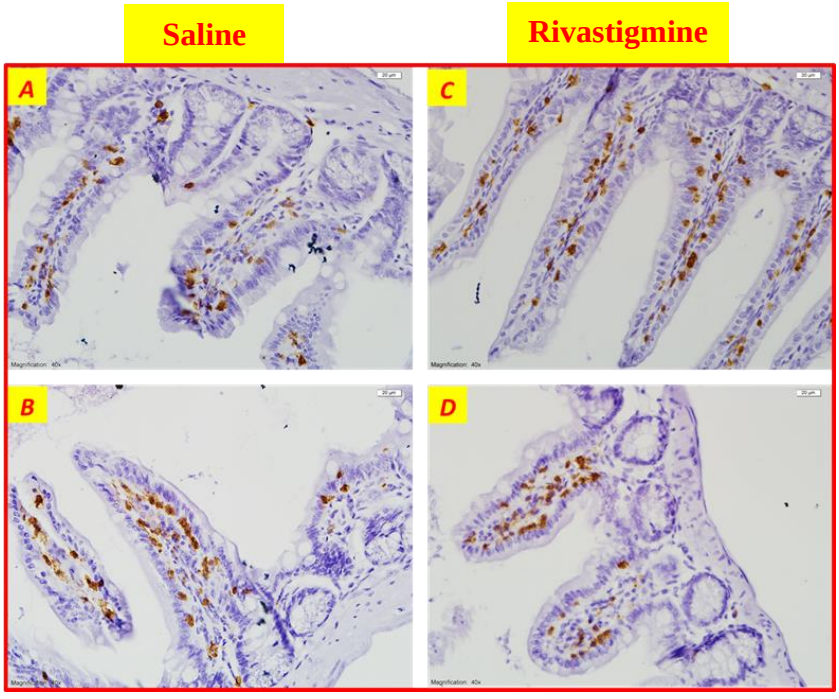

Figure 19: Immunohistochemical analysis of CD8$^+$ cells in the small intestine. Ileum sections obtained from saline (A-B) and rivastigmine (C-D) treated mice were stained with anti-CD8 α-chain antibody. Representative pictures are shown where positively stained cells are brown. Hematoxylin was used for counterstain. Original magnification 200x (A-C) and 400x (B-D). L: lumen. Photos are representative of 3-5 mice/group/experiment.

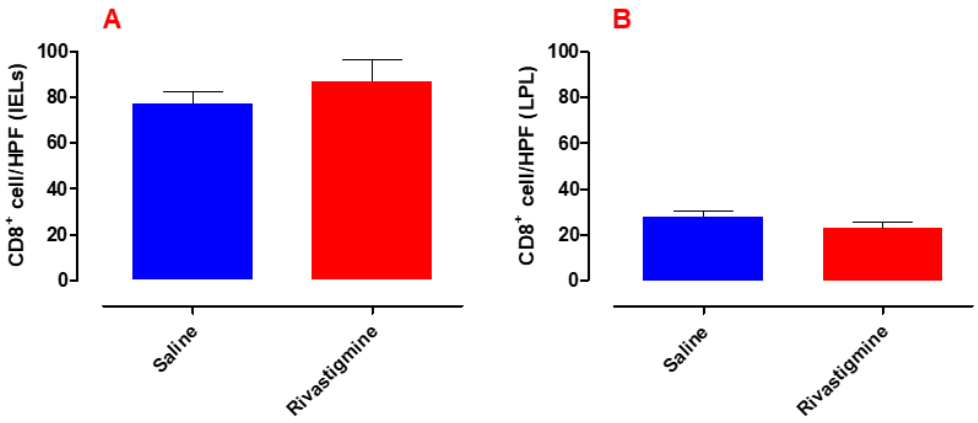

Figure 20: Quantitative analysis of $CD8^+$ cells in the intestinal mucosa. Brown labeled cells ($CD8^+$) present in the intestinal epithelium (A) and in the lamina propria (B) of intestinal sections were counted in 10 HPF/section and 2-3 sections/mouse, then, averaged. The values represent the mean ± SEM of 4-5 mice/group and the results are pooled from 3 independent experiments.

5.6.1.3 Macrophages were only detected in the lamina propria of the intestinal mucosa

By immunohistochemistry, the presence of macrophages identify using an anti-F4/80 antibody. Despite the high background obtained with this antibody, results showed few $F4/80^+$ cells in the intestinal lamina propria of saline (Figure 21A) and rivastigmine-treated mice (Figure 21B). When positive cells were quantified, no significant differences between the two groups were found (Figure 21C). No $F4/80^+$ cells could be observed in the intraepithelial compartment.

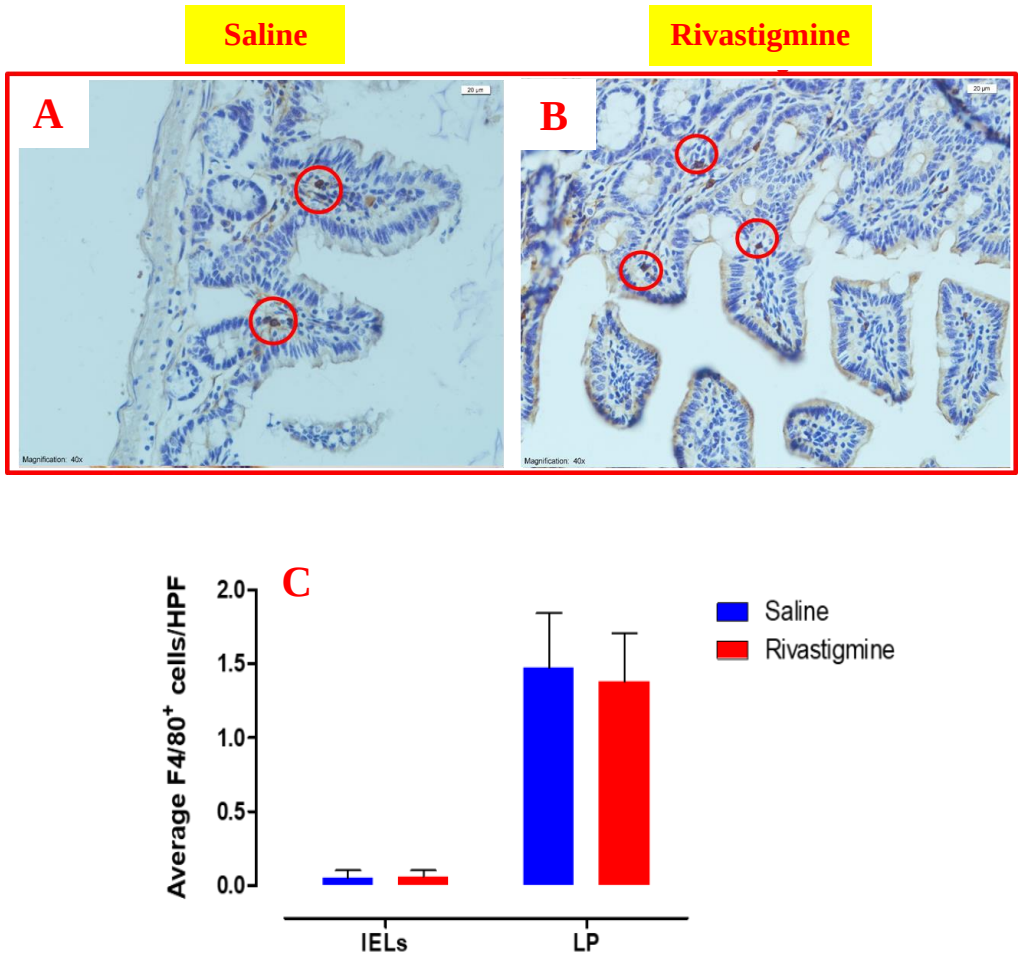

Figure 21: Presence of macrophages in the intestinal lamina propria. Ileum sections obtained from (A) saline and (B) rivastigmine-treated mice were stained with anti-F4/80 antibody to detect macrophages, and counterstained with hematoxylin. Representative images of 3-5 mice/group/experiment are shown. Original magnification 400x. L: lumen. (C) F4/80^{+} cells were counted in 10 HPF/section and 2-3 sections/mouse, then, averaged. The values represent the mean ± SEM of 4-5 mice/group and the results are pooled from 2 independent experiments.

5.6.1.4 Abundant presence of IFNγ-producing cells in the ileal mucosa

Given the critical role of IFNγ in resistance to *Salmonella* infection (Ingram, Brodsky, & Balachandran, 2017) the presence of IFNγ-producing cells analyzed in the mucosa of the ileum by immunohistochemistry. In both the saline and rivastigmine groups, IFNγ^{+} cells were observed within the epithelial layer at the basolateral surface of

adjacent epithelial cells and in the lamina propria (Figure 22). Rivastigmine treatment seems to increase the number of IFNγ^+ cells (Figure 22B) compared to the saline group (Figure 22A). This was based on quantification of the number of IFNγ^+ cells in both compartments. Based on the staining, it was difficult to accurately determine whether these cells were in the LP or in the epithelial layer. This fact was partially confirmed by quantification of IFNγ^+ cells in the tissue sections, although the increment did not reach statistically significant difference (Figure 22C).

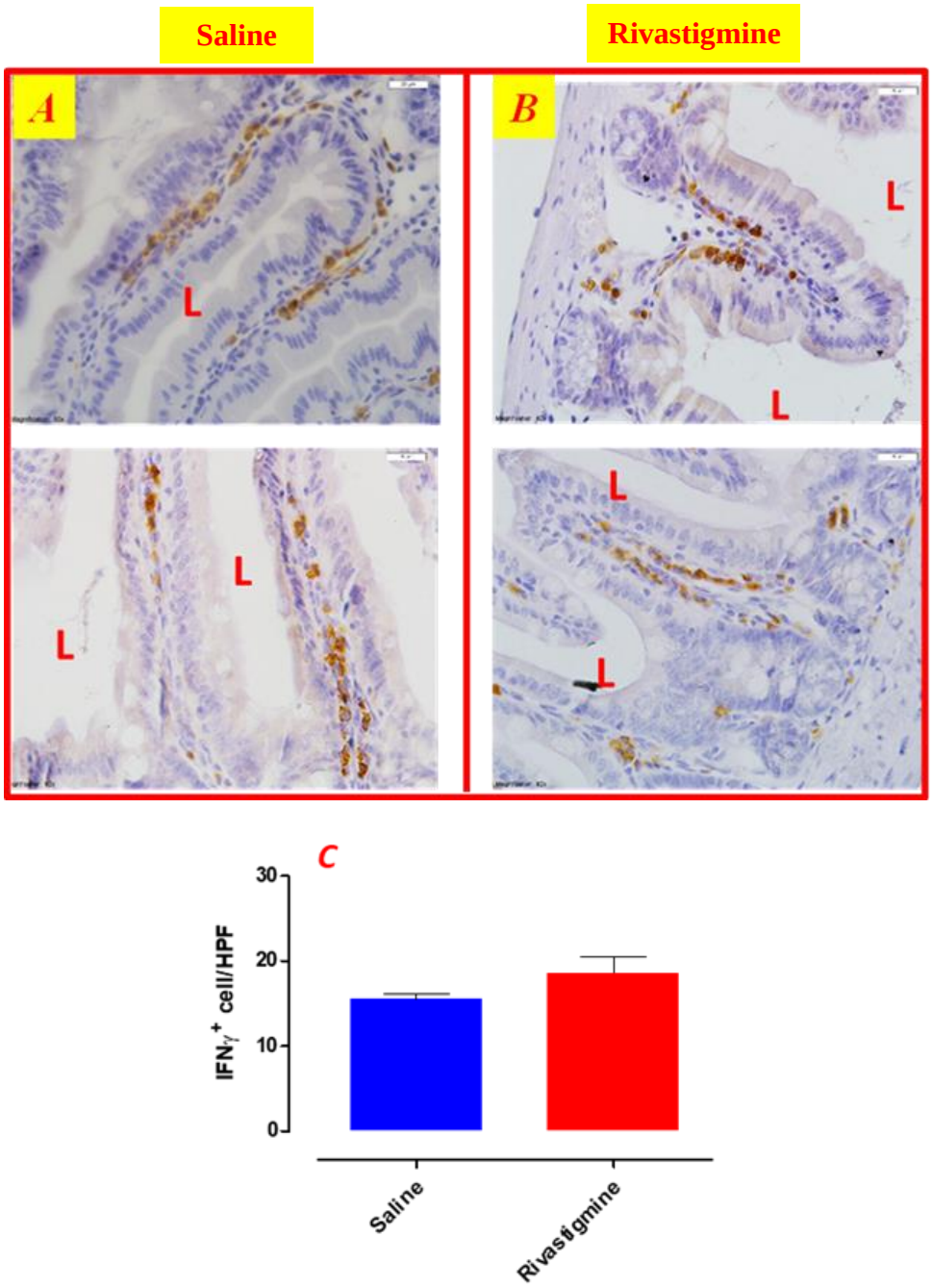

Figure 22: IFNγ producing cells in the small intestine mucosa. Ileum sections obtained from (A) saline and (B) rivastigmine-treated mice were stained with anti-IFNγ antibody and counterstained with hematoxylin. Representative images (600x) from two independent experiments (four mice/group/experiment) are shown. L: lumen. (C) Quantitative estimation of the number of IFNγ cells in intestinal sections of saline and rivastigmine groups. Data are shown as the mean ± SEM of the number of positive cells per high power field. The values represent the mean ± SEM of 4-5 mice/group and the results are pooled from 2 independent experiments.

5.7 Phenotypic characterization of the immune population in the intestinal epithelium and lamina propria following rivastigmine or paraoxon administration.

In order to see if there is any difference between the effect of paraoxon and rivastigmine treatment on the immune population, further investigated the phenotypic composition of the immune cells present in the intestinal mucosa by flowcytometry. The intestinal tissue was excised from mice treated with either saline, paraoxon or rivastigmine, and processed for isolation of two populations of lymphoid cells based on their location: intraepithelial lymphocytes (IELs) and lamina propria leukocytes (LPLs). Representative dot plots with gating strategy for IELS from each group (saline, rivastigmine and paraoxon) are shown in Figures 23 and 24. Date from rivastigmine group are preliminary as it is based on one experiment only. For the saline and paraoxon groups, the data is representative of 3 experiments.

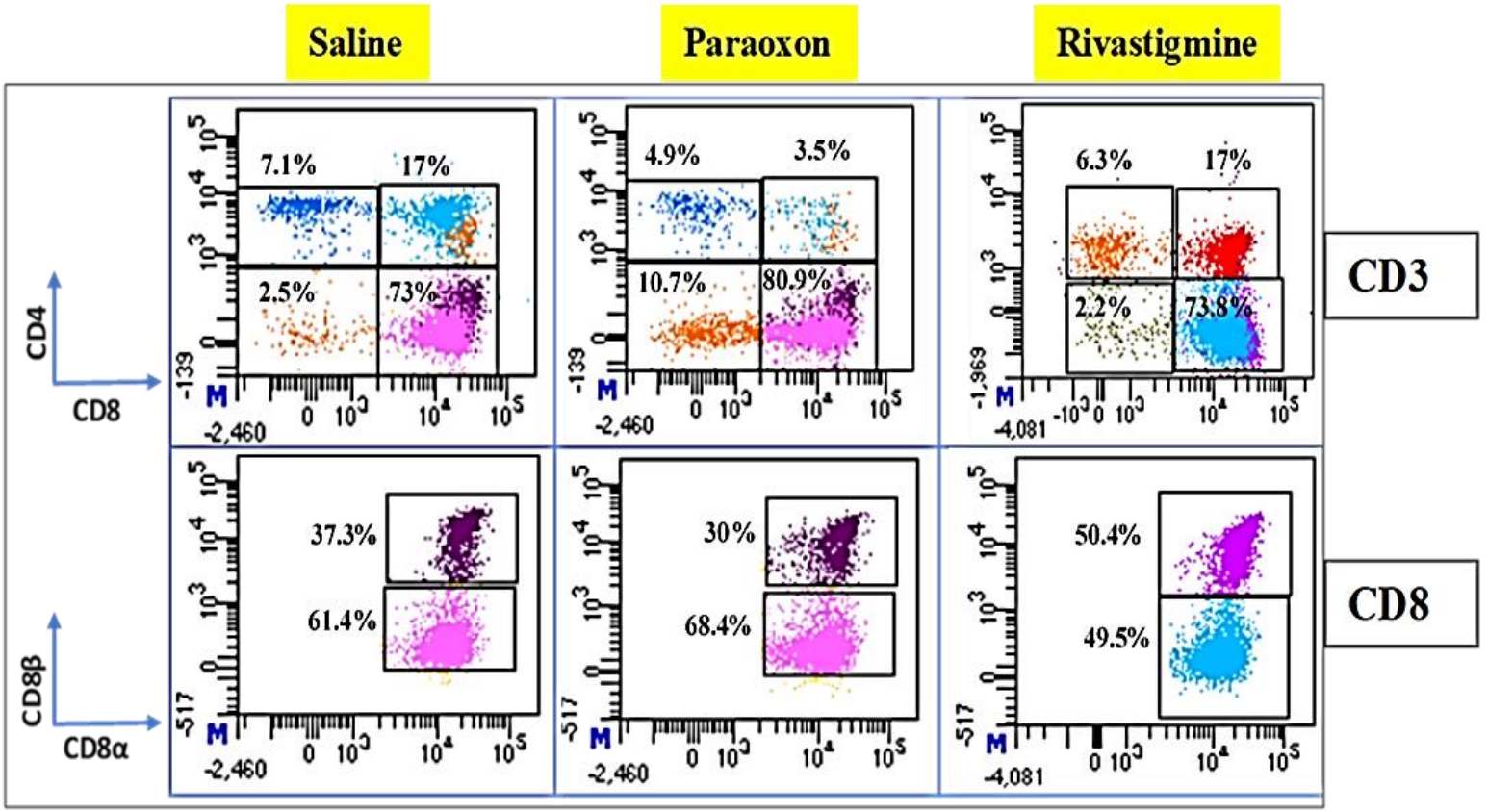

Figure 23: Gating strategy for intraepithelial T cells. $CD3^+$ intraepithelial cells of saline, paraoxon and rivastigmine were analyzed for CD4 and CD8 positivity. $CD8^+$ cells were further analyzed for the expression of α or β chain, and two population were discerned $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$.

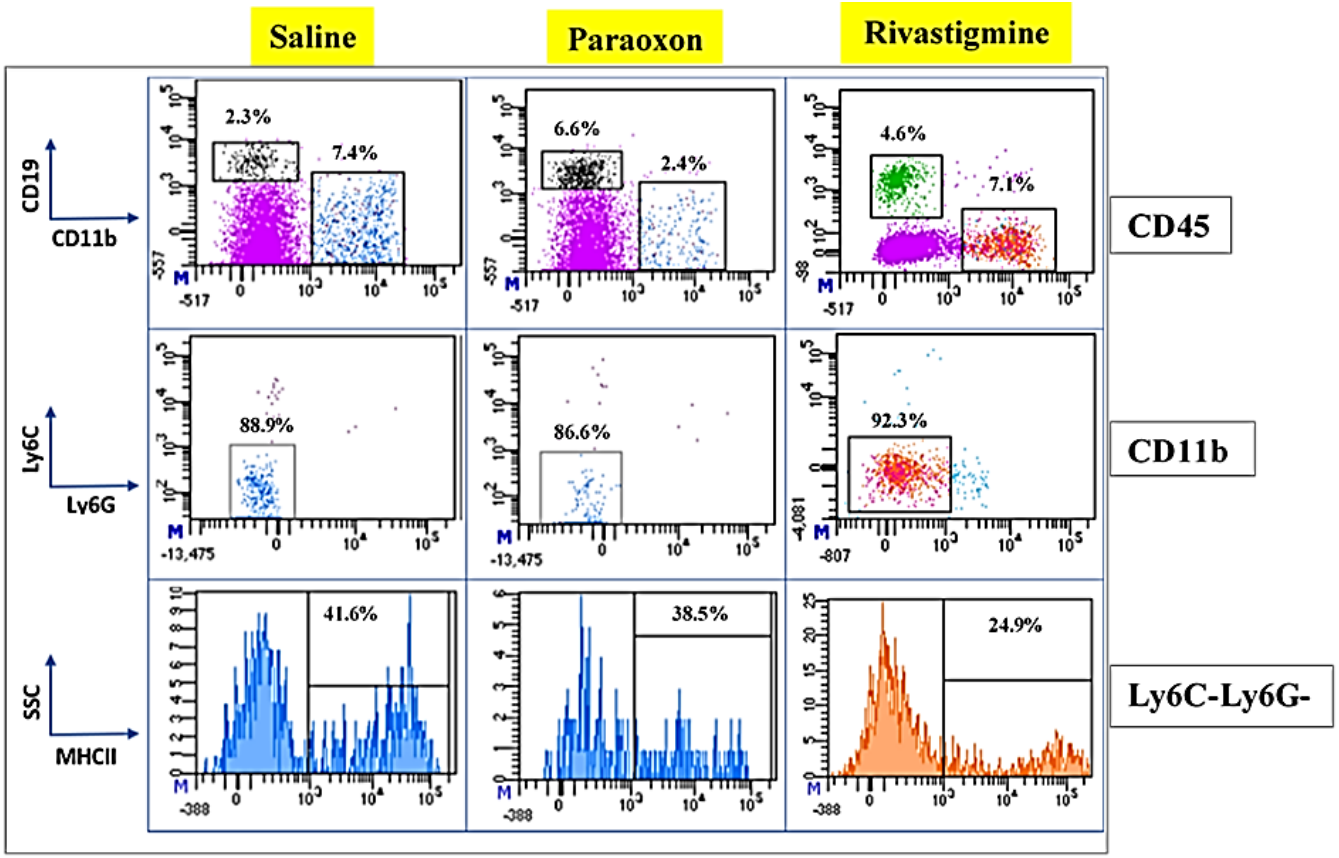

Figure 24: Gating strategy for intraepithelial myeloid cells. CD45+ intraepithelial cells of saline, paraoxon and rivastigmine were analyzed for CD19 and CD11b positivity. $CD11b^{+}$ cells were further analyzed for the expression of Ly6G and Ly6G molecules. $Ly6C^{-}Ly6G^{-}$ population were further analyzed for the expression of MHCII activation marker. Representative dot plots for individual mice of each group are shown.

Analysis of immune cell populations in the intestinal epithelium of saline-treated mice revealed that almost 80% of the haemopoietic ($CD45^+$) cells are T cells ($CD3^+$) with much lower percentage of B cells ($CD19^+$) and myeloid cells ($CD11b^+$) (Figure 25A-D). Rivastigmine (Figure 25D) or paraoxon (Figure 25A) treatment did not induce any change in any of the major cell populations, except a significant reduction in myeloid cells of paraoxon treated group (Figure 25A). In all three groups, cytotoxic lymphocytes ($CD8^+$) constituted the majority of T cells (11-fold more than $CD4^+$ or double positive cells) (Figures 25B-E). CD8 is a cell surface dimeric glycoprotein that can be expressed as a heterodimer (CD8α with CD8β) or as a homodimer (two CD8α chains). In saline-treated mice, $CD8\alpha\alpha^+$ cells represent 56% compared to 40% of $CD8\alpha\beta^+$ (Figures 25C-F). Paraoxon treatment did not change the proportion between these two subpopulations (Figure 25C), however, rivastigmine treatment induced a statistically significant increase in the percentage of $CD8\alpha\beta^+$ cells (naïve CD8 cells) and, consequently a decrease in the $CD8\alpha\alpha^+$ population (Figure 25F). Finally, looked at the myeloid populations present in the intestinal epithelium compartment and observed that the majority of the $CD11b^+$ cells were $Ly6C^-Ly6G^-$ in all groups (~75% in saline and paraoxon; ~85% in rivastigmine group) which represented ~ 5% , 4% and 6% of the total $CD45^+$ cells, respectively (Figures 26A-C). Only rivastigmine treatment significantly increased the percentage of myeloid cells in the intestinal epithelium (Figure 26C). When the activation state was analyzed, it observed that ~40% of the $Ly6C^-Ly6G^-$ cells expressed MHC class II in the saline group (Figures 26B-D). This percentage was not altered after paraoxon (Figure 26B) or rivastigmine administration (Figure 26D).

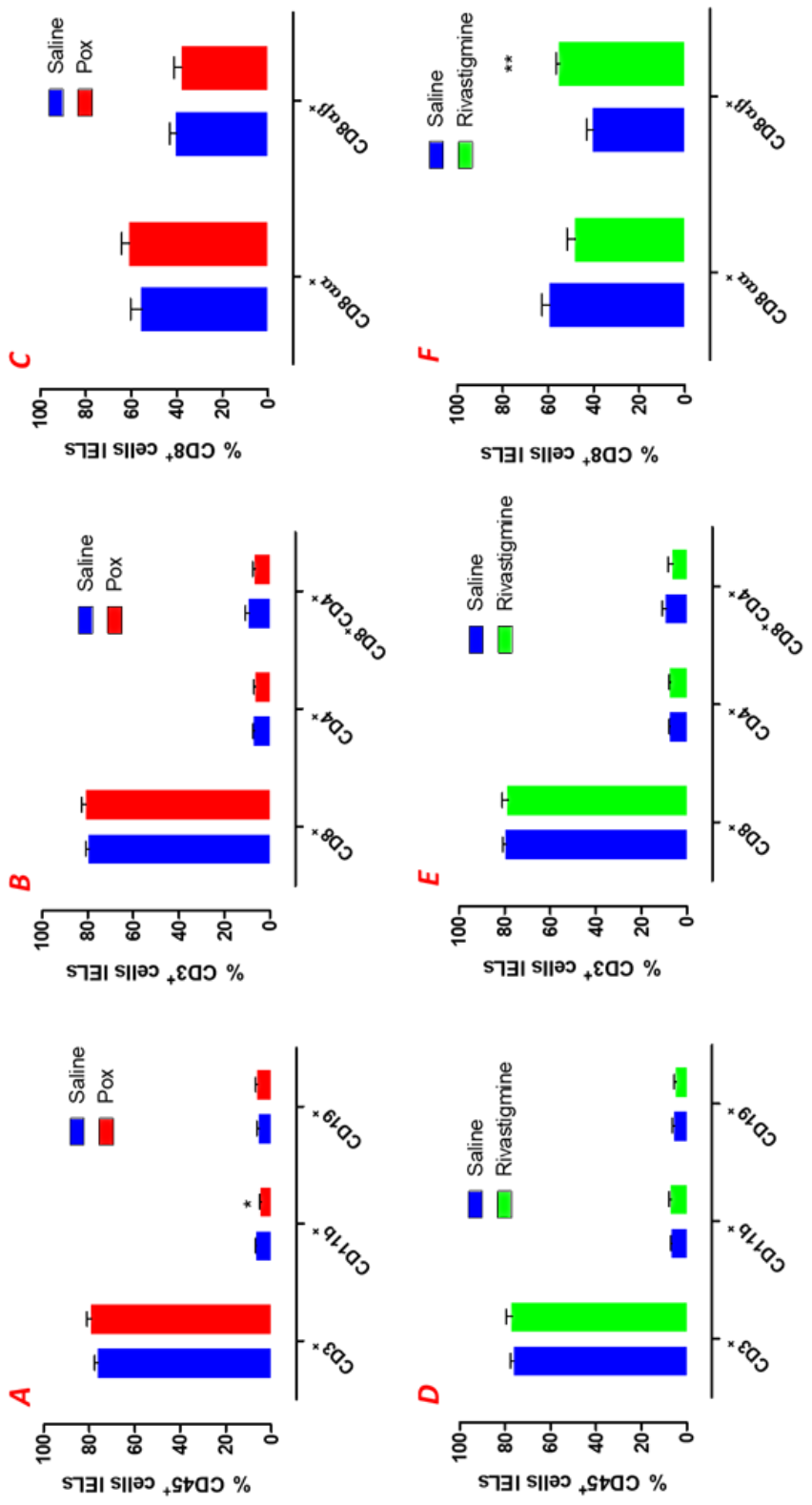

Figure 25: Rivastigmine treatment but not paraoxon increased significantly the percentage of CD8αβ$^+$ T cells in the intestinal epithelium. Most of hematopoietic cells in IELs are CD3$^+$ cells. Majority of these cells are CD8$^+$ cells. Treatment with paraoxon (A, B, C) or rivastigmine (D, E, F) did not make any change in either the percentage of these population nor the phenotype.

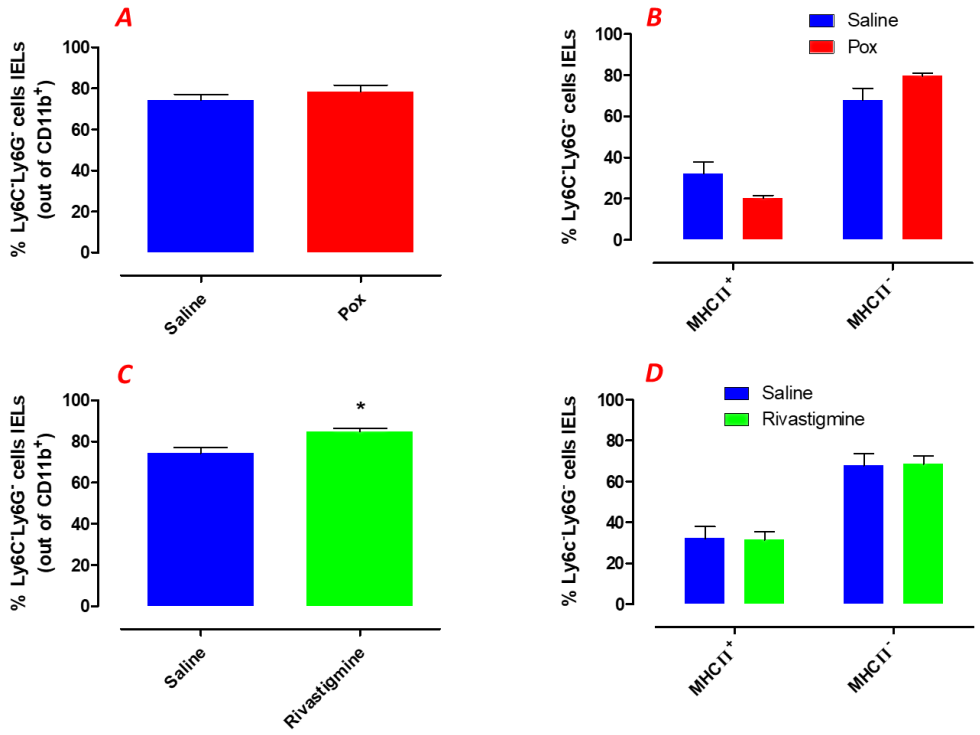

Figure 26: Rivastigmine treatment but not paraoxon increase significantly $Ly6C^{-}Ly6G^{-}$ myeloid cells in the intestinal epithelium. The majority of myeloid cells are $Ly6C^{-}Ly6G^{-}$. MHC^{+} molecule represent around 41% of these cells. Treatment of paraoxon (A-B) did not change the percentage of these cells. Rivastigmine treatment (C-D) significantly increase the percentage of $Ly6C^{-}Ly6G^{-}$ and no change in the expression of MHC^{+}.

Analysis of the same lymphoid populations in the lamina propria (see Figures 27 and 28 for gating strategy), revealed that the percentages of the different $CD45^{+}$ cell populations were different to the ones in the epithelium. The percentage of T cells ($CD3^{+}$) was lower (~40%) and the percentage of B cells and myeloid cells was increased to ~9% and ~26%, respectively, in saline group (Figures 29A-D). In this group, T cells were distributed: ~50% $CD8^{+}$, ~20% $CD4^{+}$ and ~17% $CD8^{+}CD4^{+}$ (Figures 29B-E). Paraoxon treatment induced a significant increase in the percentage of T cells compared to saline (Figure 29A), that was not detected after rivastigmine treatment (Figure 29D). However, both treatments resulted on higher percentage of

$CD8^+$ cells and, lower percentage of $CD4^+$ and $CD4^+CD8^+$ cells (Figures 29B-E). In all groups, ~50% of the $CD8^+$ cells expressed CD8αα and the other half CD8αβ (Figures 29C-F). Similar to what was observed in the epithelium compartment, ~80% of the $CD11b^+$ cells in the lamina propria were $Ly6C^-Ly6G^-$ in all the three groups, representing ~7% of the hematopoietic $CD45^+$ cells. Only treatment with paraoxon reduced the percentage of $Ly6C^-Ly6G^-$ cells expressing MHC class II (Figure 30B). Rivastigmine treatment did not induced any change in MHC class II expression in comparison to the saline treatment (Figure 30D).

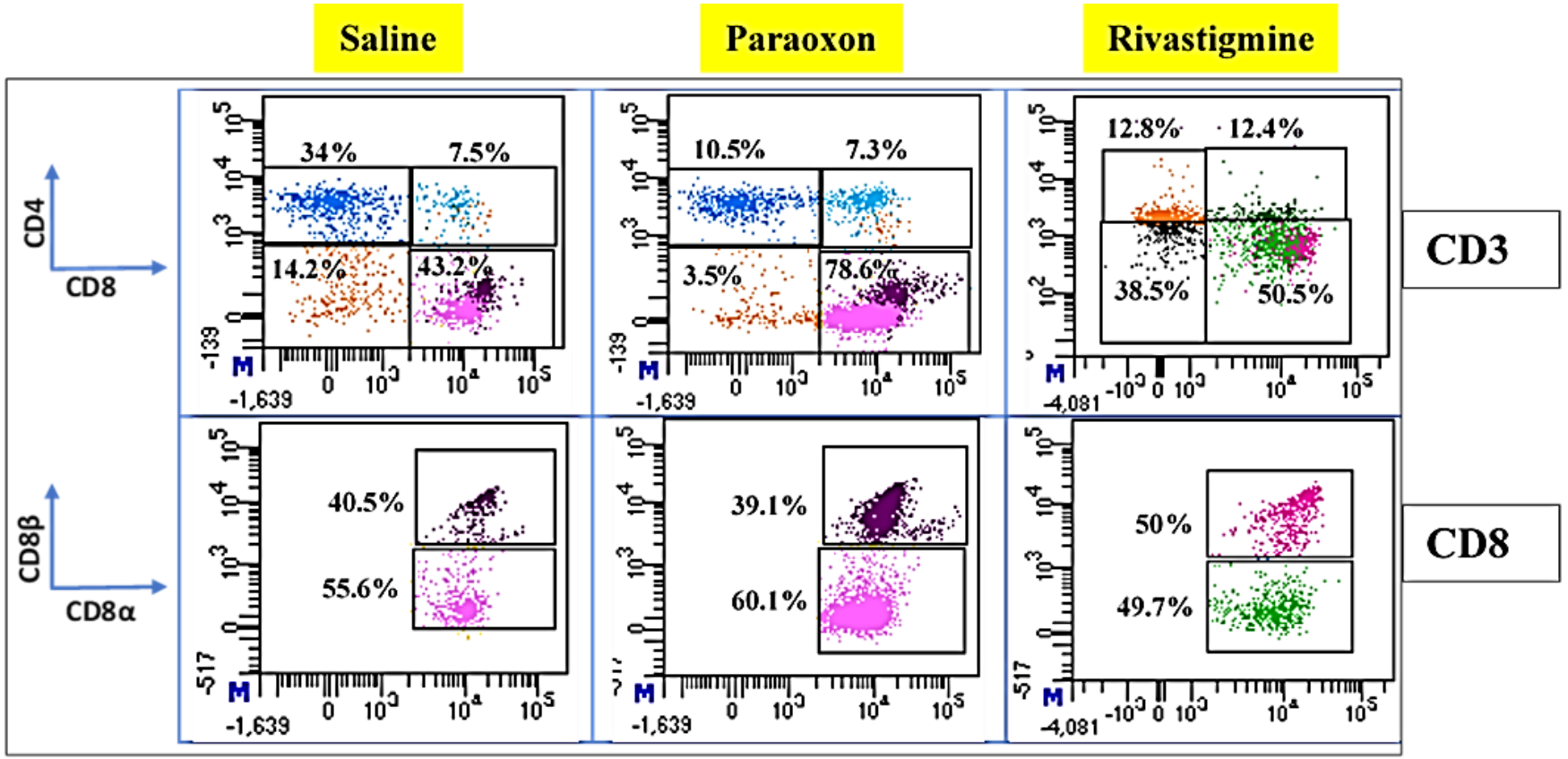

Figure 27: Gating strategy for T cells in the lamina propria. $CD3^+$ intraepithelial cells of saline, paraoxon and rivastigmine were analyzed for CD4 and CD8 positivity. $CD8^+$ cells were further analyzed for the expression of α or β chain, and two population were discerned $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$. Representative dot plots for individual mice of each group are shown.

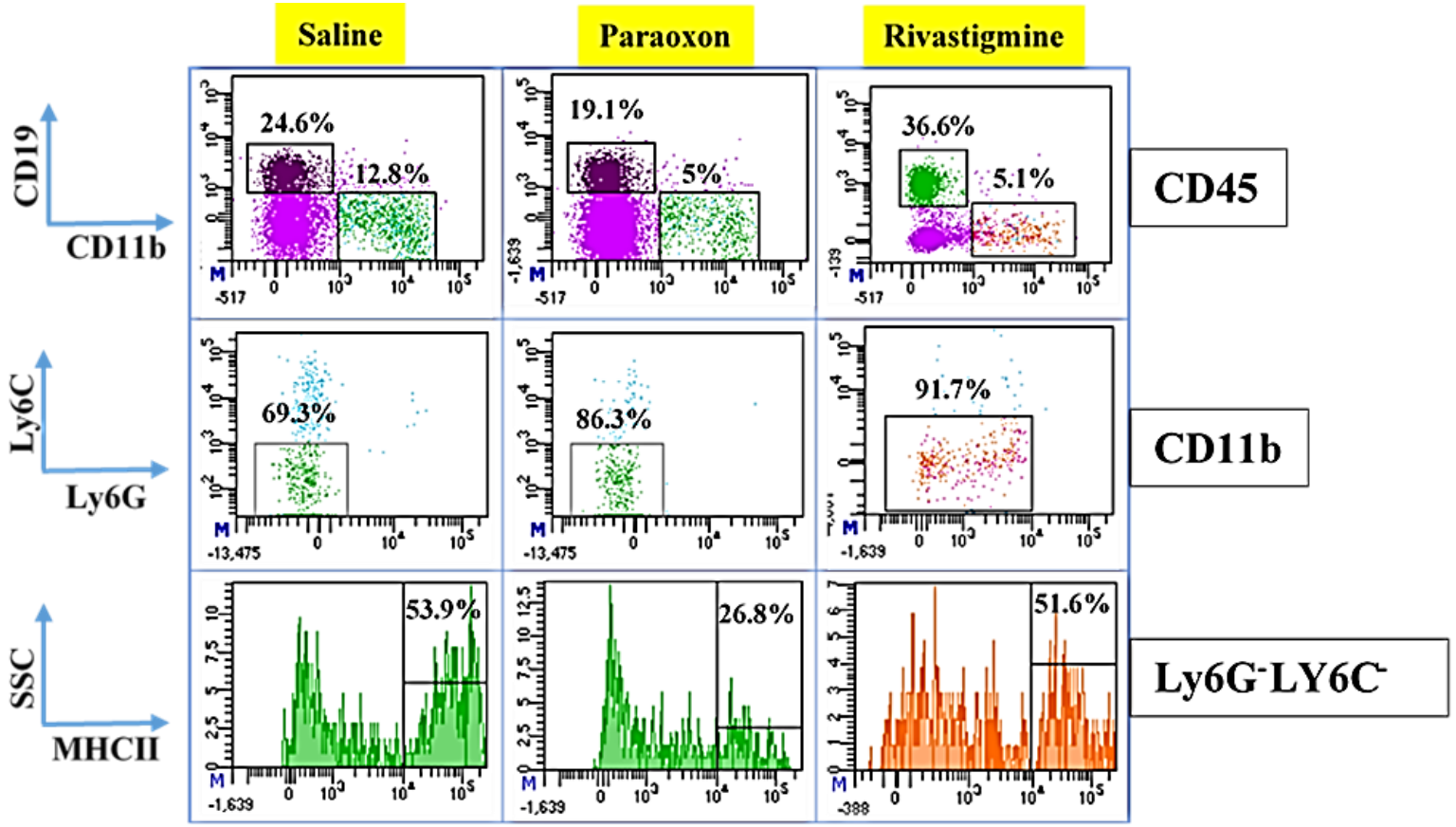

Figure 28: Gating strategy for myeloid cells in the lamina propria. $CD45^+$ intraepithelial cells of saline, paraoxon and rivastigmine were analyzed for CD19 and CD11b positivity. $CD11b^+$ cells were further analyzed for the expression of Ly6G and Ly6G molecules. $Ly6C^-Ly6G^-$ population were further analyzed for the expression of MHCII activation marker. Representative dot plots for individual mice of each group are shown.

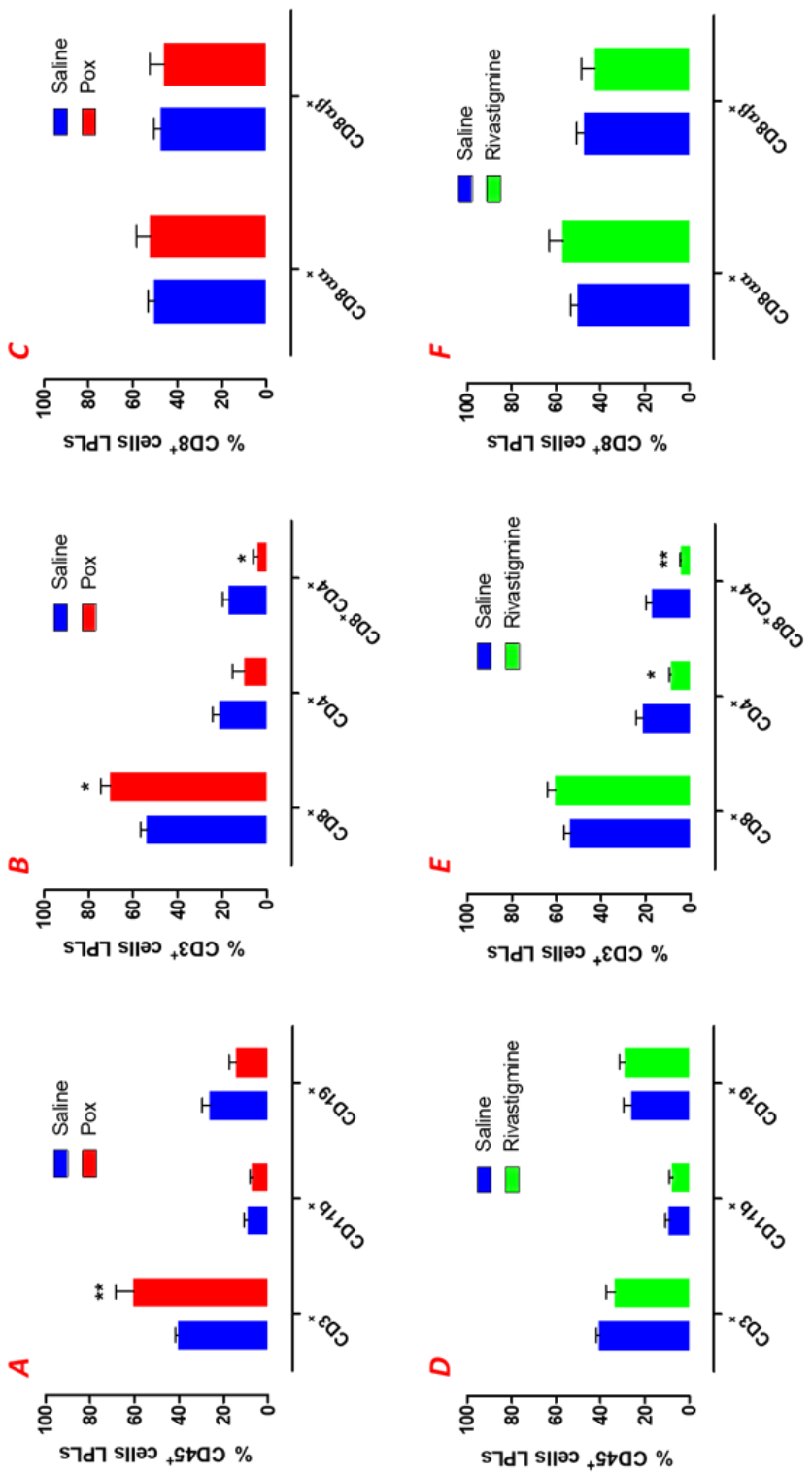

Figure 29: Paraoxon treatment increased the percentage of $CD8^+$ cells in the lamina propria. Most of hematopoietic cells in LPL are $CD3^+$ cells. Majority of these cells are $CD8^+$ cells. Treatment with paraoxon (A-B-C) increase significantly the $CD3^+$ and $CD8^+$ cells. Rivastigmine treatment (D-E-F) did not make any significant change in either the percentage of these population nor the phenotype.

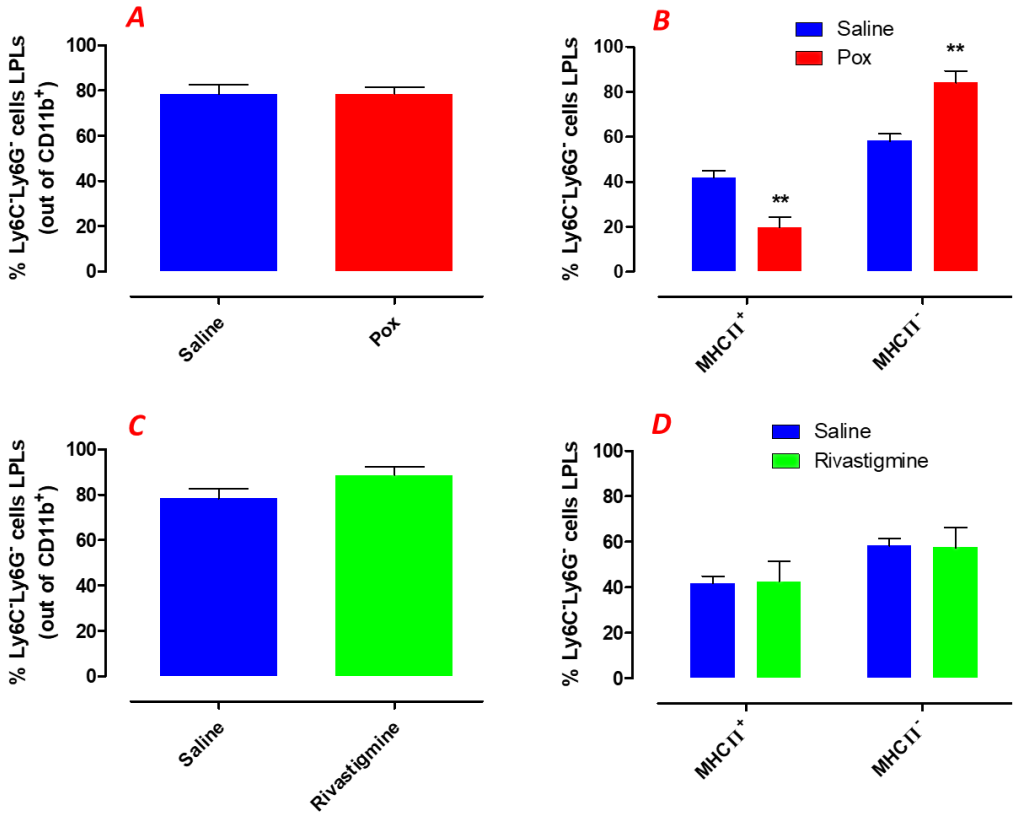

Figure 30: Paraoxon treatment decreased the expression of $MHCII^+$ in lamina propria myeloid cells. The majority of the myeloid cells are $Ly6C^-Ly6G^-$. MHC^+ molecule represent around 42% of these cells. Treatment with paraoxon (A) did not change the percentage of $Ly6C^-Ly6G^-$ cells, However MHC expression decrease significantly in these cells (B). Rivastigmine treatment (C) did not change the percentage of $Ly6C^-Ly6G^-$ cells and no change in the expression of MHC^+ (D).

These results indicate that paraoxon and rivastigmine induced different effects on the immune population in the intestinal mucosa. Paraoxon induced an increase in the percentage of $CD8^+$ cells and a downregulation in the activation markers on DCs ($Ly6C^-Ly6G^-$) in the lamina propria. In contrast, although there was no significant change in the total percentage of lamina propria $CD8^+$ cells, rivastigmine-treatment induced a shift in the phenotype of conventional $CD8^+$ cells by increasing the percentage of $CD8^+\alpha\beta^+$ in the epithelium.

Chapter 6: Discussion

Previously, it was demonstrated that exposure to paraoxon, a highly specific AChE inhibitor, enhanced survival of orally infected mice with a virulent strain of *Salmonella enterica* serovar *Typhimurium* (Fernandez-Cabezudo et al., 2010). In further studies (Al-Barazie et al., 2018), it was shown that this protection was correlated with decreased bacterial load in systemic target organs, like spleen and liver, and mediated by an enhanced innate immune responses in the intestinal mucosa. This study reported that cholinergic stimulation induced the degranulation of intestinal secretory cells, goblet and Paneth cells, enhancing the innate immune defenses at the intestinal barrier and preventing the translocation of bacteria to systemic organs (Al-Barazie et al., 2018). The current study is aimed to investigate whether rivastigmine, a more physiological ACh inhibitor, can also protect against an oral infection with virulent *Salmonella*.

In order to monitor the effectiveness of rivastigmine as AChE inhibitor, the activity of AChE in blood after 24 hours of its administration measured (data not shown), but could not detect any AChE inhibition. In agreement with showed results, a recent study reported no changes in the AChE activity in plasma of adult mice exposed to various concentrations of rivastigmine (Basaure, Peris-Sampedro, Cabré, Reverte, & Colomina, 2017). This is probably due to the short half-life in plasma that rivastigmine has, only 1 hour, and its pharmacodynamic effect of only 10 hours (Schmeltz & Metzger, 2007). It has been reported that cholinergic stimulation induces a transient hyperactive behavior in animals as well as faster peristaltic movements, which lead to a retarded body growth in young treated animals (Al-Barazie et al., 2018; George et al., 2016; Satapathy et al., 2011; Stahl, 2006). Thus, animals' body weight followed in

order to monitor the rivastigmine treatment. Results confirmed a slower body growth during the treatment period with rivastigmine indicating an effective cholinergic stimulation. In agreement with these results, AChE inhibition induced by paraoxon treatment also reduced significantly the body weight growth (Al-Barazie et al., 2018; Fernandez-Cabezudo et al., 2008; Fernandez-Cabezudo et al., 2010). Knowing that rivastigmine treatment was effective, study aimed to see if this treatment could enhance survival of mice orally infected with virulent *Salmonella*. Surprisingly, rivastigmine treatment did not protect the mice from the lethal infection and all mice died by day 23 post-infection. However, rivastigmine treatment significantly enhanced survival compared to the control saline-treated mice where the survival time was 11 days.

To gain further insight, bacterial load at mucosal sites investigated (intestine, feces and intestinal content) and systemic organs like spleen and liver, at day 3 post-oral infection with high dose of *Salmonella*. Data showed that in rivastigmine-treated mice most of the bacteria were located in feces and intestinal content with few in the systemic organs. The opposite was found in saline-treated mice where spleen and liver presented higher bacterial load than feces and intestinal content. However, the bacterial load in the whole intestine (tissue + bacterial content) was similar in both groups (saline and rivastigmine). The fact that, in the saline-treated group, low number of bacteria were found in the intestinal content compared to the number in systemic organs could indicate that *Salmonella* in this group was already translocated from the intestinal lumen to the intestinal mucosa which also explains the presence of higher number of bacteria in the systemic organs. On the other hand, in rivastigmine-treated mice most of the bacterial load found in the whole intestine seemed to be located in the intestinal content which could indicate a stronger intestinal barrier and, therefore,

more difficulties for *Salmonella* to penetrate the intestinal mucosa and disseminate to systemic organs.

Results suggest that rivastigmine treatment is able to initially control bacterial translocation across the mucosa and effectively delay bacterial dissemination into the spleen and liver. However, this effect appears to be rather transient because pathogenic bacteria were able to eventually cross the epithelial barrier and disseminate into systemic organs. In contrast, paraoxon was able to effectively inhibit bacterial translocation and block their dissemination, and hence improving log-term host survival (Al-Barazie et al., 2018). To try to explain these different treatment outcomes after *Salmonella* infection, study focus into the mechanism of action of each of two AChE inhibitors. Both paraoxon and rivastigmine target the cholinesterases AChE and BChE (butyrylcholinesterase) and are able to cross the blood-brain barrier. Paraoxon is an irreversible cholinesterase inhibitor with a plasma half-life of 20 hours, and binds to the active site of the enzyme making it unable to hydrolyze ACh (Musilova, Kuca, Jung & Jun, 2009). On the other hand, rivastigmine is a non-competitive reversible cholinesterase inhibitor with a plasma half-life of 1 hour, although it has a longer action in the brain (Hofmann, Hock, Smelser, & Baltes, 2001; Schmeltz & Metzger, 2007). The reversible mode of its binding to the AChE enzyme allows rivastigmine to quickly dissociate from the enzyme, which in turn regains its function of hydrolyzing ACh, leading to a reduction in the levels of ACh. However, paraoxon binds in an irreversible manner, through strong covalent bonds, to the catalytic site of the AChE enzyme, preventing its action on ACh, and, therefore, increasing the levels of ACh. So, as speculation, lower levels of ACh available in rivastigmine-treated mice compared to paraoxon-treated mice leads to reduced cholinergic stimulation and lower protection against oral bacterial infections.

Next, wanted to see if rivastigmine treatment had any effect on the intestinal mucosal epithelial cells as their proper function and integrity is essential for the elimination of microbes. Absorptive enterocytes, goblet cells, Paneth cells and enteroendocrine cells constitute the majority of cells in the intestinal mucosal epithelium (Kim & Ho, 2010). In this study, two of these types of mucosal epithelial cells analyzed, goblet and Paneth cells. Results showed that rivastigmine treatment clearly induced degranulation of goblet and Paneth cells and increased the mucus barrier in the intestinal lumen. These findings are similar to what previously reported with paraoxon (Al-Barazie et al., 2018).

Goblet cells represent a major cellular component of the innate defense in the intestine and play an important role in maintaining the intestinal homeostasis (McCauley & Guasch, 2015). They are responsible for the secretion of mucins, one of the components of the mucus barrier. Several factors control the release of mucins in the intestine including autonomic neural pathways mediated by cholinergic receptors on goblet cells (Specian & Neutra, 1980). Mucin secretion from goblet cells was found to be abrogated by atropine (reversible antagonist of the muscarinic acetylcholine receptors) indicating that the effect of ACh on mucus secretion is mediated by muscarinic receptors (Specian & Neutra, 1982). The presence of a mucus layer in the intestinal lumen is an extremely important defense mechanism against oral pathogens like *Salmonella*, as the bacteria get trapped and removed by peristalsis, avoiding their adhesion to the surface of the epithelium (Elphick & Mahida, 2005). So, the increase on mucins secretion by cholinergic stimulation induces the accumulation of mucus in the intestinal lumen and, therefore, increases the mucus barrier which is the first line of defense against bacterial infection. Results showed a significant increase in the thickness of the mucus layer in the lumen of colon tissue following rivastigmine

treatment compared to tissue from saline-treated mice. Moreover, both AChE inhibitors (rivastigmine and paraoxon) observed to induce a comparable accumulation of mucus.

As mentioned above, data also showed that rivastigmine treatment induced degranulation of Paneth cells and release of lysozyme. Paneth cells are important in the innate intestinal defense as they contribute to the extra-epithelial defense barrier by secreting antimicrobial proteins and peptides (AMPs) such as lysozyme and defensins. Paneth cells contribute to regulate the intestinal microbial density and to the protection of the stem cells also located in the crypts of Lieverkuhn (Elphick & Mahida, 2005). Upon exposure to bacteria or bacterial products, Paneth cells release the AMPs to the intestinal lumen in order to control microbial growth and prevent microbial invasion into the crypt (Ayabe et al., 2000). A recent study reported the presence of mAChR (M2) in the intestinal crypt, probably present on the Paneth cells (Muise, Gandotra, Tackett, Bamdad, & Cowles, 2017) although a more extensive investigation is needed. Results suggest that rivastigmine treatment induces a more robust defense barrier in the intestinal lumen containing more mucin to trap bacteria and more lysozyme to fight against pathogens. This mechanism could explain the initial protection that rivastigmine treatment exerted on *Salmonella*-infected animals where bacteria was present mostly in the intestinal content and feces, and almost absent from systemic organs. Previously reported similar results using paraoxon as AChE inhibitor (Al-Barazie et al., 2018). However, while paraoxon treatment induced long term protection in 80% of the mice following a lethal *Salmonella* infection, rivastigmine treatment prolonged animal survival by up to 11 days, after which all mice succumbed to the infection. This indicates that the reinforcement of the luminal barriers induced by cholinergic stimulation is not enough to protect mice from a lethal

bacterial infection. Therefore, decided to look at the possible effect that treatment with AChE inhibitors exerted on the intestinal mucosa (epithelium and lamina propria) where the innate and adapted immune responses to invading pathogens take place.

The epithelial layer in the intestine is considered a barrier that protects and avoids the entrance of microbiota and food antigens inside the body. When a pathogen breaks this barrier a disruption in the normal balance of the intestine takes place which triggers a coordinated immune response to eliminate the pathogen. *S. Typhimurium* successfully invades the host and stablishes an infection by disrupting the tight junctions that maintain the epithelial cells closely adhered (Ashida, Ogawa, Kim, Mimuro, & Sasakawa, 2012) to promote its translocation to the intestinal lamina propria. In the lamina propria, *S. Typhimurium* will encounter many different types of effector cells including B cells, T cells, dendritic cells, macrophages and natural killer cells that will coordinate an effective immune response (Patel & McCormick, 2014). As part of this immune response, effector cells activated in the MLN by DCs migrated from PP will arrive to the lamina propria. Additionally, resident immune cells in the lamina propria will be able to mount an initial an immediate immune response.

First, looked at the spatial location of the main lymphoid cells in the intestinal tissue by immunohistochemistry and results showed that the main lymphoid populations are located, as expected, in the intestinal mucosa which is comprised by the epithelium and the lamina propria.

Quantitative analysis of staining reveled that in the epithelium, $CD8^+$ cells were the most abundant T cell population with few $CD4^+$ T cells (~4:1 ratio) in both groups, saline and rivastigmine. In the lamina propria, much lower number of $CD8^+$ cells were present and a discreate higher number of $CD4^+$ T cells compared to the epithelium compartment. Although no significant differences were found between saline and

rivastigmine group, a slight increase noticed in the number of T cells (CD4 and CD8) present in the epithelium and a slight decrease in the lamina propria, after rivastigmine treatment. Few macrophages could only be observed in the lamina propria with no differences between saline and rivastigmine groups.

In a detailed phenotypic analysis of the immune cells that populate each of the effector compartments of the intestinal mucosa (epithelium and lamina propria), not only saline and rivastigmine-treated groups included but also mice treated with paraoxon in order to see if could detect any difference between paraoxon and rivastigmine treatment that could explain the different levels of protection against oral *Salmonella* infection. The intestinal epithelium contains not only the typical columnar cells but also a population of lymphocytes named intraepithelial lymphocytes (IELs) (Paul, 2003) located between the basolateral surface of the epithelial cells. IELs are continuously exposed to antigens that cross the epithelial barrier and, therefore, participate in the induction and regulation of the immune response in the intestinal mucosa. T cells, specially $CD8^+$ cells in the intestinal epithelium provide the first line of immune defense against invading pathogens (Jabri & Abadie, 2015). The intestinal epithelium contains one of the most abundant T cell populations in the body which are responsible for the continuous surveillance of the intestine in order to maintain the homeostasis and prevent the entrance of microbes and food particles (Van Konijnenburg et al., 2017).

In agreement with previous studies (Cheroutre, 2004; Guy-Grand et al., 1991; Lefrancois, 1991; Lin et al., 1994), results showed that in the saline control mice, the majority of the IELs were T cells which around 80% expressed CD8 and only 15% CD4. Neither rivastigmine nor paraoxon treatment changed the percentage of $CD8^+$ cells in the epithelium compartment, compared to the saline control treatment.

However, the proportion of CD8αα$^+$ cells decreased after rivastigmine treatment inducing an increase in the percentage the CD8αβ$^+$ population.

The presence of CD8 effector memory cells are essential for a rapid control of any pathogens invading the intestinal mucosa. Two types of CD8 T cells were found among the IELs: CD8αα homodimer and CD8αβ heterodimer. CD8αβ$^+$ cells are T cells that have been activated in the lamina propria or MLNs and then, migrated to the epithelium where their function is to protect the mucosal barrier either as a effector cells or tissue resident memory T cells (Montufar-Solis, Garza, & Klein, 2007). They have been reported to express strong cytotoxic T lymphocyte (CTL) activities and produce large amounts of IFN-γ upon TCR-dependent stimulation (Emoto, Emoto, & Kaufmann, 1996). Intraepithelial CD8αβ$^+$ T cells are also TCRαβ$^+$ and express constitutively granzyme B, CD103 (integrin β7), CD69 and produce lower amounts of TNFα and IFNγ than conventional CD8 T cells (Masopust, Vezys, Wherry, Barber, & Ahmed, 2006). In the other hand, CD8αα$^+$ T cells are functionally distinct population with the capacity to produce greater levels of TNFα and IFNγ upon stimulation, and perforin than their CD8αβ$^+$ counterparts (Walker et al., 2013). Therefore, results suggest that by reducing the percentage of CD8αα$^+$ IELs, rivastigmine treatment induced a deficit of professional fast-responder CD8 T cells that rendered the host more susceptible to pathogen invasion.

Histological study also showed the presence of IFNγ producing cells mostly located at the base of the epithelium and few in the lamina propria. IFNγ is produced not only by T lymphocytes but by other several immune cells including macrophages, NK cells, NKT cells (Rhee, Walker, & Cherayil, 2005). Another important cell type in the intestinal mucosa are the innate lymphoid cells (ILCs) which are a novel family of

tissue resident cells (Ebbo, Crinier, Vely, & Vivier, 2017; Mjösberg & Spits, 2016) that lack antigen-specific receptor. They are able to detect any changes that may occur in the microenvironment and respond by secreting cytokines. Specifically, ILC1s secrete IFNγ in response to IL-12, IL-15 and IL-18 (Panda & Colonna, 2019) and are the most abundant type of ILCs in the intraepithelial compartment (Han et al., 2019). The potential role that the neuronal system could play in the activation of ILCs to produce cytokines has been indicated but not confirmed (Han et al., 2019). As speculation, in this study the IFNγ-producing cells detected in the intestinal mucosa could be $CD4^+$ cells, $CD8^+$ ($\alpha\alpha$ or $\alpha\beta$) cells or ILC1s, but further experiments are needed to clarify this point.

Lamina propria is the layer of connective tissue underlying the intestinal epithelium. Its cellular population differs from the epithelium in that it contains more B cells, more $CD4^+$ cells, more myeloid cells and the presence of new additional sub-population of $CD8^+$ cells, $CD8\alpha\beta^+$ $TCR\gamma\delta^+$ T cells that was absent in the epithelium compartment. $TCR\gamma\delta^+$ cells are usually defined as $CD8\alpha\alpha^+$ or $CD8^-$ (Hayday, Theodoridis, Ramsburg, & Shires, 2001; Vantourout & Hayday, 2013) but not $CD8\alpha\beta^+$. Only in $TCR\beta^{-/-}$ mice $CD8\alpha\beta^+$ $TCR\gamma\delta$ were described (French, Roark, Born, & O'Brien, 2009) and, more recently in humans where were identified in blood and, intestine (Kadivar, Petersson, Svensson, & Marsal, 2016). This $CD8^+$ T cell subset expresses cytotoxic mediators, responds to IL-2, produces IFNγ and TNFα, and they may play a role in the gut homeostasis and mucosal healing in bowel diseases (Kadivar et al., 2016). In this model, a more exhaustive analysis of the lamina propria immune populations is needed to be able to detect this controversial subpopulation.

This results also showed that ACh inhibition with paraoxon increased the percentage of the total T cell population compared to the control, but not rivastigmine. $CD8^+$ cells are responsible of this increase in T cells and, it could be due to a recruitment of $CD8^+$ cells to the lamina propria or to an active proliferation of existing CD8. The presence of more $CD8^+$ cells indicates an increase in the surveillance and recognition capacity which allows a more rapid and efficient response to any invading pathogen. It has been shown that $CD8^+$ T cells are essential for survival to primary infection with *Y. enterocolitica* $CD8^+$ and prevent early systemic dissemination of the bacteria (Siefker & Adkins, 2017).

On the other hand, rivastigmine treatment did not increase the percentage of total T cells but increased the percentage of $CD8^+$ cells among the T cell population and, therefore, decreased the percentage of $CD4^+$ and double positive $CD4^+CD8^+$ cells. Knowing the important role that $CD8^+$ cells play in *Salmonella* clearance (Hess, Ladel, Miko, & Kaufmann, 1996), an increase on these cells will suppose an advantage for the host.

It is good to mention that the changes induced on immune cells by AChE inhibitors can be due to direct effect (ACh+ AChR) or indirect (cytokine driven). Without discarding the possibility that the increase of mucus secretion and AMP induced by AChE administration would have also altered the microbiota composition in the intestine which could influence the development of some T cell populations.

Paraoxon treatment, but not rivastigmine, showed to have an effect downregulating the expression of MHCII on lamina propria resident DCs while no changes were detected on this population after rivastigmine exposure. The major histocompatibility complex class II molecules (MHC-II) are constitutively expressed by immune antigen-presenting cells (APCs) like B cells, monocytes, macrophages and dendritic cells

(DCs). MHC-II molecules are essential for the initiation of the antigen-specific immune response during which APCs, specially DCs, present processed antigens to naïve $CD4^+$ T cells and, therefore, stimulate them. In the intestinal lamina propria resident DCs ($Ly6C^-Ly6G^-$) founded that patrol their microenvironment searching por antigens that internalize to generate complexes peptide-MHCII that eventually will be present to a naïve T cell (Trombetta & Mellman, 2005). During *Salmonella* infection, DCs recognize LPS or flagellin, antigens expressed on *Salmonella*, and respond by increasing the expression of MHC class II and the co-stimulatory molecules CD80, CD86, and CD40 (McSorley, Ehst, Yu, & Gewirtz, 2002; Salazar-Gonzalez et al., 2007), therefore, resting unstimulated cells could be of more use to fight a new infection than already stimulated ones. Downregulation of the MHC-II molecules expression on DCs present in the lamina propria found in paraoxon-treated mice can also be explained by (1) the presence of a more robust luminal barrier (with more mucins and AMP) that leads to less antigens transposing the epithelial barrier and (2) the presence of more $CD8^+$ cells that will take over the immune defense of the lamina propria. Despite the fact that rivastigmine treatment also induced a stronger luminal barrier, could not appreciate any MHC-II downregulation in DCs.

This study did not analyze the innate lymphoid cells (ILCs). Although it is well known that ILCs are important regulators of early infection at mucosal barriers and they provide an immediate immune response through the production of cytokines that limit pathogen invasion (Colonna, 2018). In future studies one of the plans to evaluate the effect of cholinergic stimulation on these immune populations.

Chapter 7: Conclusions

Rivastigmine treatment enhanced host survival after a lethal oral infection. The observed effect seems to be partially due to an increased in degranulation of goblet and Paneth cells in the epithelium of the GI tract which enhanced the innate immune mucosal defense mechanism as showed in Figure 31C. This could explain the reduction in the initial bacterial translocation and systemic dissemination of oral *Salmonella* leading to enhanced survival. However, rivastigmine treatment failed to increase the total $CD8^+$ population in charge of surveillance as it was observed in mice treated with paraoxon.

To conclude, protection against lethal bacteria in the GI tract depends on the extent of cholinergic pathway activation induced by AChE inhibitors. Cholinergic stimulation with rivastigmine induced a deficit of professional fast-responder CD8αα T cells within the intestinal epithelium, which could render the host more susceptible to pathogens. In the lamina propria, rivastigmine treatment did not induce any significant change in the $CD8^+$ population (Figure 31C). In contrast, paraoxon increased the total $CD8^+$ population in charge of surveillance which facilitates a more rapid and efficient response to invading pathogens (Figure 31B).

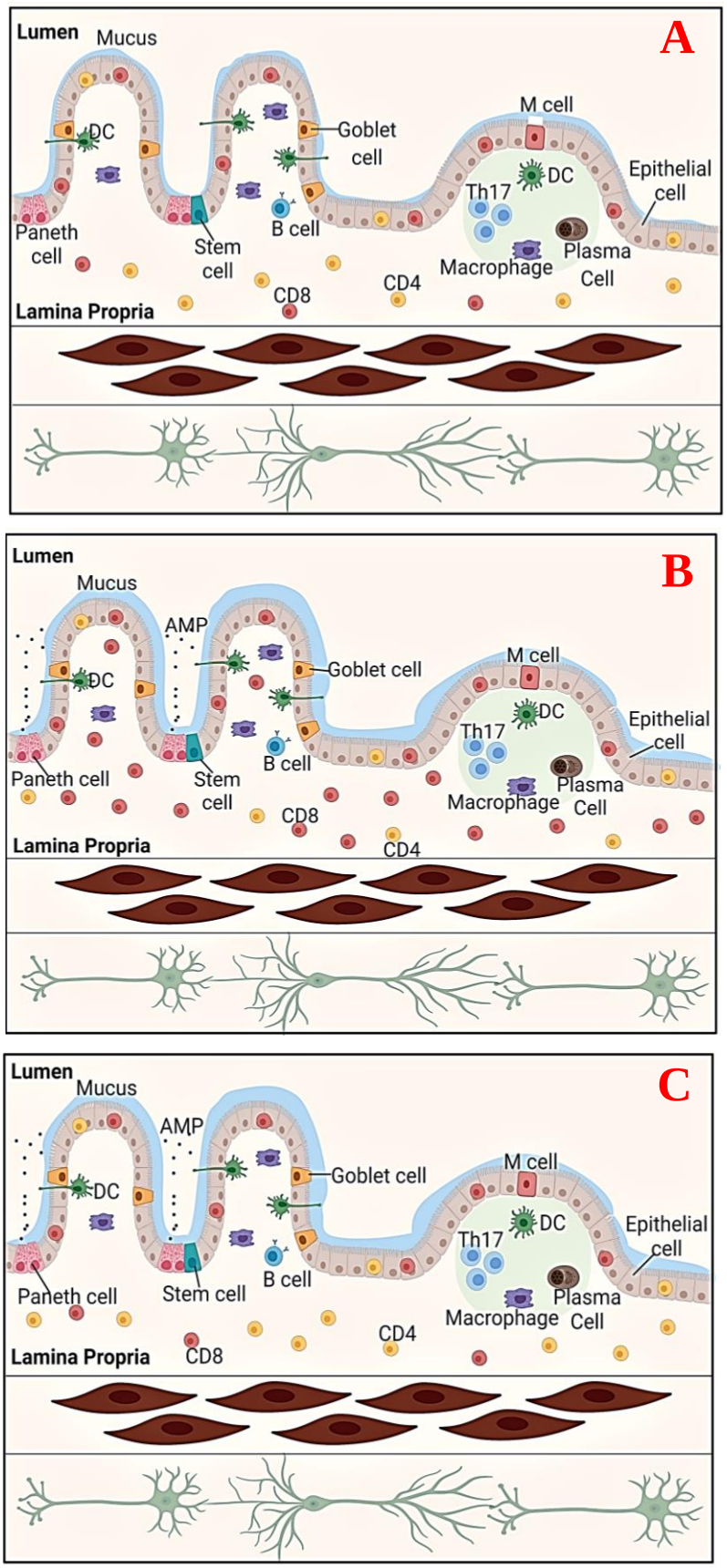

Figure 31: Effect of AChE inhibition on the intestinal mucosa. Schematic representation of the intestinal immune defense in mice treated with (A) saline, (B) paraoxon and (C) rivastigmine.

7.1 Future directions

Many different experiments have been left for the future due to lack of time, and some questions need to be answer. Such as, what is the mechanism by which ACh modulates CD8+ cells in the intestinal lamina propria? What is the effect of cholinergic stimulation on other immune cell populations like DCs and ILCs? How cholinergic stimulation modulates the response of these populations to an in vivo infection? In order to administer these questions, the aimed will be to:

- Study the effect of AChE inhibitors on the intestinal immune population after infection.
- Investigate the different $CD8^+$ subpopulations based on the different TCR expression as well as the intestinal innate lymphoid cell (ILC) population and their role in protection against infection.
- Study, in vitro, the effect of ACh on CD8 T cell differentiation and proliferation.

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