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United Arab Emirates University

College of Medicine and Health Sciences

MECHANISMS UNDERLYING CONTROL OF ANTI-MICROBIAL IMMUNITY BY ACETYLCHOLINESTERASE INHIBITION

Ray M. Esam Al-Barazie

This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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

April 2015
Declaration of Original Work

I, Ray M. Eşam Al-Barazie, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "Mechanisms underlying control of anti-microbial immunity by acetylcholinesterase inhibition", hereby, solemnly declare that this dissertation is an 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 been previously formed as the basis for the award of any academic degree, diploma or a similar title at this or any other university. The materials borrowed from other sources and included in my dissertation have been properly cited and acknowledged.

Student's Signature __________________________ Date 11/12/2015
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Approval of the Doctorate Dissertation

This Doctorate Dissertation is approved by the following Examining Committee Members:

1) Advisor (Committee Chair): Maria J. Fernandez-Cabezudo
   Title: Associate Professor
   Department of Biochemistry and Molecular Biology
   College of Medicine and Health Sciences
   Signature [Signature]
   Date 30/4/2015

2) Member: Basel al-Ramadi
   Title: Professor and chair
   Department of Medical Microbiology and Immunology
   College of Medicine and Health Sciences
   Signature [Signature]
   Date 30/4/2015

3) Member: Eric Mensah-Brown
   Title: Professor
   Department of Anatomy
   College of Medicine and Health Sciences
   Signature [Signature]
   Date 30/4/15

4) Member (External Examiner): Rosa P. Gomariz
   Title: Professor
   Department of Cellular Biology
   Institution: Faculty of Science, Complutense University, Madrid, Spain
   Signature [Signature]
   Date 30-4-15
This Doctorate Dissertation is accepted by:

Dean of the College of Medicine and Health Sciences: Professor Dennis Templeton

Signature [Signature] Date 25-5-2015

Dean of the College of the Graduate Studies: Professor Nagi T. Wakim

Signature [Signature] Date 11-6-2015

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Inflammation is a crucial defense mechanism that protects the body from the devastating effects of invading pathogens. However, an unrestrained inflammatory reaction may result in systemic manifestations with dire consequences to the host. The extent of activation of the inflammatory response is tightly regulated through immunological and neural pathways. Previously, we demonstrated that cholinergic stimulation confers enhanced protection in experimental animals orally infected with a lethal dose of *Salmonella typhymurium*. In this study, we investigated the mechanism by which this enhanced protection takes place. We showed that cholinergic stimulation enhanced host survival following oral-route infection, which correlated with significantly reduced bacterial load in target organs, including livers and spleens. Enhanced protection was not due to increased gut motility or rapid bacterial clearance from the GI tract. Moreover, protection against bacterial infection was lost when the animals were infected systemically, suggesting that the acetylcholine-mediated protective effect was mostly confined to the gut mucosal tissue. *In vivo* imaging demonstrated more localized infection and delay in bacterial dissemination into systemic organs in mice pre-treated with acetylcholinesterase inhibitors. Morphological analysis of the small intestine (ileum) showed that acetylcholinesterase inhibition induced the degranulation of goblet cells and Paneth cells, two specialized secretory cells involved in innate immunity. Our findings demonstrate a crucial pathway between neural and immune systems that acts at the mucosal interface to protect the host against invading pathogens.

**Keywords:** acetylcholinesterase inhibition, *Salmonella*, neuro-immune, intestine-mucosal barrier, goblet cells, Paneth cells.
Title and Abstract (in Arabic)
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Espeical thanks to my parents, sister and brother for their unconditional support, continued encouragement and endless patience during all these years.

Finally, many thanks to my dear friends and colleagues in the laboratory whom were always there for me, listen, advised and kept supporting me.
Dedication

To my beloved parents and family
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<tr>
<td>2-ME</td>
<td>2-Mercaptoethanol</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-Aminoactinomycin D</td>
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<td>ACh</td>
<td>Acetylcholine</td>
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<td>AChE</td>
<td>Acetylcholinesterase</td>
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<td>AMPs</td>
<td>Anti-Microbial Peptides</td>
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<td>Ang</td>
<td>Angiogenin</td>
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<tr>
<td>BLI</td>
<td>Bio-Luminescence Imaging</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CFUs</td>
<td>Colony Forming Units</td>
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<td>ChAT</td>
<td>Choline Acetyltransferase</td>
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<td>ConA</td>
<td>Concanavalin A</td>
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<td>CRS</td>
<td>Cryptdin Related Sequence</td>
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<td>Defensin</td>
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<td>E.coli</td>
<td>Escherichia Coli</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>FAE</td>
<td>Follicle-Associated Epithelium</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>GALT</td>
<td>Gut-Associated Lymphoid Tissue</td>
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<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<td>GIT</td>
<td>Gastrointestinal Tract</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Hb</td>
<td>Hemoglobin</td>
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<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
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<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid</td>
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<tr>
<td>IFN</td>
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<td>mAChR</td>
<td>Muscarinic Acetylcholine Receptor</td>
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<td>MHC II</td>
<td>Major Histocompatibility Complex Class II</td>
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<td>Mesenteric Lymph Node</td>
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<td>MMP-7</td>
<td>Matrix Metalloproteinase (matrilysin)</td>
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<td>MNL</td>
<td>Mononuclear Leukocytes</td>
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<tr>
<td>nAChR</td>
<td>Nicotinic Acetylcholine Receptor</td>
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<tr>
<td>NK</td>
<td>Natural Killer</td>
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<td>NO</td>
<td>Nitric Oxide</td>
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<tr>
<td>Nramp1</td>
<td>Natural Resistance Associated Macrophage Protein 1</td>
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<tr>
<td>PAMPs</td>
<td>Pathogen Associated Molecular Patterns</td>
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<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<td>Peritoneal Exudate Cells</td>
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<td>Paraoxon</td>
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<td>Peyer’s Patches</td>
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<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<td>RBCs</td>
<td>Red Blood Cells</td>
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<tr>
<td>RT-PCR</td>
<td>Réverse Transcriptase Polymerase Chain Reaction</td>
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<td><em>Salmonella Typhymurium</em></td>
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<td>Stem Cells Antigen-1</td>
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<td>SED</td>
<td>Sub-Epithelial Dome</td>
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<tr>
<td>TLR</td>
<td>Toll Like Receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrotic Factor</td>
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<td>VACChT</td>
<td>Vesicular Acetylcholine Transporter</td>
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Chapter 1: Introduction

1.1 Acetylcholine

Acetylcholine (ACh) is an important neurotransmitter in the autonomic nervous system. It is the pre- and postganglionic neurotransmitter in the parasympathetic nervous system as well as the preganglionic neurotransmitter in the sympathetic nervous system. ACh is synthesized from acetyl CoA and choline in the presynaptic neurons by the enzyme choline acetyltransferase (ChAT). At that point the vesicular acetylcholine transporter (VACHT) is responsible for leading the ACh into the vesicles to be stored until it is needed. When a nerve is stimulated, ACh containing vesicles will be mobilized by the influx of calcium ions through the membrane, fuse with the presynaptic membrane and release ACh into the synaptic cleft, in the case of neurons, or into the neuromuscular junction in muscles. ACh will, then, bind to its receptors which are expressed on the post-synaptic membrane.

Two types of ACh receptors are known: the muscarinic acetylcholine receptors (mAChR) and the nicotinic acetylcholine receptors (nAChR). mAChRs are G-protein coupled receptors which are classified into five subtypes M1, M2, M3, M4 and M5 which vary in their distribution in the central and peripheral nervous systems, and different organs and cells (1). nAChRs are pentameric ligand-gated ion channels with different types of subunits depending on whether the receptors are located in neurons or in muscles. In neurons, the nAChR subunits can be either α (ranging from α2 to α10) or β (ranging from β2 to β4) and they can be arranged as homomeric receptors like α7 nAChR or heteromeric receptors like (α4)2(β2)3. The nAChR in muscles is an heteromeric structure composed of 2 α1 subunits and one of each of β1, γ (or ε) and δ.
(1,2). To finalize the ACh signaling, ACh is hydrolyzed by acetyl cholinesterase (AChE) to acetic acid and choline. Choline is then reabsorbed into the pre-synaptic neuron via a high-affinity choline transporter to be reused in synthesizing ACh (2). Diagram 1 shows ACh life cycle.

Diagram 1: Acetylcholine life cycle

Cholinergic nerve transmission is terminated by the enzyme acetylcholinesterase (AchE). AchE is found both on the post-synaptic membrane of cholinergic synapses and in other tissues e.g. red blood cells. Acetylcholine (Ach) binds to AchE and is hydrolysed to acetate and choline. This inactivates the Ach and the nerve impulse is halted.

Obtained from
https://www.cnsforum.com/educationalresources/imagebank/dementia_cholinergic/rept_sys_ach_esterase
1.2 Non-neuronal cholinergic system

The existence of a cholinergic system in many non-neuronal mammalian cells is well known. These cells contain the main components of the cholinergic system which are:

- ACh
- ChAT thus the ability to synthesize ACh
- AChE hence the ability to hydrolyze ACh
- n-AChRs and m-AChRs for ACh to interact

Furthermore, the presence of ACh and ChAT has been demonstrated in evolutionary primitive life like fungi and bacteria (E.coli, Staphylococcus aureus and Bacillus subtilis) and multicellular organisms for example sponge as well as in plants such as bamboo shoot, eggplant and mushrooms (3).

Examples of non-neuronal mammalian cells that express ChAT are:

- Human placenta and airway epithelial cells which manifest ChAT activity in plasma membranes and associated structures such as microvilli, cilia, caveolae, cytosol and nucleus. The expression of ChAT in such important and functional areas is linked with the cellular functions (ciliary activity, proliferation, differentiation and organization of the cytoskeleton) of the non-neuronal ACh produced by these cells (4).
- Murine embryonic stem cell line (CGR8) which is able to synthesize and release ACh involved in the regulation of phenotype-specific cell function and in
cellular homeostasis. Moreover, CGR8 cell line express functional nicotinic and muscarinic ACh receptors (5).

- Mice cardiac myocytes which have the capability to synthesize ACh. In these cells ACh has been shown to reduce cellular oxygen consumption, accelerate angiogenesis and up-regulate cardiac ischemic tolerance factor HIF-1α (hypoxia-inducible factor-1 alpha) which all lead to the prevention of cardiac myocyte ischemia thus has a protective role (6). Moreover, Oikawa et al. (7) demonstrated that the non-neuronal cholinergic system is able to regulate the levels of ATP and survival of cells under serum deprived conditions. ACh in ChAT-expressing cells can increase cellular ATP levels and increase glucose up-take by inducing the expression of HIF-1α, a transcription factor responsible for glucose utilization, glucose up-take and glycolysis, thus maintaining cell energy and protecting it from death (7).

1.3 **Cholinergic system in the immune cells**

In the immune system, immune-competent cells contain all the components of the cholinergic system as they have the ability to synthesize, release and metabolize acetylcholine on their own, independently of the nervous system, although the expression of these components vary from cell to cell. It has been demonstrated that 60% of ACh present in blood is indeed localized in circulating mononuclear leukocytes (MNL) (8). Additional studies on cell lines have revealed that human T-lymphocytes express ChAT which is identical to the ChAT expressed in the nervous system indicating that the origin of ACh in the blood is from the MNLs (9).
The expression of muscarinic ACh receptors in the MNL was evaluated repeatedly, yet conflicting data were obtained. Two studies (10,11) found that MNL express M3, M4 and M5 subtypes while M1 and M2 mRNA were not detected. However, another study performed on MNL from different donors showed that M4 and M5 were expressed in all the individuals but the expression of M1, M2 and M3 varied among them (12). It was also demonstrated the presence of neuronal nicotinic ACh receptors in MNL with α2, α5 and α7 subunits but not α3, α4, β3 and β4 subunits. In some subjects α6 and β2 were expressed indicating the diversity of muscarinic receptor subtypes and nicotinic receptors subunits expressed by MNL among the different individuals (12).

The presence of different cholinergic components has been described in murine MNLs, dendritic cells (DCs) and macrophages. ChAT was not expressed in resting MNLs, DCs and macrophages; however, upon stimulation ChAT expression was induced in MNLs and DCs but not macrophages. Furthermore, all subtypes of muscarinic receptors (M1-M5) were expressed on the three cell types. In addition, α2, α5, α6, α7, α10 and β2 nicotinic receptors subunits were expressed in the three cell types while α4, α9 and β4 expression varied. Interestingly, α3 and β3 encoding mRNA was not detected in any of the cell types. Moreover, AChE was expressed by the three cell types indicating that ACh is either synthesized by these cells or act on these cells through AChRs (13).

1.4 Cholinergic modulation of the immune cells

Several studies have shown contradictory effects of cholinergic stimulation on different immune cells. In human leukemic cell lines it has been demonstrated that ACh, released from T cells acted (through M1 ACh receptor) in an autocrine/paracrine manner on T
and B lymphocytes elevating intracellular free calcium levels and subsequently increasing c-Fos mRNA expression (14). Furthermore, when human peripheral blood mononuclear cells, monocytes and whole blood were treated with nicotinic agonists and then stimulated with a TLR agonist, pro-inflammatory responses were inhibited but not the production of the anti-inflammatory cytokine IL-10. Surface expression of TLR2 and TLR4 were also down-regulated and these responses were found to be mediated by activation of the Jak2/STAT3 pathway (15).

Studies on IL-4 stimulated pulmonary endothelial cell line revealed that ACh, nicotine and muscarine inhibited the expression of the adhesion molecule E-selectin and vascular endothelial growth factors (16). Similarly, in a mouse in-vivo model, cholinergic agonist and vagus nerve stimulation were found to block leukocytes/endothelial adhesion and leukocytes migration. These effects were found to be partially mediated through n-AChRs (17).

Activation of muscarinic receptors has been shown to have a pro-inflammatory effect on mice as they induced an antibody response and T cell proliferation, they did not prevent leukocytes infiltration and resulted in tissue injury. Moreover, these effects were inhibited by muscarinic antagonists (18). Nicotinic receptors in particular, α7, inhibited the release of TNF-α from macrophages and neutrophil recruitment resulting in an anti-inflammatory effect in mice (19).

Furthermore, the effect of cholinergic stimulation varies in different immune cell types. Nicotine administration reduced the surface expression of CD11b in neutrophils by suppressing F-actin polymerization, the surface expression of β2-integrin and leukocyte
chemotaxis (20). Cholinergic activation in dendritic cells induced an increase in the expression of CD86 and the release of TNF-α and IL-8. When cholinergic activated dendritic cells were stimulated with LPS, a reduction of T cell priming ability, TNF-α and IL-12 production were observed (21). Additionally, nicotinic receptor activation on stimulated CD4+CD62L+ T cells resulted in up-regulation of IFN-γ and down-regulation of IL-17 production. However, muscarinic receptor activation on these cells up-regulated IL-10 and IL-17 production and down-regulated IFN-γ (22). CD8 T cells were also showed to be regulated by ACh as M1 mAChR was found to be engaged in the differentiation of CD8 cells to cytotoxic lymphocytes (23). Moreover, α7 nAChR in B cells was found to be coupled to CD40 and to have an inhibitory effect, on the contrary α4β2 nAChR coupled to IgM, has a stimulatory effect (24).

α7 nAChR are involved in the regulation of IgG1 and inflammatory cytokines like IL-6, TNF-α and IFN-γ production most likely in an inhibitory manner (25). In contrast, activation of M1 and/or M5 mAChRs elevate the production of inflammatory cytokines like IL-6 and IgG1 production by mediating antibody class switching from IgM to IgG1 (26).

It has been shown that some parasitic infection can regulate the inflammatory responses through non-neuronal cholinergic system. *Trypanosoma evansi* parasite infection induced an increase in the production of AChE in rat lymphocytes during the acute phase of infection that is characterized by high number of circulating parasites. High levels of AChE reduce the levels of ACh and promote inflammatory responses. In the chronic phase of the disease where the number of circulating parasites decreased AChE
levels were reduced and therefore promoted ACh mediated anti-inflammatory responses (27).

Table 1: Cholinergic modulation on the immune cells

<table>
<thead>
<tr>
<th>AChR</th>
<th>Subtype</th>
<th>Immune cell affected</th>
<th>Function modulated</th>
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<tbody>
<tr>
<td>nAChR</td>
<td>Monocytes</td>
<td></td>
<td>pro-inflammatory cytokine production (15)</td>
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<td>TLR2 and TLR4 expression (15)</td>
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<td>IL-10 (15)</td>
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<td>Leukocytes</td>
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<td>leukocyte/endothelial cell adhesion (17)</td>
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<td>Leukocyte migration (17)</td>
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<td>Chemotaxis (20)</td>
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<tr>
<td></td>
<td>α-7 Macrophages</td>
<td></td>
<td>Inflammatory cytokine production (19, 28)</td>
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<tr>
<td></td>
<td>Neutrophils</td>
<td></td>
<td>Neutrophil recruitment (19)</td>
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<td></td>
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<td>CD11b expression (20)</td>
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<td>β2 integrin expression (20)</td>
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<td>CD4CD62L T cells</td>
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<td>IFN-γ production (22)</td>
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<td>IL-17 production (22)</td>
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<td></td>
<td>α-7 B cells</td>
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<td>IL-6 and IgG1 production (25)</td>
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<td>mAChR</td>
<td>Leukocytes</td>
<td></td>
<td>Leukocyte infiltration (18)</td>
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<tr>
<td></td>
<td>DCs</td>
<td></td>
<td>TNF-α, IL-8 and IL-12 production (21)</td>
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<td>CD86 expression (21)</td>
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<td>Proliferation (18)</td>
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<td>CD4CD62L T cells</td>
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<td>IL-17 and IL-10 production (22)</td>
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<td>IFN-γ production (22)</td>
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<td></td>
<td>M1 CD8 T cells</td>
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<td>Differentiation to cytotoxic cells (23)</td>
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<td></td>
<td>M1 and/or M5 B cells</td>
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<td>IL-6 and IgG1 production (26)</td>
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<td>Antibody response (18)</td>
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1.5 The inflammatory reflex

The presence of muscarinic and nicotinic receptors on immune cells and the fact that vagus nerve (a major parasympathetic nerve) innervates lymphoid tissue suggested that ACh may act as a neuro-immunomodulator in the interactions between the nervous and immune systems. Tracy and group demonstrated for the first time that the interaction between the immune and nervous systems is vital for modulating immune responses and controlling inflammation (28). Initially it was thought that the cholinergic stimulation of the splenic macrophages, mediated by the α7 AChR, was directly via the vagus nerve and it was named “cholinergic anti-inflammatory pathway” (28,29). Later investigations demonstrated that the vagus nerve does not directly innervate the spleen instead it innervates the celiac ganglion and stimulates the adrenergic splenic nerve which relays the stimulation to the splenic macrophages through splenic T cells (30).

So the link between the nervous system being the master of the body and the immune system has been well established and therefore the immune responses are regulated by the two arms of the autonomic nervous system through what is called the “inflammatory reflex”. In the inflammatory reflex, the presence of endogenous or exogenous molecular products of inflammation in the periphery can stimulate the afferent action potential and relay the signal to the central nervous system particularly the brain (31). Then, an efferent action potential will be sent through the vagus nerve and results in the release of ACh in the celiac ganglion. There ACh will bind to the α7 nAChR on the adrenergic splenic nerve (30,32). Subsequently, splenic nerve will convey the signal by the release of the neurotransmitter noradrenaline in the spleen which will bind to β2-adrenergic receptors (33) expressed on the surface of CD3⁺CD4⁺CD25⁻ T cells. A specific subset of
these T cell population (CD4⁺CD44⁺CD62L⁻) has been found to be responsible for the production of ACh upon adrenergic stimulation (34.35). Then, ACh will bind to α7 nAChR expressed on the macrophages (29) resulting in the inhibition of the production of anti-inflammatory cytokines like IL-1 and TNF-α thus reducing the inflammatory responses (28). A summary of the inflammatory reflex is shown in diagram 2.

Diagram 2: The inflammatory reflex

The prototypical reflex circuit regulating immunity is comprised of afferent and efferent signals transmitted in the vagus nerve in response to the molecular products of infection and injury, including cytokines, eicosanoids, DAMPs, and PAMPs. The activation of adrenergic neurons in the spleen culminates in the release of norepinephrine in the vicinity of T cells that are capable of secreting acetylcholine. Acetylcholine crosses the marginal zone and enters the red pulp, where it interacts with α7 nAChR expressed on cytokine producing macrophages. α7 nAChR signal transduction suppresses the synthesis and release of TNF, IL-1, IL-18, HMGB1, and other cytokines.

Obtained from: Ulf Andersson and Kevin J. Tracey. Neural reflexes in inflammation and immunity, June 2012. JEM vol. 209 no.6 1057-1068. (36)
Activation of the inflammatory reflex by vagus nerve stimulation, selective α7 nAChR agonist and other ACh mimicking agents has been studied in murine endotoxemia and sepsis models. Cholinergic stimulation improved the survival of mice through reducing TNF-α circulating levels thus protecting the vital organs from TNF-induced tissue injury during sepsis and preventing multiple organs failure (28,36).

The splanchnic artery occlusion (SAO) shock model is characterized by systemic inflammatory responses due to an increase in the production of TNF-α, an accumulation of neutrophils in the different tissues, especially in lungs and liver, and eventually organ failure. Vagus nerve stimulation in a SAO animal model resulted in a reduction in the NF-κB activation and reduces mRNA expression of TNF-α with subsequent reduction of TNF-α level in the circulation. Moreover, vagus nerve stimulation caused a reduction in leukocytes accumulation in the ileum and lungs which could be a result of the amelioration of inflammatory cascade through reducing circulation TNF-α. These modulations through vagus nerve stimulation improved the mice survival time and rate which lead to a protective effect (37).

In animal models of inflammatory diseases like acute kidney injury, obesity and collagen-induced arthritis has been shown that cholinergic stimulation lead to a reduction in the inflammatory responses and had a protective role (38–40).

The effect of the cholinergic stimulation in a live infectious model seems to be controversial. In a peritonitis model with live bacteria (E.coli) cholinergic stimulation reduced the pro-inflammatory cytokines and cell recruitment in mice, however it worsen the clearance of bacteria and survival rate of the animals in comparison to control mice.
These effects were reversed in α7 nAChR deficient mice which have faster neutrophils recruitment (41). Similarly, when the anti-inflammatory reflex pathway was inhibited by eliminating the noradrenergic neurons in an murine model of peritonitis induced by *Klebsiella pneumonia*, the animal survival was improved (42). On the contrary, previous administration of AChE inhibitor improved the survival of mice after a lethal infection with *S. typhimurium* (43); moreover, M3 m-AChR activation improved Th1 adaptive immune responses in *Salmonella* infection by inducing IFN-γ production and Th2 adaptive immune responses in *Nippostrongylus brasiliensis* infection by inducing IL-13 production, resulting in a protective effect (44).

### 1.6 Immuno-physiology of the gastrointestinal tract

The main role of the gastrointestinal tract (GIT) is digesting and absorbing different nutrients that are needed for survival. GIT also confers the first line of defense against orally ingested microorganisms and food antigens. The defensive factors vary from saliva, gastric acidity, mucus intestinal flora, peristaltic movement epithelial cell membrane to intracellular junctional complexes that prevent the foreign matters from crossing the epithelium and eliminate them (45).

The intestine has different innate immune mechanisms (mechanical, chemical and cellular) to protect the gut mucosa from microbial infections: the first is a mechanical mechanism that includes the epithelium, peristalsis and mucus coating; the second is a chemical mechanism given by specific anti-microbial peptides, proteins, PAMs (pathogen recognition molecules) and cytokines secreted by the intestinal cells; and finally, there is a third mechanism, the cellular mechanism, which includes the epithelial...
cells, macrophages, dendritic cells, NK cells and γδ T cells. These three components act at different levels: extra-epithelial, epithelial and subepithelial levels (46). The components that act at the extra-epithelial level include anti-microbial proteins and peptides secreted by a group of cells located at the base of the crypts of Lieberkühn, in the distal part of the small intestine, called Paneth cells. The Paneth cells are rich in secretory granules containing microbicidal peptides and proteins, like lysozyme, phospholipase A. cryptdins, cryptdin-related sequence peptides (CRS), RegIIIγ and angiogenin 4, that contribute to the intestinal innate immunity (47,48). It has been demonstrated that the interaction between the pathological microorganisms and the Paneth cells results in an increased concentration of the anti-microbial peptides in the intestinal lumen (49). Secretory phospholipase A2 is released upon stimulation by bacterial products and it has been shown to have bactericidal activity against Salmonella typhimurium and Listeria monocytogenes (50,51). It has been also demonstrated that angiogenins are present in the secretory granules of the Paneth cells and are released upon stimulation by bacterial products (52).

Among all the antibacterial products secreted by the Paneth cells, cryptdins (alpha-defensins in humans) are the most abundant accounting for 70% of the released peptides with anti-bacterial activity (49). Cryptdins are small cationic cysteine-rich anti-microbial peptides belonging to the alpha-defensin family and specific to Paneth cells. They are synthesized as pre-peptides and, then, processed to active peptides by matrix metalloproteinase matrilysin (MMP-7). Their bactericidal activity of the cryptdins is due to their chemical structure characterized by tridisulfide array and amphipathicity (53–55) which induce the formation of membrane spanning pores in the bacterium leading to
its lysis (46,47). Several studies have shown the essential role that cryptdins play in the mucosal immune defense to *Salmonella* infection. Transgenic mice that overexpressed cryptdins in the Paneth cells are more resistant *Salmonella enterica serovar typhimurium* infection (56) whereas mice with deficiency in cryptdins processing are more sensitive to oral infection by *Salmonella* (57).

Moreover, goblet cells located in the crypt are known to synthesize and release mucin to form the thick mucus layer in response to mechanical insult, chemical irritation and bacterial toxins. Goblet cells morphologically composed of theca containing mucus granules that lay beneath the apical membrane (58). Mucus layer that covers the intestinal lumen is made of a mesh-like viscous, permeable and gel forming glycoprotein (mucin) (59). This mucus layer not only contains mucin but also anti-microbial peptides secreted by Paneth cells and secretory IgA (60). Mucus layer acts as a barrier to protect the host from invading oral pathogens and also it act as a lubricant (59,60).

Gut-associated lymphoid tissue (GALT) (Diagram 3) is composed of different immunological structures which are important in the host intestinal defense. Those structures are organized lymphoid tissues such as mesenteric lymph nodes and Peyer’s patches and diffusely scattered lymphocytes in the intestinal lamina propria and intraepithelium (61).

Peyer’s patches (PPs) are organized lymphoid tissues that vary in number: 8-10 in mice and hundreds in humans (62). In general, PPs are composed of three regions: the follicle-associated epithelium (FAE), subepithelial dome (SED) and follicular region.
PPs have efferent but not afferent lymphatic vessels thus to compensate, the FAE of PPs which is a specialized epithelial region contains specialized antigen-sampling microfold cells (M cells). M cells are enterocytes that exhibit at their apical surface a poorly organized brush border with short microvilli and thin mucus layer. Their cytoplasm contains small endocytic vesicles with transcytosis capability which allows these cells to sample the contents in the lumen (particulate matters and pathogens) and transport them across the epithelium to the basolateral membrane (49,50,51).

SED region has abundancy of dendritic cells (DCs) which can engulf the antigens transported by the M cells, process them and present them to the mucosal T and B cells in order to generate antigen-specific response. DCs constitute around 10% of the total population of the PPs. B cells located at the follicular region are the most abundant cells in the PPs, constituting around 60% of the total population. Additionally, T cells comprise around 25% of the PPs in which 45% of the T cells are CD4+, 35% are CD8+ and 20% are CD4-/CD8- T cells. T cells are mostly located at the interfollicular region (IFR) (66).

Mesenteric lymph nodes (MLN) are present at the intestinal mesentery. Morphologically MLN have three compartments: the cortex which has follicles that composed B cells and dendritic cells, paracortex that contains high percentage of T cells and dendritic cells and lastly the medulla that contains B cells and T cells (67). Dendritic cells carrying the antigen, migrate from the intestinal epithelium to the MLNs where they present the antigen particles to the naïve T cells in the paracortex which results in activation of the adaptive immune responses. Afterwards, the effector T cells will leave the MLNs and
migrate to other organs (67,68). B cells in MLN contribute to the release of secretory IgA in the gut lumen (69).

Finally, the intestinal lamina propria (LP), located between the epithelium and muscularis mucosa, and it contains a variety of lymphoid cells like IgA producing plasma cells, T cells, dendritic cells, macrophages and mast cells. In the intraepithelial space between the tight junctions and the basal membrane of the gut epithelium, intraepithelial lymphocytes are located. These lymphocytes are T cells both, CD8+ and CD4+ cells that do not need antigen presentation; instead they can release cytokines upon direct interaction with the antigen (70).

Diagram 3: Schematic representation of the lymphoid elements of the intestinal immune system

The organized tissues of the Peyer's patches and mesenteric lymph nodes (MLNs) are involved in the induction of immunity and tolerance, whereas the effector sites are scattered throughout the lamina propria and epithelium of the mucosa. Both the Peyer's patches and villus lamina propria are drained by afferent lymphatics that go to the MLNs. SED, subepithelial dome; TDA, thymus-dependent area.

1.7 Effect of the cholinergic system on the intestinal cells

Sympathetic and parasympathetic autonomic nervous systems are well known to innervate the gastrointestinal tract (GIT) and regulate GI motility, secretions and material absorption. Furthermore, the existence of non-neuronal cholinergic system has been established in the GI epithelial cells and the intestinal resident immune cells. Anatomical studies revealed that cholinergic enteric neurons innervate and are located in close proximity to macrophages, plasma cells and lymphocytes present in the lamina propria in addition to the lymphocytes in the Peyer’s patches. Epithelial cells in the gallbladder and lower intestine along with M cells and T cells in the Peyer’s patches were shown to express non-neuronal ChAT thus they were able to synthesize ACh (71). Furthermore, cholinergic as well as adrenergic neurons were found to be present in a close proximity to secretory epithelial cells in the intestinal crypt and IgA producing cells in the intestine (72). The postoperative ileus is an animal model characterized by a localized bowel inflammation that results in dysfunctional bowel movement, accumulation of gas, abdominal distention and other gastric symptoms. In this model it was shown that intestinal inflammation activated the afferent vagus nerve which in turn activated the efferent arm of vagus nerve, mainly the neurons innervating the inflamed area in the intestine (73) which indicates that the cholinergic nervous system could modulate the immune responses in the intestinal area.

The inflammatory response was found to be initially induced by the resident intestinal muscularis macrophages (74). Moreover, it was also shown that specific α7 nAChR agonist as well as vagus nerve stimulation had an anti-inflammatory effect that was mediated through the reduction in the transcriptional activity of NF-κB and the
induction of STAT3 phosphorylation (activate Jak2/STAT3 pathway) in the macrophages thus inhibiting the inflammatory responses (75). This anti-inflammatory effect of the vagus nerve stimulation was mediated by the direct interaction of the vagus nerve with the gut resident intestinal muscularis macrophages and independent of the spleen (76). Additionally, vagus nerve stimulation was shown to enhanced luminal uptake as well as the phagocytic ability of the lamina propria resident macrophages despite of inhibiting the activation of NF-κB and reducing the production of pro-inflammatory cytokines. These effects were due to the binding of ACh to the α4β2 nAChRs on macrophages (77).

Several evidence showed that intestinal epithelium synthesizes ACh that regulates growth and differentiation of epithelial stem cells via muscarinic ACh receptors (78). Moreover, it was demonstrated that ACh stimulation induced degranulation of mucin granules causing a massive release of mucin to the intestinal lumen (79).

Additionally, Paneth cells were also able to respond to ACh through their muscarinic AChRs. ACh stimulation induced massive exocytosis of the AMPs containing granules into the lumen (80,81). A study on the ultra-structural analysis of the Paneth cells from rats subjected to bilateral subdiaphragmal trunkal vagotomy, revealed a profound ultrastructural re-arrangement in the cytoplasm and an increase in the total amount of secretory granules (82). Moreover, the secretion of Paneth cells was blocked when mice were subjected to atropine treatment (81).

In humans, it has been shown the importance of Paneth cells and the secretion of defensins on maintaining healthy intestinal mucosa. A reduction in the antibacterial
activity in the intestinal mucosal extracts and a decrease in the expression of alpha-
defensins by Paneth cells has been reported in patients with Crohn’s disease (83) resulting in luminal bacterial overgrowth and increase in the number of Paneth cells, not only in the small intestine but also in the large intestine (metaplasia). These observations confer to Paneth cells a role in the pathogenesis of intestinal inflammation.

In response to the presence of an antigen in the gastric mucosa, plasma B cells present in the intestinal epithelium (Peyer’s patches, isolated lymphoid follicles and lamina propria) and MLN were able to produce secretory IgA that act as one of the defensive mechanisms in the gut (69,84,85). As mentioned earlier, cholinergic and adrenergic neurons are present in close proximity to IgA producing plasma cells which could indicate the ability of these neurons to regulate the secretory IgA production. It has been demonstrated that ACh and adrenaline, through muscarinic ACh receptors and adrenergic β2 receptors, respectively, expressed on the surface of the epithelial cells in the crypt and intestine, stimulated the release of secretory IgA to the lumen suggesting a protective role of these neurotransmitters in the intestine (72).

1.8 Acetyl cholinesterase inhibitors

Acetyl cholinesterase is an enzyme present in the synaptic cleft that hydrolyzes the ACh in order to terminate its action (86). The administration of AChE inhibitors increases the concentration of ACh in the synaptic cleft and therefore, induces the hyper-stimulation of target cell. Acetyl cholinesterase inhibitors are administered to patients with myasthenia gravis and Alzheimer’s disease to reduce the deteriorating symptoms (87). In animal models the effect of AChE inhibitors on the immune system is not clear and it
seems to be contradictory. Most probably, this variability is due to the use of different experimental models, different AChE inhibitors with different dosages and exposure time. In a murine model of experimental autoimmune encephalomyelitis (EAE), rivastigmine an AChE inhibitor was found to reduce neurons axons demyelination and inflammatory infiltration of T cells and microglial cells which subsequently ameliorated the clinical symptoms and improved the spatial memory in mice. Moreover, rivastigmine has been shown to reduce the production of pro-inflammatory cytokines IL-17, IFN-\(\gamma\) and TNF-\(\alpha\) which was mediated by the activation of \(\alpha7\) nAChRs (88). Likewise, galantamine, another AChE inhibitor, and through \(\alpha7\) nAChRs reduced TNF-\(\alpha\) production and improved mice survival in a murine endotoxemia model (89). In addition, AChE inhibitors like tacrine, rivastigmine and neostigmine reduced the LPS induced production of IL-1\(\beta\) in the brain and blood (90). On the other hand, in a murine model it was demonstrated that the administration of AChE inhibitor pyridostigmine bromide did not inhibit the production of IL-2 or IFN-\(\gamma\) by T cells (91). Likewise, in mice, the sub-chronic administration of organophosphorus compound dimethoate (another AChE inhibitor) resulted in a pro-inflammatory effect in the brain and it enhancement the neuro-inflammatory response upon LPS administration (92).
1.9 Experimental model

1.9.1 Paraoxon as an AChE inhibitor

In our experimental model we use paraoxon as AChE inhibitors. Paraoxon (O, O-diethyl O-p-nitrophenyl phosphate) is the bioactive metabolite of the organophosphate pesticide parathion (O, O-diethyl O-p-nitrophenyl phosphorothioate). De-sulphuration of parathion by liver enzymes, or sunlight, results in the formation of paraoxon, which inhibits AChE activity. Paraoxon mechanism of action is through a non-reversible binding to the active site of the AChE which makes the enzyme unable to hydrolyze the endogenous substrate (ACh) and therefore inhibits its activity. The effect of paraoxon on the immune system is not well defined and still controversial. In-vitro studies showed that paraoxon induced apoptosis in EL4 cell line (93,94). In rats, administration of paraoxon was found to induce the production of free radicals in different organs such as brain, liver and spleen (95). In other studies paraoxon has shown to be immune-suppressant and acts as a protective agent that regulates immune responses (96,97). Our group demonstrated in a murine model, that chronic exposure to paraoxon did not affect the normal immune responses and had no influence over the survival of mice following a lethal bacterial infection model (98). Later studies showed that sub-chronic exposure surprisingly improved the mice survival significantly in the same lethal infection model (43).
### 1.9.2 *Salmonella* infection model

*Salmonella enterica* is a facultative intracellular gram negative bacterium that causes an enteric disease in humans as well as in mice. It is transmitted through oral-fecal route via contaminated food and water. Different serovars cause different diseases, such as human restricted *S. enterica* serovar Typhi causes Typhoid fever however; *S. enterica* serovar Typhimurium (*S. typhimurium*) causes a self-limited gastroenteritis in human while in mice it causes a systemic disease resembles the manifestations of Typhoid fever in human (99). After oral ingestion, *Salmonella* adheres to the epithelial cells in the intestinal wall and uses different mechanisms to invade (99). The major invasion mechanism of *Salmonella* is through the intestinal microfold cell (M cells). *Salmonella* induces the ruffling of the M cells membrane which helps the bacteria to get internalized causing the death of the M cells. Afterwards, bacteria will travel either laterally along the basal lamina or deeper into the dome of the follicle (100). *Salmonella* can be also internalized through non-phagocytic enterocytes using virulence-associated type 3 secretion system encoded by *Salmonella* Pathogenicity Island 1 (SPI-1) (99). Specific subtype of DCs/macrophages that express the chemokine CX3CR1 are involved in the uptake of *Salmonella* from the lumen to the lamina propria. Some studies suggested that CX3CR1 expressing phagocytes were responsible for the luminal sampling and destruction of the invading bacteria rather than transportation as the CX3CR1-deficient mice had higher bacterial load in the organs and higher susceptibility compared to the wild type (101,102). The invasion mechanisms of *Salmonella* are shown in diagram 4.

Initially during *Salmonella* infection, innate immune mechanisms in the intestine such as the presence of mucus released from goblet cells in the crypt, along with the release of
anti-microbial peptides (AMPs) from Paneth cells residing at the bottom of the crypt, will be the first barrier encountered by *Salmonella*. Mucus layer composed of mucins (a family of glycoproteins) covers the surface of the epithelium resulting in a barrier that needs to be penetrated before bacteria gain access to the epithelial cells and penetrate them (99). The bactericidal action of the anti-microbial peptides resides in their capacity to disrupt the integrity of the bacterial cell membrane. The expression of some of these peptides like RegIIIβ/γ was increased in streptomycin pre-treated mice infected with *Salmonella* (103). Moreover, IL-23 produced by the epithelial cells was able to induce the production of IL-22 that subsequently lead to the induction of angiogenin 4 (Ang4) (104). Despite the fact that AMPs are lethal to *Salmonella*, this bacterium was found to reduce the expression of cryptdins (defencin in humans) and lysozyme in Paneth cells along with their granules content, however an increase in the number of Paneth cells, mitotic events in the crypt and apoptotic events in the villi was observed simultaneously (46,105).

*Salmonella* that passed the first line of defense at the intestinal epithelium will encounter macrophages and dendritic cells which are the first responders in the intraepithelial tissue and part of the innate immune system. Cell wall components of the *Salmonella* which are called PAMPs (pathogen associated molecular patterns) will be recognized by the surrounding cells through PRRs (pathogen recognition receptors) present on their surface examples of PAMPs and TLRs recognize it are: lipopolysaccharide (LPS) by TLR4, bacterial lipoproteins by TLR1/2/6, flagellin by TLR5 and DNA by TLR9 (99). Once the cells recognize the pathogen they will produce inflammatory cytokines (IL-1,
TNF-α, IL-12, IL-18 and IL-6) and chemokines to recruit immune cells to the site of injury.

During *Salmonella* infection, macrophages will produce IL-12 which is one of the major inducers of IFN-γ production by T cells and natural killer (NK) cells, as well as an essential cytokine for the differentiation of T helper cells to Th1 cells. Additionally, macrophages are also able to produce IL-18 which was showed to be involved in the induction of IFN-γ production (as IL-12) but it is unable to induce T cells to develop to Th1. IL-18 and IL-12 have been reported to have synergistic effect, although IL-12 was more effective during *Salmonella* infection. The IL-12, IL-18 and IFN-γ axis was found to be crucial to limit bacterial burden in the systemic organs and improve overall host survival (106-108). Together, TNF-α produced by macrophages and IFN-γ produced by T cells and NK cells are essential in the host defense against *Salmonella* due to their ability to activate the bactericidal activity in macrophages which leads to the reduction of live bacterial load in the different organs (110).

A critical factor that is involved in the ability of macrophages to control the replication of intracellular pathogen is the presence of functional *Nramp1* (natural resistance-associated macrophage protein) molecule which is a transmembrane protein expressed on macrophages that acts as iron transporter (111). The absence of functional *Nramp1* in mice lead to a reduction in the bactericidal activity of macrophages and subsequently an increased susceptibility to invading *Salmonella* such as BALB/c strain (112) that we use in our study.
IL-10 is an anti-inflammatory cytokine that plays a central role in the *Salmonella* as it limits the immune response to pathogen by inhibiting the production of radical oxygen and nitrogen species as well as TNF-α and IL-12 cytokines. The levels of expression of IL-10 mRNA during *Salmonella* infection varies depending on the genotype of the host, but in both resistant and susceptible mice the levels of IL-10 correlates with the bacterial load in the organs; the more bacterial burden the higher the levels of IL-10. However IL-10 does not play a key role in the susceptibility to *Salmonella* infection (113).

Cytotoxic CD8 T cells that are MHC Ia restricted were found to have a protective role during *Salmonella* infection as they limited the bacterial growth by producing perforin a pore forming molecule and granzyme A and B which has a proteolytic action and kill the infected cells (114).
Diagram 4: Schematic representation showing the different routes Salmonella can take in order to invade the intestinal mucosa

(1) The major pathway of invasion is through M cell mediated transcytosis at the Peyer's patches. (2) An alternative route is via uptake by enterocytes. (3) This route requires the injection of bacterial effector proteins by the SPI-1 Type 3 Secretion System that induce ruffling and uptake of the bacterium. Uptake through intercalating CX3CR1+ macrophages/DCs might represent an additional route of invasion.

Chapter 2: Hypothesis

Following infection with virulent *Salmonella*, inflammatory cytokines are secreted as a response to the infection. An excess in the production of inflammatory cytokines (cytokine storm) leads to septic shock and death of the host.

We hypothesize that by increasing the ACh levels, the inflammatory reflex is stimulated to control the cytokine storm produced after lethal infection, and, therefore, protect the host from septic shock. Other unknown mechanisms mediated by ACh could also be involved in suppressing the progression of the infection and protection of the host.

**Diagram 5: Project hypothesis**

Schematic diagram shows the hypothesized mechanism of protection by AChE inhibition which could be through the activation of the inflammatory reflex or through other unknown mechanism by limiting the infection progression.
Chapter 3: Aim and Objectives of the Study

We have previously demonstrated that inhibition of AChE enzyme by paraoxon increases host resistance to an oral infection with the Gram-negative bacterial pathogen *Salmonella enterica* serovar Typhimurium. The overall aim of the current study was to investigate the cellular and molecular mechanism(s) by which the cholinergic nervous system influences the immune response to infection.

The specific objectives were to:

- Utilize multi-colour flow cytometry to evaluate the effect of AChE inhibition on the cellular phenotype and activation status of lymphoid and myeloid cell populations at mucosal immune system sites, represented by mesenteric lymph nodes, as well as systemic sites, including spleen and peritoneal exudate cells.

- Use quantitative PCR (qPCR) to investigate alterations in gene expression profiles in splenic immune cells following inhibition of AChE.

- Characterize the immune response in paraoxon-pretreated mice, or their control counterparts, following oral or systemic infection with either attenuated or virulent strains of *Salmonella typhimurium*.

- Investigate the kinetics of bacterial dissemination following oral infection using *in vivo* bioluminescent imaging technology.

- Investigate the mucosal innate immune responsiveness of paraoxon-pretreated or control mice, focusing on the extent of production of several types of antimicrobial peptides and other effector molecules.
Investigate possible alterations in intestinal mucosal epithelial tissue and associated cell types (Paneth cells and goblet cells) as a consequence of paraoxon-administration and infection with *Salmonella*. 
Chapter 4 : Materials and Methods

4.1 Materials

4.1.1 Experimental animals

BALB/c mice were purchased from Harlan Olac (Bicester, UK) and bred in the animal facility at the College of Medicine and Health Science, United Arab Emirates University (UAEU). Male mice aged 8-12 weeks and weighed 22-26 grams were used for this study. Animals were housed in plastic cages with a controlled light and dark cycle of 12 h each at 24-26°C and received rodent chow and water ad libitum. 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).

4.1.2 Solutions

0.1 M phosphate buffer

solution 1: 17.8g of Na₂HPO₄.2H₂O in 1L distilled water

solution 2: 2.27g of KH₂PO₄ 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 100ml phosphate buffer. Stored at -20°C

6 mM ethoprazine

20.94 mg in 10ml of 12 mmol/L HCl. Stored at -20°C
**Transformation buffer**

200mg potassium ferricyanide, 50 mg potassium cyanide, 1g sodium bicarbonate and 500 µl 100% Triton X-100 in 1L distilled water

**RBC’s lysis buffer**

8.3g ammonium chloride, 1g potassium bicarbonate and 1.8ml of 5%EDTA in 1L of distilled water and filtered through 0.2µm filter

**Supplemented RPMI-1640**

5% heat inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 mM HEPE§, 0.075% sodium bicarbonate, 1x essential amino acids, 0.5x non-essential amino acids, 50 µg/ml gentamicin, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5x10-5M 2-mercaptoethanol (2-ME).

**Griess reagent**

2.35 ml of phosphoric acid (86%), 1 g Sulfanilamide and 0.1 g Naphthylethylene diamine hydrochloride in 100 ml of distilled water.

**10x PBS**

87.66g NaCl, 2.56g NaH₂PO₄·H₂O and 11.94g Na₂HPO₄ dissolved in 1L of distilled water
ELISA reagents

0.1M sodium carbonate coating buffer
7.13g of sodium bicarbonate and 1.59g of sodium carbonate in 1L distilled water, pH9.5

0.2M sodium phosphate coating buffer
12.49g of disodium hydrogen phosphate and 15.47 sodium dihydrogen phosphate in 1L of distilled water, pH6.5.

Washing buffer
1xPBS (80g sodium chloride, 11.6g Disodium hydrogen phosphate, 2g potassium chloride and 2g potassium dihydrogen phosphate in 10L distilled water) and 0.05% Tween-20, pH7.

Assay diluent
10% FBS in 1x PBS pH 7.

AutoMACS buffer
0.5% BSA and 2mM EDTA in 1x PBS pH7.2.

Staining buffer for flow cytometric analysis
1% FBS and sodium azide in 1x PBS

Extraction buffer
1% bovine serum albumin, protease inhibitors cocktail III (1:100 dilution) and 30mM EDTA.
**IgA ELISA**

*Washing buffer*

1x PBS and 0.05% Tween-20, pH 7.2-7.4.

*Blocking buffer for total IgA*

2% BSA in 1x PBS pH 7.2-7.4

*Blocking buffer for anti-Salmonella IgA*

5g sucrose, 0.05g sodium azide and 1g BSA in 100ml 1x PBS pH 7.2-7.4

*Reagent diluent for anti-Salmonella and total IgA*

1% BSA in 1x PBS. pH 7.2-7.4

**HBSS**

0.4g KCl, 0.09g Na₂HPO₄, 7H₂O, 0.06g KH₂PO₄, 0.35g NaHCO₃, 0.14g CaCl₂, 0.1g MgCl₂, 6H₂O, 0.1 MgSO₄, 7H₂O, 8g NaCl and 1g D-glucose in 1L distilled water, adjust pH to 7.4 and stored at 4°C.
### 4.1.3 Summary of antibodies used for flow cytometry

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<th>Dilution</th>
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### Continue Summary of antibodies used for flow cytometry

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<th>Antibody (all anti-mouse)</th>
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### 4.1.6 List of tissue culture reagents

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<th>Tissue culture reagents</th>
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<td>Hyclone</td>
<td>SH.30027.01</td>
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### 4.1.7 List of RNA-PCR reagents

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4.2 Methods

4.2.1 Paraoxon preparation

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

4.2.2 Experimental protocol

Male BALB/c male mice of matched age and weight were randomly assigned into two groups:

1. Control animals were treated daily with i.p. injection of pyrogen-free saline for 3 weeks
2. Experimental group received daily injection of 40 nmols of paraoxon for 3 weeks

Mice were weighed weekly and blood was also collected and analyzed for acetyl cholinesterase (AChE) activity. At the end of the third week of treatments, animals were either sacrificed for the collection of different internal organs, or infected with one of the two strain of *S. Typhimurium* (BRD509 (115) or SL1344 (43)) and sacrificed at
indicated time points. Serum from every sacrificed mouse was obtained and stored frozen at -20ºC until further analysis.

4.2.3 Red blood cells AChE activity

Venous blood was collected from the tail (20µl), diluted in 2ml of 0.1 M phosphate buffer and stored at -20ºC (for not more than 2 months) until analyzed. After thawing, 1ml of the sample was incubated with 2ml of phosphate buffer, 100µl of 10 mM DTNB (5,5′-dithiobis-2-nitrobenzoic acid) and 10µl of 6 mM ethoprazine for 20 minutes in a water bath set at 37ºC. Then, 50µl 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, 1ml of the blood sample was incubated with 1ml of transformation buffer for 10 minutes at room temperature. Then absorption at 546nm 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%).
4.2.4 Spleen and mesenteric lymph nodes (MLN) cell preparation

After the aseptic removal of spleen and MLN, organs were gently teased in 1x PBS using the frosted ends of microscopic slides in order to get single cell suspensions. Cells were spun at 1200 rpm for 5 minutes at 4°C and re-suspended in cold 1x PBS. In order to lyse the RBCs, spleen cells were incubated with cold RBCs lysis buffer (10 ml/spleen) for 3 minutes. Then, cells were spun at 1,200 rpm for 5 minutes at 4°C and washed with 5 ml cold 1x PBS, spun and re-suspended in 1x PBS. Cells were, then, counted on a hemocytometer and cell viability was determined by trypan blue dye exclusion method.

4.2.5 Isolation of peritoneal cells

Peritoneal exudate cells (PECs) were retrieved by peritoneal lavage. Briefly, peritoneal cavity was washed with 10 ml of cold 1x PBS (Mg²⁺ and Ca²⁺ free) and centrifuged at 1200 rpm for 5 minutes at 4°C. Cells were re-suspended in 1ml cold 1x PBS and counted using hemocytometer. Cell viability was determined by trypan blue dye exclusion method. If required, 300μl were kept for bacterial load determination.

4.2.6 Cellular ex-vivo culture

Erythrocyte-depleted splenocytes and PECs single cell suspensions were prepared in supplemented RPMI-1640. Splenocytes (5x10⁶ cells/ml or 3x10⁶ cells/ml) were cultured, in 24-well plates, in the absence or presence of ConA (2.5-5μg/ml) or LPS (10 μg/ml). PECs (1.5x10⁶ cell/0.5ml) were cultured in the absence or presence of LPS at 10μg/ml concentration, in 24-well plates. Then, cells were incubated for 24 - 48 hours at 37°C in 5% CO₂. Culture supernatants were collected spun free of any cells and kept at -20°C until assayed for cytokines and nitric oxide content.
4.2.7 Nitric oxide determination

Production of nitric oxide (NO) was measured by detecting levels of nitrite (NO$_2^-$), a stable metabolite of the reaction of NO with oxygen, according to the Griess method. The nitrite concentration was determined from a standard curve prepared using sodium nitrite (5-100 µM), 5 µM being the minimum limit of detection. Standard curve was prepared from 100mM sodium nitrite stock solution by 1-fold serial dilution. In 96-well plates, 50µl of the standard or samples were added to the wells, in duplicates. Subsequently, 50µl of Griess reagent were added to each well. Color was developed within 5 minutes and absorbance measured at λ = 492 nm using a Sunrise TECAN microplate reader (Maennedorf, Switzerland).

4.2.8 Cytokine analysis

Supernatants of ex-vivo-cultured cells and serum samples were analyzed for cytokine content using BD OptEIA ELISA mouse kit (BD, Franklin Lakes, NJ, USA) and Duoset ELISA development kits (R&D, Minneapolis, MN, USA). All cytokine determinations were carried out in non-adherent 96-well plates (Nunc, Roskilde, Denmark). Plates were coated overnight with 100µl/well of capture antibody diluted in 0.1M sodium carbonate coating buffer or 0.2M sodium phosphate coating buffer at 4°C. After overnight incubation, plates were washed with washing buffer and then blocked with assay diluent (250µl/well) for 1 hour at room temperature. Afterwards, plates were washed, standards were diluted in assay diluent to appropriate dilutions to make the standard curve, and samples were added in the appropriate dilutions. Standards and samples were added in a total volume of 100µl/well and incubated for 2 hours at room temperature. After washing, plates were incubated with 100µl of the detection antibody
and streptavidin-horseradish peroxidase conjugate (SAv-HRP reagent) for 1 hour in the dark at room temperature. Plates were then washed and 100μl of Tetramethylbenzidine (TMB) substrate solution was added. After 10-30 minutes, the reaction was stopped by 2N sulphuric acid and absorbance was measured at λ=450nm by TECAN microplate reader (Maennedorf, Switzerland). Magellan3 software (TECAN) was used to calculate the results. The sensitivity of detection was 30pg/ml for IFN-γ and IL-10, 15pg/ml for IL-6 and IL-12/IL-23p40.

4.2.9 Bacterial strains and preparation

Two strains of Salmonella enterica serovar Typhimurium (S. typhimurium) were used in this study: the wild-type virulent strain SL1344 and the attenuated double auxotrophic strain aroA/aroD mutant designated BRD509E (115). Due to deletions in the aroA and aroD genes, BRD509E strain lacks the ability to produce essential prokaryotic aromatic compounds which makes its growth weak and slow. Additionally, SL1344::lux, a bioluminescent bacterium tagged by expression of the lux operon from Photorhabdus luminescens was generously provided by Prof. Christopher H. Contag, (Stanford University School of Medicine, Stanford, California).

To grow the bacteria, thawing aliquots of frozen bacteria stock were plated on Salmonella Shigella (SS) agar plates with (BRD509E) or without (SL1344) ampicillin (100μg/ml). For bacterial dosage preparation, three to five colony-forming units (CFU) were cultured in 10 ml of triptase soy broth (TSB) with (BRD509E) or without (SL1344) ampicillin (1μg/ml) overnight and then diluted 1:5 in fresh TSB with (BRD509E) or without (SL1344) ampicillin (1μg/ml) and grown for two hours shaking at 200rpm and 37°C. To determine bacterial concentration, 1 ml of the culture was read
on a spectrophotometer at $\lambda = 600$ against TSB as blank. Appropriate dilutions of the log-phase bacterial suspension were made in pyrogen-free 1x PBS to prepare the indicated doses and their verification. Bacterial dose was confirmed by CFUs plate counts. For systemic infection, 0.5ml/mouse of BRD509E at the indicated dose was injected intraperitoneally (i.p.). For oral inoculation, 200µl of 7.5% sodium bicarbonates were given 5 minutes prior to the 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.

4.2.10 Determination of bacterial load in organ homogenates and fecal pellets

Fecal pellets were freshly collected from infected animals at different time points, weighed and homogenized in 0.5 – 1 ml cold 1x PBS. Then, 50-100 µl from the homogenate or appropriate dilution were plated on SS agar plates with Streptomycin (200 µl/ml) for the pellet collected from SL1344 infected animals, or Streptomycin (200 µl/ml) and ampicillin (100 µl/ml) for the pellet collected from BRD509E infected mice. CFUs counts were determined after overnight incubation at 37°C.

Mice were sacrificed at different time points after i.p. or oral infection. Spleen, liver and mesenteric lymph node (MLN) were aseptically removed at indicated time points and homogenized in 1-2 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 (BRD509E) or without (SL1344) ampicillin plates where CFUs counts were determined after overnight incubation at 37°C.
To determine the bacterial loads in the freshly collected peritoneal fluid, 100 μl were plated following the same above described protocol.

4.2.11 In vivo Bioluminescence Imaging (BLI)

Mice treated with paraoxon or saline for three weeks were infected by oral gavage with SL1344::lux (1 x 10⁵ CFUs/200μl/mouse). Five minutes previous to the infection, 200 μl of 7.5% sodium bicarbonate were administered to the animals by oral gavage. Bacterial dissemination and, therefore, progression of the disease was followed by in vivo bioluminescence imaging (BLI) using in-vivo imaging system (IVIS) Lumina II (Caliper Life Sciences, Hopkinton, Massachusetts, USA).

Mice were initially placed in a chamber where they inhaled a mixture of the anesthetic isoflurane (Baxter, Deerfield, IL, USA) and oxygen. Afterwards, mice were moved to the platform in the imaging chamber where their noses were put at the anesthetic-gas ports to maintain anesthesia. At different time points, mice were imaged for 8 minutes to detect the light emitted by the bioluminescence bacteria which is transmitted through the mouse tissues. Data acquisition and analysis were performed using living image software (Caliper Life Sciences).

4.2.12 Splenic macrophages purification

Splenocytes were magnetically labeled to mouse microbeads conjugated with monoclonal anti-mouse CD11b antibodies (MACS Milteni Biotec). Cells were labeled using 10μl of the beads per 1 x 10⁷ cells and incubated at 4°C for 15 minutes. Then, cells were washed with 1-2 ml/10⁷ cells of AutoMACS buffer and spun for 10 minutes at 4 °C at 1500rpm. Afterwards, cells were re-suspended in 500μl of AutoMACS buffer and
positively selected by magnetic separation using the autoMACS separator (MACS Miltenyl Biotec, Bergisch Gladbach, Germany). Flow cytometric analysis of purified cells showed an approximate of 80-90% purity.

4.2.13 Flow cytometry

Cells, at a concentration of 0.5x10^6 cells/well, were incubated in U shaped bottom 96-well plate (BD) for 30 minutes at 4°C in 50μl of staining buffer containing anti-CD16/CD32 monoclonal antibody (clone 2.4G2) to block the FcyII/III receptors to prevent any non-specific binding of the antibodies. Then, the plate was spun at 750rpm for 3 minutes at 4°C, decanted and cell incubated with different combinations of conjugated monoclonal antibodies (from BD or Biolegend or eBioscience as detailed in table I) in a total volume of 100μl/well. 7-AAD (7-amin-actinomycin D) (eBioscience, San Diego, CA, USA), a fluorescent DNA intercalator, was used with each combination for exclusion of non-viable cells. For each antibody, appropriate isotype control was used. All antibodies were titrated in preliminary experiments and used at saturating concentrations. After 30 minutes of incubation at 4°C, plates were spun, decanted and cells washed twice with staining buffer. In the first wash, 100μl/well of staining buffer was added to the cells that were then mixed, centrifuge and decanted. In the second wash, 200μl/well of staining buffer was added to each well, where cells were mixed, the plate, then, was centrifuge, decanted and cells were re-suspended in 500 μl of staining buffer. Cells were read immediately or fixed overnight by adding 100 μl of 4% paraformaldehyde. Data were collected on 30,000 cells using BD FACS Canto II (BD biosciences, Mountain View, CA, USA) and analyzed using BD FACSDiva software (BD).
For the earlier experiments, single spleen cell suspensions and peritoneal cavity cells were stained with only two fluorescent conjugated monoclonal antibodies. Spleen cells were double stained with different combinations of directly conjugated monoclonal antibodies specific to: CD19 and CD3, Gr-1 and CD11b, CD3 and Sca-1, CD19 and Sca-1. Peritoneal cells were labeled with following combinations of directly conjugated monoclonal antibodies specific to: F4/80 and CD11b, F4/80 and Sca-1. Cells were analyzed by FACSscan (BD). Data were collected on 20,000 cells and analyzed by CELLQUEST software (BD).

4.2.14 Intracellular staining

For intracellular staining, 2x10^6/ml/well splenocytes were seeded in 24-well plate and stimulated them with PMA (100ng/ml) and ionomycin (1mg/ml) along with 1μl of BD GolgiPlug (Berfeldin A) for 4 hours at 37°C in 5% CO₂ incubator. Un-stimulated cells were cultured without PMA/ionomycin to serve as a negative control. Cells were then incubated with blocking buffer for 30 minutes at 4°C before stained for surface markers CD4 and CD8 as mentioned earlier. After that cells were permeabilized using 100μl permeabilization/fixation buffer (BD Cytofix/Cytoperm Plus) for 20 minutes at 4°C. Cells were washed twice with 200μl 1x permeabilization buffer and then stained with 100μl of anti-mouse IFN-γ antibody conjugate for 30 minutes at 4°C. Cells were washed with 200μl permeabilization buffer and re-suspended in 300μl permeabilization buffer. Data acquisition was done on 30,000 cells using BD FACSDiva software.

4.2.15 Collection of fecal samples for the estimation of fecal IgA

Mice pre-treated with saline or paraoxon, were infected i.p. with BRD509E bacteria and sacrificed at different time points. Fecal pellets were freshly collected, weighed and
suspended in extraction buffer (400μl of extraction buffer per 100mg of feces). The suspension was homogenized in extraction buffer. Each 100 mg of feces pellet was homogenized in 400μl of extraction buffer and then samples were placed on ice for 1.5-2 hours. Afterwards, the homogenates were centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatants were collected and stored at -20°C until they were analyzed for total or anti-Salmonella IgA antibody levels by ELISA.

4.2.16 Estimation of fecal IgA by ELISA

MaxiSorp 96-well plates (Nunc) were coated with 100μl/well of goat anti-mouse Ig H+L [IgA, IgG and IgM] antibody diluted in 1xPBS (0.1μg/ml) (Southern Biotech, Birmingham, AL, USA) and incubated overnight at 4°C for total IgA. For detection of anti-Salmonella IgA plates were coated with 100μl/well of heat killed Salmonella diluted in 1xPBS (1x10^6 CFUs/ml) and incubated overnight at room temperature. For both assays (total IgA and anti-Salmonella IgA), the rest of the protocol remains identical. Next day, plates were washed with washing buffer and blocked with 300μl/well blocking buffer for 2 hour at room temperature. After washing, serial dilutions of test samples were prepared in individual Eppendorf tubes and 100μl aliquots transferred to the respective wells and incubated for 2 hours at room temperature. Afterwards plates were washed and incubated with 100μl/well of biotin conjugated anti-mouse IgA antibody (Southern Biotech), diluted in reagent diluent for 2 hours at room temperature. After further washing, plates were incubated with 100μl of streptavidin-HRP (R&D) for 40 minutes in dark at room temperature. Finally, color was developed by adding 100μl of TMB substrate solution for 10-15 minutes and the reaction was
stopped with 2N sulphuric acid (H2SO4). Plates were read with the TECAN microplate reader at λ=450nm.

4.2.17 Intestinal transit

Mice, treated with paraoxon or saline for three weeks, were fasted overnight 2 days after the last injection. The following day, mice received 100 µl of 5% Evans blue suspension by oral gavage. 15 minutes later, animals were sacrificed by cervical dislocation and the small intestine was removed and spread on a flat surface. The distance travelled by the Evan blue dye was measured in centimeters and calculated as a percentage of the total length of the small intestine from duodenum to cecum.

4.2.18 Isolation of intestinal epithelial cells

Mice were treated with paraoxon or saline for three weeks. Two days after the last injection, mice were fasted overnight. The following day, mice were sacrificed, their abdomen was opened and the small intestine immediately removed. Ileum (distal part of the small intestine) was separated from the rest of the intestine, cut into small pieces, opened and washed with 1x PBS. Intestinal pieces were, then, immersed in 3 ml of 30mM EDTA in 1x PBS and shaken at 4°C for 45 minutes. Afterwards, samples were spun at 3,000 rpm at 4°C for 10 minutes, supernatants aspirated and pellet incubated with 250µg of collagenase (Sigma, St. Louis, MO, USA) in 5ml HBSS at 37°C for 10 minutes with gentle shaking. After incubation, tissue debris was removed with a pipette and cells washed with 3 ml of HBSS and re-suspended in 1ml Trizol for RNA extraction and stored at -80°C.
4.2.19 Phagocytosis assay

Phagocytic capability of purified splenic macrophages (CD11b+ cells) was measured using Vybrant Phagocytosis Assay Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) which contains Fluorescein-labeled Escherichia coli K-12 BioParticles. Fluorescent E. coli Bioparticle suspension was prepared as per the manufacturer's protocol instructions. One vial of BioParticles (5mg of solid powder) was thawed, suspended in 0.5 ml of 10x Hanks' balanced salt solution (HBSS) and briefly sonicated. Suspension was then transferred to a glass tube containing 4.5 mL of deionized water and sonicated until all the fluorescent particles were homogeneously dispersed.

Previously purified CD11b+ spleen cells (1x10^5/50 μl/well, in DMEM) were added to 96-well tissue culture plate and spun at 750 rpm for 3 minutes at 4°C. Supernatants were removed by vacuum aspiration and the adherent cells were incubated with 100μl of the prepared fluorescent BioParticles suspension. After 2 hours incubation at 37°C in 5% CO₂, supernatants were vacuum aspirated and 100μl/well of trypan blue was added and kept for 1 minute at room temperature to quench the extracellular fluorescence. Trypan blue suspension was then removed by vacuum aspiration. Intracellular uptake was quantified by measuring fluorescence emitted by engulfed bacteria at 485nm excitation and 535nm emission using a Victor X3 2030 microplate reader (PerkinElmer, Waltham, MA, USA). To minimize the effect of experimental errors, measurements of negative control, positive control and experimental samples were made in 4 replicates and the averages of these replicates were used for the calculations. Results were expressed as fold increase from the control mice.
4.2.20 RNA extraction using Trizol

RNA was extracted from whole spleen cells or ileum epithelial cells using the Trizol method. Cells (2-5x10⁶ per sample) were pelleted and re-suspended in 0.5-1ml Trizol (Invitrogen) and stored at -80°C. Afterwards samples were thawed and 200µl of chlorophorm were added to the vials and mixed well. Cells were then spun down at 14,000 rpm for 10 minutes and of the 3 phases obtained, the top clear RNA layer (~500µl) was transferred to a new tube. Equal volume of 2-propanol (~500µl) was then added and mixed well to precipitate the RNA. Tubes were spun, supernatants were discarded and washed with 500µl of 70% ethanol were added. On flicking the tubes, RNA was observed as a white pellet. Tubes were spun again, supernatant was removed, dried and RNA suspended in 20-50µl nuclease free water. Quality and quantity of the RNA was determined using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA) and then, stored at -80°C.

4.2.21 Reverse Transcription Reaction

RNA was reverse transcribed using TaqMan reverse transcription reagent (Applied Biosystems, CA, USA). Each master mix reaction contained 10x RT buffer, 25mM MgCl₂, deoxy NTPs mixture (2.5mM), random hexamers (50µM), RNAase inhibitor (20U/µl) and MultiScribe RT enzyme (50U/µl). The master mix was added into separate PCR tubes (Extragene, Palos Heights, IL, USA). RNA was added (1µg per reaction) and total volume was made up to 50µl with nuclease-free water. The one step RT-PCR reaction was run on GeneAmp PCR System 2700 from Applied Biosystems, under the following conditions: hexamer incubation for 10 minutes at 25°C, reverse transcription at 48°C for 30 minutes and reverse transcriptase inactivation at 95°C for 5 minutes. The
samples were held at 4°C for a maximum of 1 hour through which the samples could be removed and stored at -20°C.

4.2.22 Real time PCR reactions

The real time PCR was performed using TaqMan gene expression assay and amplified using the 7500 Real Time PCR System (Applied Biosystems). Each 20μl PCR reaction contained 10μl of 2xTaqMan Universal Master Mix, 1μl of 20x TaqMan assay Mix (both from Applied Biosystems), 2μl cDNA and 7μl nuclease free water. A negative PCR reaction was also carried out using only the reaction mixture without cDNA in order to ensure that there was no DNA contamination. The thermal cycling conditions were as follows: 95°C for 10 minutes (Inactivation of Reverse Transcriptase and activation of TaqMan polymerase), 95°C for 15 seconds (denaturation of dsDNA) and 60°C for 1 minute (annealing/extension- fluorescent data collected during this step) for a total of 40 cycles with the threshold set as 0.2. Data was analyzed using the Ct values for each sample that were in duplicates. Results were normalized to HPRT (Hypoxanthine-guanine phosphoribosyl transferase) and the mRNA fold change was determined using the following equation:

$$\text{Fold change} = 2^{\frac{\Delta C_t (\text{treated})}{\Delta C_t (\text{control})}}$$

where $$\Delta C_t (\text{treated}) = \text{threshold cycle (Ct) for target gene after treatment} - \text{Ct for HPRT after treatment}$$ and $$\Delta C_t (\text{control}) = \text{Ct for target gene saline} - \text{Ct for HPRT saline}$$

The assay IDs of the primers (Applied BioSystem, MA, USA) used are:

- HPRT (Mm01545399_m1)
- IL-12p40 (Mm00434174_m1)
- IFN-γ (Mm01168134_m1)
### 4.2.22.1 List of encoding sequences for AMPs primers (Metabion, Steuikirohen, Germany)

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4.2.23 Light microscopy analysis of the ileum

The distal part of the intestine (ileum) was removed and immediately fixed in 10% formalin. After overnight fixation, tissues were placed in histological cassettes, dehydrated in ascending series of graded ethanol cleared with xylene and then infiltrated and embedded in paraffin (Sherwood Medical, St. Louis, Mo, USA). Blocks were trimmed and 5 μm sections cut in a Shandon Finesse 325 manual microtome (Thermo Scientific, Pittsburg, PA, USA) and placed on gelatin coated slides. Sections were then rehydrated in xylene, descending series of graded ethanol and distilled water and then stained with Instant Haematoxylin (Thermo Scientific Shandon, Pittsburg, PA, USA) for 10 minutes followed with eosin yellowish (Panreac, Spain) for 1 minute. Finally, the sections were dehydrated in ascending series of graded ethanol, cleared in xylene and mounted with DPX (Panreac, Spain). Images were captured with an Olympus BX51 microscope model V-LH100HG (Olympus Corporation, Japan).

4.2.24 Electron microscopy analysis of the ileum

The distal part of the intestine (ileum) 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. Afterward, 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 (0.5mm) sections cut in an ultramicrotome Leica EM UC7 (Leica, Welzlar, Germany) and stained with 1% aqueous toluidine blue on glass slides. After selecting the right blocks, 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 were examined and photographed with TECNAI G2 Spirit Transmission Electron Microscope (FEI, Hillsboro, Oregon, USA).

4.2.25 Statistical analysis

Statistical significance was analyzed by Mann-Whitney test or 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 AChE activity in mice exposed to paraoxon

In every experiment the effectiveness of the paraoxon as a potent AChE inhibitor, was confirmed by measuring the RBC AChE activity in blood. Blood from control and experimental animals was collected just before the treatment started and at the end of each of the three weeks that the treatment lasted. After one week of paraoxon treatment AChE enzymatic activity was reduced by 50% of baseline value. This reduction was maintained till experiment was terminated (Fig. 1). In contrast, control group showed no significant changes in the enzyme activity, from the baseline, during the three weeks of treatment. Differences in AChE activity between the experimental and control groups were significant (p ≤ 0.001) at all time points, confirming that paraoxon treatment resulted in AChE inhibition.

![Figure 1: Reduction of RBC AChE activity in paraoxon treated mice.](image)

Mice were treated with paraoxon or saline for three weeks. Enzyme activity was measured following modified Ellman’s method. All enzyme activities were expressed in percentage of the baseline activity (100%). Depicted are the mean values ± SD of data pooled from 4 independent experiments. Asterisks denote statistically significant differences between the control and experimental groups ***p<0.001.
5.2 Effect of AChE inhibition on the mouse body weight

Body weight change over the three week of treatment was also assessed. Body weights were recorded before starting the treatment with saline (control) or paraoxon and considered as a baseline. Mice were weighed once per week during the three weeks treatment and the percentage increase in body weight calculated. The results showed that the body weights of saline-treated animals gradually increased by 3.5% after the first week, 6.8% after the second week and 7.6% after the third week of treatment (Fig. 2). Paraoxon treated mice showed a 0.09% reduction in their weights after the first week of treatment. During the following week (week 2), mice experienced a modest increase in their body weight of 1.6% of their original weights. During the third week of the treatment their body weights remained constant compared with the week before.

![Graph showing body weight change over weeks of treatment](image)

**Figure 2: Retarded growth of paraoxon-treated mice.**

Mice were treated with paraoxon 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.
5.3 Response to a lethal oral infection after AChE inhibition

After three weeks exposure to paraoxon or saline, mice were inoculated orally with SL1344, a virulent strain of *S. Typhimurium* at a lethal dose of $1 \times 10^4$ CFUs/mouse. Mice were then followed for survival for up to day 60 post-infection. Results revealed that exposure to paraoxon enhanced the percentage survival, with 76.5% of the mice surviving the lethal infection and a median of $>60$ days, the maximum period of observation (Fig. 3). This was in sharp contrast to the saline-treated group where percent survival was only 24% with a median of 11 days. This difference on survival, between paraoxon-exposed and control mice, was statistically significant ($p = 0.0027$).

![Figure 3: Paraoxon-treatment enhanced host survival in a lethal oral infection.](image)

Mice were treated with paraoxon or saline for three weeks. At the end of the treatment mice were infected orally with a dose of $1 \times 10^4$ CFUs/mouse of SL1344 and followed for survival up to 60 days post infection. Depicted are the mean values ± SD of data pooled from 3 independent experiments. Asterisks denote statistically significant differences between the control and experimental groups **$p<0.01$.** Chi squared (Mantel-Cox) statistical test was used for this analysis.
5.4 Effect of AChE inhibition on spleen, mesenteric lymph nodes and peritoneal exudate cells

First, the effect of paraoxon treatment and, therefore, inhibition of the AChE activity on the lymphoid organs involved in the immune response to bacterial infection that could explain the resistance to infection of paraoxon-treated mice was evaluated. MLN and spleen were collected from paraoxon-treated mice and compared to the organs from the control group who received saline for the same period of time. Because paraoxon administration was intraperitoneal, peritoneal exudates were also investigated.
5.4.1 Spleen

5.4.1.1 Size and cellularity

Paraaxon pre-treatment did not show a significant impact on spleen weights. The average spleen size of paraaxon-treated mice was $117 \pm 4.07$ g compared to the control mice weight of $128 \pm 5.7$ g (Fig. 4A). No statistical differences were found between the groups. Furthermore, when the spleen cellularity was calculated, paraaxon-treated group, as expected, showed a lower number of cells per spleen with an average of $85 \times 10^6 \pm 5$ cells/spleen while the control group rendered an average of $112 \times 10^6 \pm 11$ cells/spleen but the differences were not significant (Fig. 4B).

![Figure 4: No change in spleen size and cellularity following paraaxon treatment.](image)

Mice were treated with Paraaxon or saline for three weeks. Then spleen weights (A) and spleen cell counts (B) were determined. Depicted are the mean values ± SD of data pooled from 2 independent experiments.
Flow cytometric analysis of the different splenic cell populations and their activation status were evaluated per spleen after three weeks of treatment with saline or paraoxon. Regarding the spleen cell population in saline-treated mice, CD3$^+$ cells (T cells) represented 33 ± 2.3%. CD19$^+$ cells (B cells) were 43 ± 2.1%. CD11b$^+$ cells (myeloid cells) were 11.4 ± 1.04% and CD11c$^+$ cells (dendritic cells) were found to be 8.5 ± 0.63% of the total spleen population (Fig. 5A and 5B). Paraoxon pre-treatment did not cause any significant alteration compared to the control mice in the percentages of the different spleen populations. The spleen of paraoxon-treated mice presented 33 ± 2.3% of CD3$^+$ cells (T cells), 42 ± 0.59% of CD19$^+$ cells (B cells), 12.5 ± 1.16% CD11b$^+$ cells (myeloid cells) and 9.5 ± 0.58% of CD11c$^+$ cells (dendritic cells). When these percentages were used to calculate the total number of cells in each spleen population (Fig. 5C), no significant differences were found between the two groups. Due to the lower cellularity of the paraoxon-treated spleens, the total numbers of T (17.7 ± 1.7x10$^6$) and B cells (22.9 ± 3.46x10$^6$) were 18% lower than in spleens of control mice (21.5 ± 0.35x10$^6$ and 27.9 ± 2.59x10$^6$, respectively). However, the total number of CD11b$^+$ (6.9 ± 1.43x10$^6$) and CD11c$^+$ (5.2 ± 1.01x10$^6$) cells in paraoxon-treated mice decreased only 6.8% and 5.5% compared with the saline treated group (7.4 ± 0.74x10$^6$ and 5.5 ± 0.58x10$^6$, respectively) which could indicate a discrete increment in myeloid cells induced by paraoxon.
Figure 5: Flow cytometric analysis of total spleen population
Mice were treated for three weeks with paraoxon or saline. Afterwards, erythrocyte-depleted spleen cells were analyzed by flow cytometry. Representative dot plots show the positive cell percentages in saline and paraoxon-treated mice (A). Bar graphs show percentage of positive cells (B) and absolute cell number (C). Graphs depicted the mean values ±SD of 3 mice/group from a representative experiment.
Next, the splenic CD3+ T cell subpopulations of CD4+ and CD8+ were studied along with the expression of the activation markers CD25 and Sca-1. Figures 6A and 6B show representative dot-plots of gated CD3+ cells and the percentages of CD4 and CD8 subpopulations in each group. In the control group, CD4+ T cells represented the majority of the cells (66.3 ± 1.2%) while CD8+ cells were 23.9 ± 1.2% of the total CD3+ T cells (Fig. 6A). Paraoxon pre-treatment did not alter these percentages of CD4+ (66.5 ± 2.3%) or CD8+ (23.8 ± 1.6%) T cells among the CD3+ cell population (Fig. 6B) compared to the saline-treated group. Figure 6C shows a bar graph represents the percentage average of population CD4+ and CD8+ T cells in each group. No differences between the control saline-treated and the paraoxon-treated animals were found. Moreover, the total number of CD4+ and CD8+ cells in each group was also calculated (Fig. 6D) and showed no significant differences of both population, in both the paraoxon-treated animals and controls which correlates with the smaller spleen size and cellularity described above. Spleens from experimental animals contained 12.6 ± 0.8x10^6 CD4+ and 4 ± 0.4x10^6 CD8+ cells, compared to the control mice that presented 13.3 ± 1.2x10^6 CD4+ and 4.8 ± 0.4x10^6 CD8+ T cells.

In order to see whether paraoxon treatment modified the activation status of T cells, the expression of CD25 and Sca-1 activation markers on both CD4+ and CD8+ populations were analyzed (Fig. 6 E-H). The expression of CD25 on CD4+ cells was only 1.3 fold higher in paraoxon group (19.6 ± 1%) compared to that in the saline group (14.9 ± 1.4%) as seen in Fig. 6E. Similarly, 14.8 ± 1.9% of the CD4+ cells were positive for Sca-1 activation marker in the control group, while in the experimental group these cells represented 17.5 ± 1.7% of the total CD4+ population (Fig. 6A). Based on these
percentages, the total number of cells in each population was calculated (Fig. 6F).

Despite the fact that the total number of CD4$^+$ cells was lower in the paraoxon treated mice than in the control group, the number of CD4$^+$CD25$^+$ cells were higher (2.2 ± 0.2x10$^6$) in the experimental group compared to that in the control (1.9 ± 0.09x10$^6$) (Fig. 6F). The number of CD4$^+$Scal$^+$ cells was approximately identical (1.9 ± 0.06x10$^6$ and 1.96 ± 0.2 x10$^6$ in the saline- and paraoxon-treated mice).

Although the statistical differences between the groups were not significant, these results suggested a specific activation of CD4$^+$ T cells in the spleen by the increase in the levels of ACh.

The activation status of splenic CD8$^+$ cells revealed that 92.2 ± 1.4% of the cells were positive for CD25 in the control group and 88.7 ± 1.7% in the experimental group. Scal antigen was positive on 61.8 ± 2.2% and 63.6% in the control and paraoxon groups, respectively (Fig. 6G). When the total number of cells in each population was calculated (Fig. 6H), spleens of paraoxon-treated mice showed lower CD8$^+$CD25$^+$ (3.5 ± 0.2x10$^6$) and CD8$^+$Scal$^+$ (2.5 ± 0.1x10$^6$) cells than the saline-treated animals (4.4 ± 0.3x10$^6$ and 2.96 ± 0.3x10$^6$, respectively). The differences between the different groups were found not to be statistically significant.
Figure 6: Flow cytometric analysis of splenic T cells and their activation status.

Mice were treated for three weeks with paraoxon or saline. Afterwards, erythrocyte-depleted spleen cells were analyzed by flow cytometry for changes in T cells subtypes and activation status. Representative dot plots show the positive cell percentages in saline (A) and paraoxon (B) treated mice. Bar graphs depicted the mean values ±SD of 3 mice/group from a representative experiment showing CD3 gated CD8⁺ and CD4⁺ cell percentages (C), total number of CD4 and CD8/spleen (D), percentage (E), and total cell number (F) of CD4⁺ cells with two different activation markers and percentage (G), and total cell number (H) of CD8⁺ cells with two different activation markers.
To investigate the activation status of B cells (CD19\(^+\)), three different surface activation markers were analyzed: CD80, CD86 and Sca-1. Fig. 7A shows representative dot plots of Sca1, CD80 and CD86 staining in saline- and paraoxon-treated mice. Figure 7C shows the quantitative analysis of each marker comparing both groups. Only a minority of CD19\(^+\) cells were positive for these markers in both control and experimental groups. While paraoxon treatment seemed to induce only a 0.2% insignificant increase in CD80\(^+\) cells, it produced significant increase (p ≤ 0.05) of 1.8% and 0.9% in CD86\(^+\) and Sca-1\(^+\) cells, respectively. These differences between the two groups disappeared when the total number of cells positive for each marker was calculated (Fig. 7D) due to the lower spleen cellularity of the paraoxon-treated mice.
Figure 7: Flow cytometric analysis of splenic B cells and their activation status.

Mice were treated for three weeks with paraxoxon or saline. Afterwards, erythrocyte-depleted spleen cells were analyzed by flow cytometry for changes in B cells activation status. Representative dot plots show positive cell percentages (A). Bar graphs depicted positive cell percentages (B) and cell number (C) of activation markers CD80, CD86 and Sca-1. Data are the mean values ±SD of 3 mice/group from a representative experiment. Asterisks denote statistically significant differences between control and experimental groups * p<0.05.
Lastly, CD11b+ cells were analyzed for their activation markers expression (CD40, CD80 and Sca-1). Figure 8A depicts representative dot plots from saline- and paraoxon-treated mice. No significant differences were observed in the expression of any of the markers between the two groups as CD40+ cells were around 6% in both groups. CD80+ cells 16.3 ± 0.8% in control and 18.1 ± 1.4% in paraoxon groups and Sca-1+ cells 6.9 ± 0.4% in control and 7.9 ± 0.3% in paraoxon groups. These results were confirmed when the average percentages of each populations were plotted (Fig.8B). The total cell numbers in each population was calculated based on the percentages which showed identical results (Fig. 8C).
Figure 8: Flow cytometric analysis of splenic macrophages and their activation status.

Mice were treated for three weeks with paraoxon or saline. Afterwards, erythrocyte-depleted spleen cells were analyzed by flow cytometry for changes in macrophages activation status. Representative dot plots show positive cell percentages (A) and bar graphs show positive cell percentage (B) and positive cell number (C) of activation markers CD40, CD80 and Sca-1. Graphs depicted the mean values ±SD of 3 mice/group from a representative experiment.
5.4.1.3 Macrophages phagocytic activity

Next, it was tested whether paraoxon treatment had any effect on the phagocytic activity of spleen macrophages. CD11b+ cells were purified from a single suspension of spleen cells from mice treated for three weeks with either saline or paraoxon. Enrichment of CD11b+ cells was verified by flow cytometry. Figure 9A shows dot plot representations of CD11b and Gr1 staining before and after purification in the control saline- and experimental paraoxon-treated mice. Spleen cells from control animals contained 11% of CD11b+ cells (from which 6% were Gr1+); in the experimental group, these cells represented 10% (out of which 5% were Gr1+) of the total spleen population. After purification, CD11b+ cells increased to 94% in the control and 92% in the experimental groups, which is an indication that there had been an 83% and 82% enrichment in the two groups. The percentages of CD11b+Gr1+ after purification increased more than 10fold in both groups.

Next, the phagocytic activity of the purified CD11b+ cells was assessed in both groups. The results showed a 3.5 fold increase in the phagocytic activity of CD11b+ cells from the paraoxon group compared with the ones from the control group. This difference in the phagocytic activity between the two groups was statistically significant (p ≤ 0.05) (Fig. 9B).
Mice were treated for three weeks with paraoxon or saline. Then, CD11b$^+$ spleen cells were purified from erythrocyte-depleted spleen cells by autoMACS. (A) Dot plot of flow cytometry represent the percent purity of purified cells. (B) Fold increase in phagocytic activity of purified splenic CD11b$^+$ cells. Graphs depicted the mean values ± SD of data from 2 independent experiments. Asterisks denote statistically significant differences between control and experimental group * \( p < 0.05 \).
5.4.1.4 Gene expression of spleen cells

It was tested whether AChE inhibition modified the expression of IFN-γ and IL-12p40, the Th1 cytokines essential for a robust immune response to *S. typhimurium* infection. RNA from single cell suspensions of spleens from control and paraoxon-treated mice was extracted. cDNA was then prepared and analyzed for the expression of IFN-γ and IL-12p40.

The relative expression of IFN-γ (Fig. 10A) was 0.024 ± 0.003 and 0.018 ± 0.0008 in spleen from control and experimental mice, respectively. Despite the fact that the reduction on IFN-γ expression in the experimental group was equivalent to 25%, this difference was not statistically significant. Figure 10B shows no differences in the relative expression of IL-12p40 between splenocytes from saline (0.0045 ± 0.0004) and paraoxon (0.004 ± 0.0004) treated mice.

So, paraoxon treatment did not upregulate the expression of either IFN-γ or IL-12p40.

![Graph](image)

**Figure 10:** Gene expression profile in splenocytes.

Mice were treated with paraoxon or saline for 3 weeks. Mice were sacrificed and spleens were collected. RNA was extracted from erythrocytes-depleted spleen cells, converted to cDNA and ran for real-time qPCR. Graphs depicted fold change in the mRNA relative expression of IFN-γ (A) and IL-12p40 (B). Mean values ±SD of data pooled from 2 independent experiments are shown.
5.4.1.5 Cytokines production upon stimulation of spleen cells

Splenocytes cellular response to mitogenic activation was evaluated. Single cell suspensions of spleen cells were prepared from saline and paraoxon-treated animals and stimulated with LPS to activate macrophages or ConA which is a mitogen to T cells. After 24 hours, culture supernatants were collected and production of pro-inflammatory cytokines (IL-12p40, IFN-γ and IL-6) measured. After LPS stimulation, IL-12p40 and IL-6 cytokine levels were evaluated and compared to that in unstimulated cells. In the control group LPS stimulation induced a 1.7 fold increase in IL-12p40 production compared to that in the unstimulated cells. Similarly, LPS stimulated spleen cells from paraoxon-treated group produced 2-fold more IL12p40 than unstimulated cells (Fig. 11A). When the production of IL-6 (Fig.11B) was analyzed, LPS stimulated spleen cells from both groups, showed an identical increase of 33.3 fold compared with that of the unstimulated cells. Production of IFN-γ was also analyzed after ConA stimulation of spleen cells. Figure 10C shows that ConA stimulated cells produced IFN-γ. In response to ConA stimulation, cells from paraoxon-treated mice produced 39% more IFN-γ than the cells from the control group. However, due to the different levels of IFN-γ production among the different members of the same group, the differences between control and experimental groups were not statistically significant.
Figure 11: Cytokines production from splenocytes after 24 hours in culture.

Mice were treated for three weeks with paraoxon or saline. Then, erythrocytes-depleted spleen cells were cultured with or without stimulation for 24 hours. Supernatants were then assayed for the production of IL-12p40 (A) and IL-6 (B) before and after LPS (10μg/ml) stimulation. Production of IFN-γ (C) before and after ConA (2.5μg/ml) stimulation was also estimated. Graphs depicted the mean values ±SD of data from 2 independent experiments.
5.4.1.6 Intracellular staining of spleen T cells

A single cell suspension of splenocytes from paraoxon- and control-treated mice were cultured and stimulated with PMA/IONOMYCIN for 4 hours. Cells were then stained for the surface molecules CD4 and CD8 and intracellularly for IFN-γ. Figure 12 represents dot plots of the flow cytometric analyses. Analysis of un-stimulated control cells showed that the percentages of CD8+ and CD4+ form the total spleen population were 8% and 21%, respectively. Of these cells, 6% of the CD8+ and 1.3% of the CD4+ cells produced IFN-γ. In the control group, after stimulation, the percentages of T cells were 7.2 ± 0.26% CD8+ cells and 23 ± 1.4% CD4+ cells while in the paraoxon-treated group, 11.6 ± 0.5% of the cells were CD8+ and 26 ± 0.6% were CD4+. These represent a 7-fold (42 ± 2.4%) and ~7.5-fold (45 ± 3.4%) increase of CD8+ cells in the control and experimental groups, respectively, after stimulation (Fig. 12). Percentages of CD4+ T cells that were positive for IFN-γ were 12% for both, control and experimental groups after stimulation (Fig. 12).

The observed increase in the percentage of CD8+ cells in the paraoxon group after stimulation represented 57% more cells than in the control group (Fig.13A). However, no differences were found when the total number of cells was calculated, due to the lower cellularity of spleens in the paraoxon-treated animals (Fig. 13B). No significant differences were found, between saline- and paraoxon-treated groups, neither in the percentage nor in the total number of IFN-γ expressing cells (Fig.13C and D).
Our results indicate that paraoxon treatment did not induce an increase in the production of IFNγ by T cells after 4 hours in vitro stimulation.

Figure 12: Production of IFN-γ by CD4⁺ and CD8⁺ splenocytes.

Mice were treated for three weeks with paraoxon or saline. Erythrocyte-depleted splenocytes were cultured and stimulated with PMA/lonomycin for 4 hours. Cells were then stained and analyzed by flow cytometry for surface markers and intracellular content of IFN-γ. Representative dot plots show percentage of CD8⁺, CD4⁺, IFN-γ producing CD8⁺ and IFN-γ producing CD4⁺ percentages. Data are from a representative experiment using 3 mice/group.
Figure 13: Quantification of IFN-γ producing T cells from spleen.

Erythrocytes-depleted splenocytes were cultured and stimulated with PMA/ionomycin. Cells were then stained for flow cytometry. Graphs show percentage (A) and absolute number (B) of CD4 and CD8 cells in the spleen of saline and paraoxon-treated mice. Graph C and D show percentage (C) and absolute cell number (D) of CD4 and CD8 IFN-γ producing cells. Graphs depicted the mean values ±SD of 3 mice/group of a representative experiment. Asterisks denote statistically significant differences between control and experimental groups *p<0.05.
5.4.2 Mesenteric lymph nodes

5.4.2.1 Size and cellularity

After 3 weeks treatment with saline or paraoxon, mice were sacrificed and mesenteric lymph nodes aseptically collected. Paraoxon treatment and therefore AChE inhibition did not have any influence on MLN weight (55 ± 5g in control mice and 58 ± 3.4g in paraoxon-treated) (Fig. 14A) or their cellularity (28 ± 4.6x10⁶ and 29 ± 3x10⁶ in control and experimental mice, respectively) (Fig. 14B).

![Figure 14: No changes in MLN following paraoxon treatment.](image)

Mice were treated with Paraoxon or saline for three weeks. Then MLN weights (A) and cell counts (B) were determined. Depicted are the mean values ±SD of data pooled from 3 independent experiments.

5.4.2.2 MLN phenotyping and activation markers

Single cell suspensions of MLN cells were prepared to evaluate whether paraoxon could have an effect on the number of the different cell populations or their activation status. First, we looked at the different cell populations present in the MLN of control animals. The flow cytometric analysis showed, as expected, that the majority of the cells (63 ± 1.4%) were CD3⁺ T cells, then 29 ± 2.7% CD19⁺ B cells, 5.9 ±1.3% CD11b⁺ and 4.1 ± 0.4% CD11c⁺ (Fig. 15A & B). Paraoxon-treatment did not show any significant changes in the cellular composition of the MLN compared to that of control animals: CD3⁺ T
cells were 66 ± 4.6%, CD19+ B cells 24 ± 2.9%, CD11b+ 5.4 ± 1.5% and CD11c+ 3.3 ± 0.5% of the total MLN population (Fig. 15A & B). Similarly, when the total number of cells in each population was calculated no significant differences were found between the two groups (Fig. 15C).

Figure 15: Flow cytometric analysis of MLN cell population.
Mice were treated for three weeks with paraoxon or saline. Afterwards, MLN cells were analyzed by flow cytometry for changes in the total cell populations. Representative dot plots show positive cell percentages (A) and bar graphs show positive cell percentages (B) and cell numbers (C). Graphs depicted the mean values ±SD of data pooled from 3 independent experiments.
Then, the expression of the activation markers on CD4<sup>+</sup> and CD8<sup>+</sup> cells was investigated. The representative dot plots showed that, in the saline group, 57% of the CD4<sup>+</sup> cells Scal<sup>+</sup> CD25<sup>+</sup>, 7% CD25<sup>-</sup>Scal<sup>-</sup> and 9% CD25<sup>+</sup> Scal<sup>-</sup> (Fig. 16B). Similar percentages were observed in the CD4<sup>+</sup> cells from the paraoxon-treated group (Fig. 16F). No statistical differences were found between saline and paraoxon-treated groups in any of the above subpopulation of CD4<sup>+</sup> cells (Fig. 17C). When the total cell number of each population was calculated, CD4<sup>+</sup>Scal<sup>+</sup>CD25<sup>+</sup> cells from the experimental group appeared to be significantly (p ≤0.05) different than the ones in the saline group (Fig. 17D).

About the CD8<sup>+</sup> cells, the dot plots showed that saline and paraoxon-treated groups presented similar percentages of CD25<sup>+</sup>Scal<sup>+</sup> cells (10 ± 6.8% vs 13 ± 4.4% respectively). The percentage of all Scal<sup>+</sup> cells was also similar in the two groups (86 ± 11.6% vs 88 ± 8.5%, respectively). However, the percentage of CD25<sup>+</sup> Scal<sup>+</sup> cells was 54 ± 2.6% in saline group and 81 ± 1.2% in the paraoxon-treated group (Fig. 16D and H) which it was a difference statistically significant (p≤0.01) (Fig. 17E). When the total cell number of each population was calculated Scal<sup>+</sup> cells from the paraoxon group was significantly higher (p ≤0.05) than in the saline group (Fig. 17F).
Figure 16: Flow cytometric analysis of MLN T cells and their activation status.

Mice were treated for three weeks with paraoxon or saline. Afterwards, MLN cells were analyzed by flow cytometry for changes in T cells subtypes and activation status. Representative dot plots show CD4$^+$ (A and E), CD4 activation markers (B and F), CD8$^+$ (C and G) and CD8 activation markers (D and H) in saline and paraoxon groups.
Figure 17: T cells population and their activation status in MLN

Mice were treated for three weeks with paraoxon or saline. Afterwards, MLN cells were analyzed by flow cytometry for changes in T cells subtypes and activation status. Bar graphs depicted CD8 and CD4 positive cell percentages (A), cell number (B), CD4+ activation markers cell percentages (C), cell number (D), CD8+ activation markers percentages (E) and cell numbers (F). Graphs depicted the mean values ±SD of 2-3 mice/group from a representative experiment. Asterisks denote statistically significant differences between control and experimental groups * p<0.05, **p<0.01.
5.4.3 Peritoneal exudate cells

5.4.3.1 Cellularity

The fact that the administration of paraoxon and saline was intraperitoneal, it was interested to investigate whether these daily injections affected the cellular composition and functionality of peritoneal cavity cells.

First, the peritoneal lavage cellularity was analyzed, as it was done previously the spleen and MLN. It was observed that paraoxon pre-treatment resulted in significantly lesser viable cell yield than the obtained in the control group as a total of 7.9 ± 0.9x10^6 (1 ± 0.1x10^6/ml) cells were retrieved from paraoxon-treated mice and 12 ± 1x10^6 (1.6 ± 0.1x10^6/ml) cells in control mice (Fig. 18A,B).

![Figure 18: Decrease in peritoneal cavity cellularity following paraoxon treatment.](image)

Mice were treated with paraoxon or saline for three weeks. Then PECs were retrieved and total cell counts (A) and cell counts/ml (B) were determined. Depicted are the mean values ±SD of data pooled from 3 independent experiments. Asterisks denote statistically significant differences between control and experimental groups *p<0.05, **p<0.01.
5.4.3.2 PECs phenotyping and activation markers

After three weeks of treatment with saline or paraoxon, PECs were retrieved from the peritoneal cavity and cultured for 24 hours with or without LPS stimulation. Afterwards, cells were collected, stained and analyzed by flow cytometry for phenotyping and activation status of the different cell populations. Peritoneal exudates from unstimulated cultures were composed of 40% B2 cells (CD19⁺CD11b⁻), 25-30% B1 cells (CD19⁺CD11b⁺) and 2-5% macrophages (CD11b⁺/F4/80⁺) cells (Fig. 19A and B) and these percentages were the same in both groups with no significant differences observed.

Figure 19: Flow cytometric analysis of PECs after 24 hours in culture.

Mice were treated with paraoxon or saline for three weeks. Then PECs were retrieved and cultured for 24 hours. Phenotypic analysis of PECs is shown in representative dot plots (A) and bar graph (B) as percentage of positive cells. Depicted are the mean values ±SD of 3 mice/group from a representative experiment.
Representative dot plots for the analysis of the diverse activation markers on B1 and B2 peritoneal cells were depicted on figure 20A. In the saline group, 10 ± 0.0005% of B2 cells were CD40⁺ in un-stimulated PECs but after LPS stimulation 29 ± 5% of B2 cells were positive for CD40. In the paraoxon-treated group this population represented 23 ± 1.8% (Fig. 20B). The differences between the control and experimental groups after LPS stimulation were statistically significant (p ≤ 0.01). All the B2 cells in both groups were MHC II negative before and after stimulation. B1 cells positive for CD40, from saline-treated mice, increased from 48 ± 1.5% on the unstimulated cells to 84 ± 1.5% on the LPS stimulated cells (Fig. 20C). In the experimental group, the percentage of CD40⁺ B1 cells before stimulation was 55 ± 1.5% vs 74 ± 1.1% after LPS stimulation (Fig. 20C).

As it happened with the B2 population, there was a statistically significant (p ≤ 0.001) difference between the percentage of CD40⁺ cells from control and experimental group after LPS stimulation. No changes in the percentages of cells positive for MHC II were observed as only around 2-3% were MHC II⁺ before and after stimulation in both groups.
Figure 20: Activation status of PECs B1 and B2 populations after 24 hours in culture.

Mice were treated with paraoxon or saline for three weeks. Then PECs were retrieved and cultured for 24 hours with or without LPS (10μg/ml). Then cells were stained for flow cytometry. (A) shows a representative dot plot of positive cell percentages of activation markers. (B) CD40+ B2 cells and (C) CD40+ and MHC II+ B1 cells. Depicted are the mean values ±SD of 3 mice/group from a representative experiment. Asterisks denote statistically significant differences between stimulated and non-stimulated cells **p<0.01, ***p<0.001.
All CD11b$^{+}$ F4/80$^{+}$ peritoneal cells (macrophages) from saline and paraoxon-treated mice were CD40$^{+}$, MHC II$^{+}$ and CD80$^{+}$ (100%) before and after LPS stimulation (Fig. 21A&B). However, Sca1 expression on CD11b$^{+}$ F4/80$^{+}$ cells was upregulated after LPS stimulation from 85-86% to 96-97%, in both groups. The expression of CD86$^{+}$ on CD11b$^{+}$ F4/80$^{+}$ cells was reduced from 97-98% to 72-74% after LPS stimulation in both groups (Fig. 21A & C). No significant differences were found between control and paraoxon-treated groups in any of the activation markers on this myeloid population.

Our results showed that paraoxon treatment impaired the capacity of the peritoneal B1 and B2 cell to upregulate the expression of CD40, but did not seem to have any effect on peritoneal macrophages.
Mice were treated with Paraaxon or saline for three weeks. Then PECs were retrieved and culture for 24 hours with or without LPS (10\(\mu\)g/ml). Then cells were stained for flow cytometry. (A) shows a representative dot plot of Positive cell percentages of activation markers, (B) MHC II\(^{+}\), CD40\(^{+}\) and CD80\(^{+}\) myeloid cells and (C) Sca-1\(^{+}\), CD86\(^{+}\) myeloid cells with or without stimulation. Depicted are the mean values ±SD of 3 mice/group from a representative experiment.

Figure 21: Activation status of PECs myeloid cells after 24 hours in culture.
5.4.3.3 Cytokine production by *in vitro* stimulated PECs

Peritoneal cells were cultured either alone or with LPS and after 24 hours supernatants were collected and assayed for the presence of IL-12p40 and IL-6 cytokines. In un-stimulated cells IL-12p40 production was below the level of detection (≤15pg/ml) in both groups, however, after LPS stimulation only paraoxon group had a slight but not statistically significant increase in the production of IL-12p40 (36 pg/ml) while in the control group the levels were still undetectable (Fig. 22A). In contrast, IL-6 production was highly induced after LPS stimulation as it went from undetectable levels (≤15pg/ml) to >4000pg/ml in both groups (Fig. 22B).

Therefore, paraoxon treatment seems to have a mild effect on stimulated peritoneal macrophages by preparing them for a better production of IL-12.

<table>
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<td><strong>IL-12p40 pg/ml</strong></td>
<td><strong>IL-6 pg/ml</strong></td>
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<td>un-stimulated</td>
<td>LPS stimulated</td>
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<td>saline</td>
<td>paraoxon</td>
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Figure 22: Cytokine production from PECs after 24 hours in culture.

Mice were treated for three weeks with paraoxon or saline. Then, PECs were cultured with or without LPS (10μg/ml) stimulation for 24 hours. Supernatants were then assayed for IL-12p40 (A) and IL-6 (B) cytokines production before and after LPS stimulation by ELISA. Graphs depicted are the mean values ±SD of data from 3 independent experiments.
5.5 Intestinal transit

The improved survival of paraoxon-treated mice following a lethal oral infection, could be attributed to enhanced intestinal motility, which implies a faster clearance of bacteria from the intestine, and not to the modulation of immune cells. In order to rule out this possibility, the effect of daily injections of paraoxon during the three weeks of treatment on intestinal motility was tested. 72 hours after the last injection, saline and paraoxon treated mice were orally inoculated with Evans blue and 15 minutes later sacrificed. Intestines were then aseptically removed and the distance travelled by the dye was measured. Fig. 23 shows no differences in the intestinal motility of paraoxon- and saline-treated animals indicating that the enhanced survival must be due to a different mechanism.

Figure 23: Paraoxon-treatment does not increase intestinal motility measured 72 hours of the last paraoxon injection.

Mice were treated with paraoxon or saline for 3 weeks then Even blue dye was given to each mouse by oral intubation. After 15 minutes mice were sacrificed and intestinal transit was measured and calculated as percentage to the total small intestine length.
5.5.1 Summary of the effect of AChE inhibition on the immune cells

Spleen:

- Paraoxon-treatment did not show any effect on spleen size, cellularity, cell phenotype and activation status or steady state cytokine gene expression.

- Spleen cells of paraoxon-treated mice showed a modest increase in IL-12 and IFN-γ production upon stimulation.

- Purified macrophages of paraoxon-treated mice showed significant enhancement in their phagocytic activity.

MLN:

- Paraoxon-treatment did not show any effect on size, cellularity or cell phenotype.

- T cells from paraoxon-treated mice had a moderate increase in their activation status particularly CD8+ cells.

PECs:

- PECs recovered from paraoxon-treated mice were significantly lower than saline-treated mice.

- Upon stimulation, a mild increase in the production of IL-12 in paraoxon group was observed.
5.6 Response to systemic infection with SL1344

5.6.1 Survival

After three weeks of saline or paraoxon treatment, mice were infected intraperitoneally (i.p.) with a virulent strain of *Salmonella* (SL1344) at a dose of 3000 CFUs/mouse. Mice were then followed for survival (Fig. 24). The survival of paraoxon treated mice was slightly increased compared with saline control, as all the animals from the control group succumbed by day 7 post-infection while in the experimental group some mice survived up to day 12 post-infection. This difference, however, was not statistically significant and the median survival for both groups was almost identical (5 and 6 days, for saline and paraoxon-treated, respectively). These results indicate that AChE inhibition did not protect mice against systemic infection with SL1344.

![Graph](image)

**Figure 24:** Paraoxon pre-treatment did not show an effect on survival after systemic infection with SL1344.

Mice pre-treated with paraoxon or saline for 3 weeks were infected i.p. with SL1344 (3x10³). Mice were followed for survival up to 60 days post infection. Depicted are the mean values ±SD of data pooled from 2 independent experiments.
5.6.2 **Bacterial load in target organs**

Saline and paraoxon-treated mice were i.p. infected with SL1344 (12x10^3 CFUs/mouse) then bacterial load in the different organs and compartments were determined at different time points. Bacteria recovered from the peritoneal cavity (Fig.25A) were \( \sim 4 \pm 2 \times 10^3 \) CFUs/ml in control group and \( \sim 14 \pm 9 \times 10^3 \) CFUs/ml in paraoxon group at 2 hours post infection with the difference being insignificant. After 20 hours of infection bacteria recovered from the peritoneal cavity increased more than 40 times \((\sim 14 \pm 14 \times 10^4 \) CFUs/ml\) in control group and only 2.5 times in the paraoxon group \((\sim 4 \pm 1.5 \times 10^4 \) CFUs/ml\). Due to the variations within each group the differences were statistically insignificant.

When bacteria recovered from peritoneal cavity was calculated as CFUs/ 1x10^6 cell (Fig. 25B) the result were identical to the ones showed in figure 25A.

Bacterial load in the spleen was also analyzed and after 2 hours of infection \( \sim 10 \) CFUs/mg were found in the spleen of control mice and increased 1000 times at 20 hours post-infection \( (\sim 10^4 \) CFUs/mg\). Spleens from paraoxon –treated group showed almost identical bacterial load as control group at both time points (Fig. 25C).

The kinetic of the bacterial load in the liver was also investigated. Similarly to the spleen, livers’ bacterial load were \( \sim 10^3 \) CFUs/g at 2 hours post infection, in both groups, that increased to \( \sim 4 \pm 2 \times 10^5 \) CFUs/g in control group and \( \sim 9 \pm 5.7 \times 10^5 \) CFUs/g in paraoxon group after 20 hours (Fig. 25D). Statistically the differences were not significant.
Figure 25: Bacterial load in peritoneal cavity and target organs following systemic infection with SL1344.

Mice pre-treated with paraoxon or saline for 3 weeks were infected i.p. with SL1344 \( (12 \times 10^3) \). Mice were sacrificed at the indicated time points. Peritoneal fluid retrieved and bacterial load was determined as CFUs/1ml volume (A) and CFUs/\( 1 \times 10^6 \) cell (B). Spleen and liver homogenates aliquots were plated and bacterial loads were determined. Spleen CFUs/mg (C) and liver CFUs/g (D) were determined. Depicted are the mean values ±SD of a representative experiment.
5.6.3 Spleen and PECs cellularity

At the same two time points after infection (2 and 20 hours), spleens were removed and weighted. Results showed a similar spleen weight (100-110 mg) in the animals from saline and paraoxon treated groups at 2 hours post-infection. After 20 hours of infection, the average weight of the spleen increased to 135 ± 11 mg in control treated mice and 160 ± 6 mg in paraoxon treated mice (Fig. 26A). Statistical analysis revealed a significant \((p \leq 0.01)\) increase in the spleen size between 2 and 20 hours only in the paraoxon-treated group.

Cell viability of spleen cells was also analyzed after infection. Viability of the spleen cells was slightly decreased in both groups after 20 hours of infection, probably due to the higher bacterial load. Figure 26B shows that at 2 hours post-infection were 53-62x10⁶ viable cells/spleen and after 20 hours of infection these numbers were 45-49x10⁶ cells/spleen in both groups. No statistically significant differences were found between saline and paraoxon group.

Cell viability was also analyzed in the cells from the peritoneal cavity. In the control group, viable cells retrieved from the peritoneal cavity were around 6 ± 1x10⁶ cells after 2 hours of infection and it was a slight but significant increased after 20 hours of infection to 12 ± 1.1x10⁶ cells (Fig. 26C). In the paraoxon treated group, the number of viable cells recovered after 2 and 20 hours of infection was around 10 ± 2x10⁶ cells (Fig. 26C).
Figure 26: Changes in spleen and PECs after systemic infection with SL1344.  
Mice were treated with paraoxon or saline for three weeks and then infected i.p. with the virulent strain SL1344 (12x10^3 CFUs/mouse). After 2 and 20 hours post infection spleen weight (A), spleen cell counts (B) and PECs cell counts (C) were determined. Depicted are the mean values ±SD of data from a representative experiment. Asterisks denote statistically significant differences between 2 and 20 hours * p<0.05, **p<0.01.
5.6.4 Cytokine production

Finally, it was desired to assess whether infected animals pre-treated with paraoxon were able to secrete more IL-12p40 than the controls. IL-12 is an important cytokine that initiate the Th1 immune response needed to fight a *Salmonella* infection. First, IL-12p40 was measured in the serum of infected animals and the results showed a significant elevation in IL-12p40 production after 2 hours of infection from ~500pg/ml (in both groups) to 1200 ± 400pg/ml and ~1900 ± 500pg/ml in control and paraoxon groups, respectively (Fig. 27A). This difference between the two groups was statistically not significant. After 20 hours of infection, IL-12p40 levels decreased to similar levels to the ones found in un-infected mice (500-600pg/ml) in both groups.

To further assess the mouse response to infection, IL-12p40 levels were estimated in culture supernatants from spleen cells cultured for 24 hours. Spleen single cell suspension was prepared from infected saline- and paraoxon-treated animals after 2 and 20 hours of infection. Results indicated that spleen cells from saline and paraoxon group increased the production of IL-12p40 after 2 hours of infection from undetectable levels (<15pg/ml) to 100-200pg/ml and remained on these levels after 20 hours of infection (Fig.27B). Despite the fact that the average amount of IL12p40 produced by spleen cells from paraoxon-treated animals after 2 hours of infection was higher than in the control mice, the differences were not significant (p value was 0.159).
Figure 27: Production of IL-12p40 after systemic infection.

Mice pre-treated with paraaxon or saline for 3 weeks were infected i.p. with SL1344 \((12 \times 10^3)\). Mice were then sacrificed at the indicated time points and IL-12p40 content assayed by ELISA in serum (A) splenocytes were obtained and cultured for 24 hours (B). Depicted are the mean values ±SD of data from a representative experiment. Asterisks denote statistically significant differences between 2 and 20 hours * \(p<0.05\), ** \(p<0.01\).
5.7 Response to systemic infection with BRD509E

5.7.1 Bacterial load in peritoneal exudate cells and target organs

Saline- and paraoxon-treated mice were infected intraperitoneally with the attenuated *Salmonella* strain BRD509E (0.27x10^6) then bacterial load in the different organs and compartments were determined at different time points. Being the site of infection, bacteria recovered from the peritoneal cavity (Fig. 28A) by peritoneal lavage were \( \sim 7.5 \pm 5.8 \times 10^3 \) CFUs/ml in control group and \( \sim 5 \pm 1.3 \times 10^3 \) CFUs in paraoxon-treated group after 2 days of infection. At day 7 post-infection bacteria recovered from the peritoneal cavity were maintained to \( \sim 7 \pm 3.3 \times 10^3 \) CFUs/ml in control group while in the experimental group, a reduction of 38% (\( \sim 3 \pm 0.9 \times 10^3 \) CFUs/ml) in comparison to day 2 was observed. No statistical differences between control and experimental groups were found at any time point. To confirm these results, bacteria recovered from the peritoneal cavity were also expressed as CFUs/1x10^6 (Fig. 28B). Results confirmed no differences between the two groups with a discrete reduction in the bacterial load by day 7 post-infection in paraoxon-treated group.

Then, bacterial load in the spleen of infected animals was estimated. At day 2 post infection, bacterial load in the total spleen of control infected animals was \( \sim 1.8 \pm 0.4 \times 10^4 \) CFUs (Fig. 28C). In contrast, bacterial load in total spleen from paraoxon-treated animals was \( \sim 7 \pm 2.7 \times 10^4 \) CFUs, at day 2 post-infection, which was significantly lower than in the control group (Fig. 28D). At day 7 post-infection, bacterial load in the total spleen was increased in both groups, to \( 5 \pm 1.4 \times 10^5 \) CFUs (2.8-fold increase) in control group and to \( \sim 2.7 \pm 0.8 \times 10^5 \) (3.9 fold increase) in paraoxon-treated group. At
this time point the differences in the spleen bacterial load between the two groups were not significant.

Finally, bacterial load in the liver was also determined. After 2 days of infection, the liver of control animals showed a bacterial load of \( \sim 2 \pm 1 \times 10^5 \) CFUs/g which slightly increased to \( \sim 2.4 \pm 0.9 \times 10^5 \) CFUs/g at day 7 post-infection (Fig. 28E). Similarly, liver of paraoxon-treated group showed, at day 2 post-infection, \( \sim 1.5 \pm 0.5 \times 10^5 \) CFUs/g bacterial load and \( 1.6 \pm 0.4 \times 10^5 \) CFUs/g) at day 7 post-infection. No significant differences were found between the two groups at any time point.
Figure 28: Bacterial load in PECs and target organs after systemic infection with BRD509E.

Mice pre-treated with paraoxon or saline for 3 weeks were infected i.p. with BRD509E \(1.2 \times 10^5\). Mice were sacrificed at the indicated time points. Peritoneal fluid retrieved and bacterial load was determined as CFUs/ml volume (A) and CFUs/\(1 \times 10^6\) cell (B). Spleen and liver homogenates were prepared, aliquots were plated and bacterial load were determined. Spleen total CFUs (C), spleen CFUs/mg (D) and liver CFUs/g (E) were determined. Depicted are the mean values ±SD of data pooled from 2 independent experiments. Asterisks denote statistically significant differences between control and experimental groups \(*p<0.05\).
5.7.2 Spleen size

Spleens from infected saline-treated and paraoxon-treated animals were obtained at day 2 and 7 post-infection. Spleen weights from the two different groups were compared at the two different time points. At day 2 post-infection, the average weight of spleens from saline treated animals was $230 \pm 10 \text{mg}$ which it was significantly larger than the average size spleen from the paraoxon-treated group ($180 \pm 9 \text{mg}$) (Fig. 29). This smaller size spleen correlates with the lower bacterial burden (Fig. 28C) in the experimental group at day 2 post-infection. At day 7 post-infection, splenomegaly was observed in both groups with spleen weights reaching $>400 \text{mg}$.

![Figure 29: Splenomegaly after systemic infection with BRD509E.](image)

Mice pre-treated with paraoxon or saline for 3 weeks then infected i.p. with BRD509E ($1.2 \times 10^5$). Mice were sacrificed at the indicated time points and spleen weights were measured. Depicted are the mean values $\pm$SD of data pooled from 3 independent experiments. Asterisks denote statistically significant differences between control and experimental groups $** p<0.01$. 
5.7.3 Cellular phenotypic analysis

5.7.3.1 Peritoneal exudate cells

Next, it was needed to evaluate whether paraaxon pre-treatment can modify the phenotype of the macrophages present in the peritoneal cavity and, therefore, the response to a systemic infection. Peritoneal lavage was collected after 2 and 7 days of i.p. infection (with BRD509E strain) from saline- and paraaxon-treated mice. The percentages of various cell populations at day 2 and 7 post-infection are depicted in figure 30. Fig. 30A shows no significant differences between control and experimental groups in the recruitment of macrophages (F4/80+) to the peritoneal cavity: in both groups, the maximum recruitment took place at day 7 post-infection, when F4/80+ cells reached about 40% of the total peritoneal cells. It was also at day 7 when these cells were more activated (Fig. 30B) with no differences between the saline- and paraaxon treated groups. The percentage of CD11b+ cells other than macrophages (Fig. 30C) was reduced ~50% at day 7 post-infection in relation to day 2, probably due to the recruitment of macrophages indicated above.
Figure 30: Flow cytometric analysis of PECs after systemic infection with BRD509E.

Mice were treated for three weeks with paraoxon or saline. Afterwards, then infected i.p. with BRD509E (1.2x10⁵). Mice were sacrificed at the indicated time points and PECs were stained for flow cytometry. Bar graphs show F4/80⁺ (B), F4/80⁺/Sca-1⁺ (C) and CD11b⁺/F4/80⁻ (D). Graphs depicted the mean values ± SD of 3 mice/group from a representative experiment.
5.7.3.2 Spleen

Mice, previously treated with saline or paraoxon, were intraperitoneally infected with the bacteria strain BRD50E9. At day 2 and 7 post infection, mice were sacrificed and flow cytometric analysis was performed on spleen single cell suspension. The results of this analysis are presented as individual graphs depicting the percentages of resting (Fig. 31A) and activated Sca-1⁺ (Fig. 31B) T lymphocytes, and resting (Fig. 31C) and activated Sca-1⁺ (Fig. 31D) B lymphocytes from the total spleen pool. No significant differences were found between saline and paraoxon pre-treated mice in the percentage of total or activated lymphocytes at any time point. Graphs 30A and 30C showed that at day 2 post-infection, spleens contained ~25% of T cells and ~55% of B cells. At day 7 post-infection, the percentage of both types of lymphocytes decreased (~20% of total T cells and ~38% of total B cells) which indicates a recruitment of cells other than lymphocytes to the spleen. When the activation state of these cells was analyzed, it was observed that the percentage of activated T cells was around 15% at day 2 and 7 post-infection in both groups (Fig. 31B). On the other hand, the percentage of activated B was ~30% at both days in saline and paraoxon pre-treated mice (Fig. 31D). So, difference in the response to systemic bacterial infection between control and paraoxon pre-treated mice was not detected.
Figure 31: Flow cytometric analysis of splenocytes after systemic infection with BRD509E.

Mice were treated for three weeks with paraoxon or saline. Afterwards, then infected i.p. with BRD509E (1.2x10^3). Mice were sacrificed at the indicated time points and erythrocytes-depleted splenocytes were stained for flow cytometry. Bar graphs show percentages of CD3^+ (A), CD19^+ (B), CD3^+/Sca-1^+ (C) and CD19^+/Sca-1^+ (D) Graphs depicted the mean values ±SD of 3 mice/group from a representative experiment.
5.7.4 Cytokine production

In order to assess the responsiveness to infection of the peritoneal exudate cells, peritoneal lavage from control and experimental groups was collected at day 2 and 7 post-infection. PECs were cultured for 24 hours without further stimulation. Culture supernatants were collected and assessed for nitric oxide and IL-2p40 and IL-6 cytokines. Fig. 32A shows minimum production of nitrites (<20μM) at day 2 post-infection in both control and paraoxon-treated PECs, which slightly increased at day 7 with no significant differences between the groups. When the kinetics of IL-12p40 and IL-6 production were analyzed (Fig. 32B and C), the data showed that at day 2 post-infection, PECs from both groups produced ~800pg/ml of IL-12p40 and ~1500pg/ml of IL-6. These levels were reduced after 7 days of infection (~200pg/ml IL-12p40 and ~1000pg/ml IL-6) in both groups probably due to the migration of the bacteria to systemic organs. Differences between paraoxon and control group showed to be not significant at both time points.
Figure 32: Functional response of PECs after systemic infection with BRD509E.

Infected mice previously treated with paraoxon or saline for 3 weeks were sacrificed at the indicated time points. PECs were retrieved and cultured. 48 hours supernatants were assayed for nitrite content by the Griess method (A). 24 hours supernatants were assayed for IL-12 (B) and IL-6 (C) by ELISA. Depicted are the mean values ±SD of data pooled from 2 independent experiments.

Furthermore, the functional ability of the spleen cells was assessed by the production of nitric oxide and IL12p40, IL-6 and IFN-γ cytokines. Spleen single cell suspensions were prepared from infected animals pre-treated with saline or paraoxon. Cells were cultured for 24 hours when supernatants were collected. At day 2 post-infection, nitrates
production by spleen cells (Fig. 33A) was <10μM in both groups and by day 7 after infection the production increased to ~30μM in both groups. When the production of IL-12p40 pro-inflammatory cytokine was investigated (Fig. 33B), spleens from control and experimental animals exhibited a 4-fold increase from day 2 to day 7, from 200 to 800-900pg/ml. The increase in the production of IL-6 (Fig. 33C) was shown to be around 10 folds (from 40 to 300-400pg/ml) higher at day 7 than at day 2 post-infection in both, saline and paraoxon-treated groups. Finally, the production of IFN-γ, an essential T cell cytokine in the immune response to Salmonella was measured. As it has been described for IL-12p40 and IL-6, spleen cells from both groups produced more IFN-γ after 7 days of infection than at day 2 post-infection (Fig. 33D). Spleens from saline-treated mice produced 92 ± 62pg/ml of IFN-γ at day 2 post-infection and 140 ± 70pg/ml after 7 days of the infection. Spleens from paraoxon-treated group at day 2 post-infection produced 30 ± 0.1pg/ml of IFN-γ and around 500 ± 200pg/ml at day 7 post-infection which represent 16 fold increase in the production of this cytokine in paraoxon-treated group. Due to the high variability among the individuals within each group, the differences between the groups were not significant at any time point.

In general, spleen cells produced more cytokines and nitrates after 7 days of systemic infection which correlates with splenomegaly and the higher bacterial load.
Figure 33: Functional response of spleen cells after systemic infection with BRD509E.

Infected mice previously treated with paraoxon or saline for 3 weeks were sacrificed at the indicated time points. Spleens were collected. Spleen single cell suspensions were cultured. 48 hours supernatants were assayed for nitrite content by the Griess method (A). 24 hours supernatants were assayed for IL-12 (B), IL-6 (C) and IFN-γ (D) by ELISA. Depicted are the mean values ±SD of data pooled from 3 independent experiments.
5.7.5 Kinetics of fecal IgA and bacterial load

Fecal immunoglobulin A (IgA) was measured after intraperitoneal infection with BRD509E \( (1.4 \times 10^5) \), an attenuated strain of *Salmonella*, at different time points by ELISA. Fecal *Salmonella* specific IgA was followed weekly for 91 days after the infection. Figure 34A shows that IgA was progressively increasing in the feces during the first 37 days after infection, when IgA levels reached the maximum and maintained constant until day 79 when they started declining. It can be observed in Fig. 34A that, although both groups exhibited similar pattern in the production of IgA, during the first 35 days the levels of IgA produced by infected paraoxon-treated animals is higher than in the infected saline group. However, no significant differences were detected between the two groups.

Fecal CFUs were also determined weekly during the first month after the systemic infection. Fecal CFUs were detected in only some of the animals at all time points and by day 29 all the animals were negative for fecal CFUs (Fig. 34B).

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**Figure 34: Kinetics of fecal IgA and bacterial load following systemic infection with BRD509E.**

Mice were treated with paraoxon or saline for 3 weeks then infected i.p. with BRD509E bacterial strain \( (1.4 \times 10^5) \). Feces were collected at the indicated time points for determining fecal IgA levels (A) and fecal bacterial load (B).
5.8 Summary of effect of paraoxon on the immune response to systemic infection

- Systemic infection with SL1344 (virulent strain):
  - Paraoxon treatment did not show any effect on mice survival or bacterial load in the different sites.
  - IL-12 production was moderately higher in paraoxon group at 2 hours post-infection in both serum and spleen cells.

- Systemic infection with BRD509E (attenuated strain):
  - Paraoxon-treated mice had a significantly lower bacterial load in the spleen at day 2 post-infection accompanied by smaller spleen size in comparison to saline-treated mice.
  - At day 7 post-infection both groups showed similar bacterial load in the different sites.
  - No differences were observed in cytokines and nitric oxide production or cell phenotype and activation status in splenocytes and PECs in both groups at both time points.
5.9  **Response to an oral infection with SL.1344**

5.9.1  **Bacterial load in the different organs**

Mice were treated with saline or paraoxon for three weeks, and then infected by oral inoculation with the virulent *Salmonella* strain SL.1344. Mice were sacrificed at day 2, 4 and 7 post-infection, and spleen, liver and MLN aseptically removed. Bacterial load was estimated in the different organs and fecal pellets.

At day 2 post-infection, bacterial load in the spleen, liver and MLN were undetectable. However, bacterial shedding was detected in the fecal pellets of some mice which were 0.2 CFUs/mg in the control group and 0.1 CFUs/mg in paraoxon-treated group. (Fig. 35A)

At day 4 post-infection, bacterial load in only two mice, one from each group, was detected in the spleen, liver, and fecal pellets and were less than 60 CFUs/mg (Fig. 35B). Bacteria were not detectable in the Peyer’s patches (PP) or MLN of any mouse. Up to this point paraoxon pre-treated mice did not show any significant differences in comparison to control group.

However, massive expansion in the bacterial growth was observed 7 days after infection (Fig. 35C). Control group presented a bacterial burden of $25 \times 10^3$ CFUs/mg in the spleen, $5 \times 10^3$ CFUs/mg in the liver, $3 \times 10^3$ CFUs/mg in the MLN and around 100 CFUs/mg in fecal pellets. On the other hand, paraoxon-treated mice showed a significantly lower bacterial burden in spleen (300 CFUs/mg 83 folds less), liver (30 CFUs/mg 16 folds less), MLN (1 CFUs/mg 3000 fold less) and feces (less than 1 CFU/mg 3 fold less) than control mice. All the mice in the control group showed bacteria in their organs:
however, only 66% mice from the paraoxon-treated group contained bacteria in their organs.

In Figure 35D summarizes the kinetic of bacterial dissemination to the different organs after oral infection.

Figure 35: Reduction in the bacterial load in target organs of paraoxon pre-treated mice after 7 days of oral infection.

Mice were treated with saline or paraoxon for 3 weeks and, then, infected orally with SL1344 ($10^4$). Mice were sacrificed and organs homogenates aliquots were plated to determine bacterial load. Bacterial loads were determined as CFUs/mg in spleen, liver, MLN and feces at day 2 (A), 4 (B) and 7 (C) post infection. (D) Shows the change in bacterial load in the different organs over the course of infection. Graphs depicted the mean values ±SD of data from 2-3 independent experiments. Asterisks denote statistically significant differences between control and experimental groups * $p<0.05$, **$p<0.01$, ***$p<0.001$. Statistical analysis was done using Mann-Whitney test. UD: un-detectable.
5.9.2 Spleen and mesenteric lymph nodes size

Spleen and MLN from infected saline- and paraoxon-treated mice were obtained and weighted at day 2, 4 and 7 post-infection. After 2 days of infection, spleens from both groups presented a normal weigh. By day 4 post-infection a non-significant increase in the size of paraoxon-treated animals was observed. It was at day 7 post-infection when spleens from saline-treated animals showed a 2 fold increase while paraoxon-treated mice showed only 1.3 fold increase. The weigh difference between the two groups was statistically significant (p<0.01) (Fig. 36A). The splenomegaly observed at day 7 in the saline group correlates with the high bacterial load found in these mice.

When the MLN weigh was analyzed at the three time points, in the control and experimental group, no differences were found between the two groups. Moreover, MLN size remained unchanged at all times points regardless of the bacterial load in the organ (Fig. 36B)

![Figure 36: Changes in spleen and MLN following oral infection.](https://via.placeholder.com/150)

Mice were treated with Paraoxon or saline for three weeks. Mice were then infected orally with a dose of $1 \times 10^4$ CFUs/mouse of SL1344. Then at day 2, 4 and 7 post infection spleen weights (A) and MLN (B) were determined. Depicted are the mean values ±SD of data pooled from 2-3 independent experiments at each time point. Asterisks denote statistically significant differences between control and experimental groups **p<0.01.
5.9.3 Spleen and MLN cell population and activation status

Single cell suspensions of spleen and MLN were prepared from orally infected mice, pre-treated with saline or paraoxon, and sacrificed at day 7 post-infection. The different cell populations were analyzed in both organs by flow cytometry. Splenocytes from non-infected mice were used as control. As shown in figure 37, uninfected spleens were constituted by 38 ± 1% CD3+ T cells, 41 ± 5% CD19+ B cells and 15 ± 2% CD11b+ cells, additionally spleens contained other cells like NK and dendritic cells in much lower percentage. The majority of those cells were negative for the activation marker Sca-1. When the spleen cell populations, from saline pre-treated mice, were analyzed a decrease in the percentage of CD3+ and CD19+ cells (17 ± 6% and 43 ± 4.3%, respectively) compared to the unstimulated group, and dramatic increase in the percentage of CD11b+ cells (35 ± 2.6%) was shown (Fig. 37). Moreover, the three cell populations had an increase in the expression of Sca-1, as 71% of CD3+ cells, 98% of CD19+ cells and 31% of CD11b+ cells were Sca-1+.

Interestingly, the analysis of the splenic cell population in infected paraoxon-treated mice (Fig. 36) showed no significant changes, compared to the non-infected mice, in the percentages of the different populations (40 ± 2.5% CD3+, 49 ± 4% CD19+ and 8 ± 1% CD11b) or in the expression of Sca-1 as 15% of CD3+ cells, 2% of CD19+ cells and 1% of CD11b+ cells were Sca-1+. 
The graphs in figure 37 depicted the average percentages of the different spleen cell populations (Fig. 38A) as well as their Sca1 expression (Fig. 38B). Additionally, the same results were expressed as number of cells/mg of spleen (Fig. 38C-D) which confirmed that the level of infection in spleens from paraoxon-treated group was very low or even did not exist in some of these animals.

Figure 37: Flow cytometric analysis of spleen population and the activation status.

Mice were treated for three weeks with paraoxon or saline. Mice were then infected orally with a dose of $1 \times 10^4$ CFUs/mouse of SL1344. At day 7 post infection erythrocytes-depleted splenocytes were stained for flow cytometry. Representative dot plots show positive cell percentage of CD3, CD19, CD11b, CD3/Sca-1, CD19/Sca-1 and CD11b/Sca-1.
Mice were treated with saline or paraoxon for 3 weeks and, then, orally infected with SL1344 (10^3). 7 days after infection, mice were sacrificed, spleen was collected and single cell suspension was stained for flow cytometry. Graphs depict the spleen total cell population percentages (A), spleen cells activation markers percentages (B), total population cell numbers (C) and activation markers cell numbers (D). Graphs depicted the mean values ±SD of data from a representative experiment. Asterisks denote statistically significant differences between groups * p<0.05, **p<0.01.

Then, the characteristics of each population were analyzed in detail. First, the two subpopulations of T cells were studied. Un-infected mice and infected paraoxon-treated mice showed similar percentages of CD4^+ (26 ± 1% vs 30 ± 0.5%) and CD8^+ (12% in both groups) cells (Fig. 39), out of the total cell population, with also similar expression of CD25 and CD69 activation markers: around 8% CD4^+ CD69^+ and CD4^+ CD25^+; on CD8^+ cells <1% were CD69^+ and <6% were CD25^+. However, in infected saline-pre-treated mice, the percentage of CD4 and CD8 populations were much lower, 10 ± 5.5% and 5 ± 1.8%, respectively, than in the other two groups which correlated with the lower
number of T cells previously described. On CD4+ cells the percentage of cells expressing CD69 was 19% (2 fold higher than in the other two groups), but no differences on the percentage of CD4+CD25+ cells were found. The percentage of CD8+ cells expressing CD69 and CD25 were similar to the un-infected and infected paraoxon-treated groups (Fig. 38). In figure 40 A-C, average of the percentages of these two subpopulations have been represented.

Figure 39: Flow cytometric analysis of spleen T cells and their activation markers at day 7 post-infection.

Mice were treated for three weeks with paraoxon or saline. Mice were then infected orally with a dose of $1 \times 10^4$ CFUs/mouse of SL1344. At day 7 post infection erythrocytes-depleted splenocytes were stained for flow cytometry. Representative dot plots show positive cell percentage of CD3/CD4, CD3/CD8 and their activation markers CD25 and CD69.
Figure 40: T cells subpopulations and their activation markers in spleen at day 7 post-infection.

Mice were treated with saline or paraoxon for 3 weeks and, then, orally infected with SL1344 \( (10^4) \). 7 days after infection, mice were sacrificed, spleen was collected and single cell suspension was stained for flow cytometry. Graphs depict T cells subpopulation percentages (A), CD8 activation markers percentages (B) and CD4 activation markers percentages (C). Graphs depicted the mean values ± SD of data from a representative experiment.

CD11b\(^{+}\)F4/80\(^{+}\) (macrophages) population was further analyzed for the expression of CD40, CD86 and MHCII surface markers. The percentage of CD11b\(^{+}\)F4/80\(^{+}\) cells was around 4% in the un-infected and infected paraoxon-treated groups, all of them were negative for the expression of CD40, around 40% were CD86\(^{+}\) and around 15% were MHCII\(^{+}\). In the infected saline-treated group, CD11b\(^{+}\)F4/80\(^{+}\) cells were around 9% (Fig. 41) of the total spleen population. Moreover, 35% of them expressed CD40, 74%
expressed CD86 and 46% were MHC II⁺ indicating that CD11b cells were highly activated (Fig. 41). In figure 42 are depicted the percentage average of each one of the subpopulation of CD11b⁺/F4/80⁺ based on the activation markers.

Figure 41: Flow cytometric analysis of splenic myeloid cells and their activation markers at day 7 post-infection.

Mice were treated for three weeks with paraoxon or saline. Mice were then infected orally with a dose of 1x10⁴ CFUs/mouse of SL1344. At day 7 post infection erythrocytes-depleted splenocytes were stained for flow cytometry. Representative dot plots show positive cell percentage of CD11b/F4/80 and their activation markers CD40, CD86 and MHC II.
Next, the cell populations in the MLNs were analyzed. Figure 43 depicted the dot plots of one representative mouse in each group (un-infected, infected saline-treated and infected paraoxon-treated). The percentages of the three main cell populations in un-infected MLN were 74% CD3+ T cells, 33% CD11b+ B cells and 2% CD11b+ cells. MLNs from infected mice (saline-and paraoxon-treated) presented similar percentages in those cell populations (Fig. 43). However, when Scal expression was analyzed, the majority of the cells in MLNs from infected saline-treated mice were activated as 70% of CD3+, 95% of CD19+ and 67% of CD11b+ were Scal+ unlike the cells in the un-infected and paraoxon-treated mice (Fig. 43). The cell percentages calculated from the flow cytometry are represented in Fig. 43A-B and they were in agreement with the result represented with the absolute cell number of each cell population (Fig. 44C-D).
Figure 43: Phenotypic analysis and activation markers of total MLN population at day 7 post-infection.

Mice were treated for three weeks with paraoxon or saline. Mice were then infected orally with a dose of $1 \times 10^4$ CFUs/mouse of SL1344. At day 7 post infection MLN cells were stained for flow cytometry. Representative dot plots show positive cell percentage of CD3, CD19, CD11b, CD3/Sca-1, CD19/Sca-1 and CD11b/Sca-1.
Mice were treated with saline or paraoxon for 3 weeks and, then, orally infected with SL1344 (10^4). 7 days after infection, mice were sacrificed, spleen was collected and single cell suspension was stained for flow cytometry. Graphs depict MLN total cell population percentages (A), activation markers percentages (B), total population cell numbers (C) and activation markers cell numbers (D). Graphs depicted the mean values ±SD of data from a representative experiment.

A further analysis of the CD3 population (Fig. 45) revealed no differences in the percentages of CD4^+ and CD8^+ cells or their activation status, represented by the expression of CD69 and CD25. Figure 46(A-C) depicted the average cell percentages in each group.
Mice were treated for three weeks with paraoxon or saline. Mice were then infected orally with a dose of $1 \times 10^4$ CFUs/mouse of SL1344. At day 7 post infection MLN cells were stained for flow cytometry. Representative dot plots show positive cell percentage of CD3/CD4, CD3/CD8 and their activation markers CD25 and CD69.
Figure 46: MLN T cell population and their activation markers at day 7 post-infection.

Mice were treated with saline or paraoxon for 3 weeks and, then, orally infected with SL1344 ($10^4$). 7 days after infection, mice were sacrificed, spleen was collected and single cell suspension was stained for flow cytometry. Graphs depict T cells sub-population percentages (A), CD8 activation markers percentages (B) and CD4 activation markers percentages (C). Graphs depicted the mean values ±SD of data from a representative experiment.
5.9.4 Cytokine production

Next, the levels of IL-12 pro-inflammatory cytokine in the serum of un-infected and infected animals, pre-treated with saline or paraoxon, at day 2, 4 and 7 post-infection were investigated (Fig. 47A). IL-12p40 concentration in the serum of un-infected mice was ~500 pg/ml. After 2 and 4 days of infection IL-12p40 concentration increased to ~800 pg/ml (1.6 fold increase), in both; saline and paraoxon-treated groups. At day 7 post-infection, the levels of IL-12p40 increased to ~1500 pg/ml (3 folds increase) in the saline-treated group, but not in the paraoxon-treated mice where the levels remained below 1000 pg/ml.

The capacity of splenocytes to produce cytokines (IL-12p40, IL-6, IL-10 and IFN-γ) was also investigated. At day 7 post-infection, splenocytes from infected mice, pre-treated with either saline or paraoxon, were cultured without any stimulation. After 24 hours, supernatants were collected and assayed for the different cytokines. Results showed (Fig. 47B-C) in general, a lower production of cytokines in the paraoxon-treated mice than in the saline-treated group although no significant differences were observed on the IL12p40 (76 ± 30 pg/ml vs 159 ± 80 pg/ml), IL-6 (20 ± 3 pg/ml vs 88 ± 50 pg/ml) and IL-10 (162 ± 120 pg/ml vs 310 ± 125 pg/ml) production. However, the production of IFN-γ by splenocytes from the saline-treated group was significantly higher (6.6 folds) than in the paraoxon-treated group (128 ± 60 pg/ml vs 851 ± 315 pg/ml).
Figure 47: Cytokines produced in serum and by splenocytes of Salmonella infected mice.

Mice were treated with saline or paraaxon for 3 weeks and, then, infected orally with SL1344 (10^4). Serum IL-12p40 was measured at indicated time points (A). 7 days after infection, mice were sacrificed and single spleen cell suspensions cultured. Culture supernatants were collected and tested for IL-12p40 and IL-6 (B) and IL-10 and IFN-γ (C). Graphs depicted the mean values ±SD of data from 2 independent experiments. Asterisks denote statistically significant differences between control and experimental groups * p<0.05.
5.9.5 IgA estimation in fecal pellets and determination of bacterial load

Fecal pellets were collected at the end of the three weeks treatment with saline or paraoxon before oral inoculation with virulent SL1344 and at different days after inoculation. Total fecal IgA content was estimated before infection and at day 4 and 7 post-infection. In figure 48A it is shown that the levels of total un-specific IgA did not change during the infection in any of the two groups for which we can conclude that paraoxon-treatment did not enhance the production of total IgA at any time point.

Additionally, specific anti-Salmonella IgA antibody was estimated after 4 and 7 days of oral infection (Fig.48B). The results showed un-detectable levels of anti-Salmonella IgA at day 4 post-infection in saline- as well as in paraoxon-treated mice. By day 7 post-infection, anti-Salmonella IgA antibody were detectable in both groups. Paraoxon group showed higher levels than the saline but the differences were, again, not significant.

Next, the effect of the inhibition of the AChE on the clearance of live bacteria at the level of the intestine, before it disseminates to the systemic organs which, could somehow protect the mice from a lethal infection was elucidated. After infection by oral inoculation of SL1344, feces were collected at different time points up to day 10 post-infection.

Early during the infection, at days 2 and 4 fecal, CFUs were very low (lower than 0.1 CFUs/mg) in both groups with no significant differences between them. At day 7 post-infection control group showed a massive increase in the fecal CFUs reaching 100 CFUs/mg, by day 10 post-infection CFUs were increased by 10 folds reaching around 900 CFUs/m (Fig. 48C). However, at day 7 post-infection, mice from the paraoxon
group showed 50 times less fecal load (around 2 CFUs/mg) than the saline group, a
difference that was statistically significant. At day 10 post-infection, paraoxon-treated
mice increased their bacterial shedding to 67 CFUs/mg which represent 10 folds less
than control (Fig. 48C).

Figure 48: Kinetic of fecal bacterial load and IgA following an oral
infection with SL1344.

Mice were treated with paraoxon or saline for 3 weeks then infected
orally with SL1344 (1x10^4). Feces were collected at the indicated time
points for determining total fecal IgA levels (A), anti-Salmonella fecal
IgA levels (B) and fecal bacterial load (C). Depicted are the mean values
±SD of data pooled from 2-3 independent experiments at each time
point. Asterisks denote statistically significant differences between
control and experimental groups* p<0.05.
Results indicated that paraoxon treatment did not induce an increase in the bacterial shedding, on the contrary, feces from paraoxon-treated mice contained less bacterial load than the ones from the control group. Therefore, the lower systemic bacterial load could not be explained by a faster clearance of the bacteria.

5.9.6 In-vivo bioluminescent imaging

After oral inoculation of the mice with a bioluminescent strain of *Salmonella* (SL1344::lux), bacterial dissemination was followed by live imaging. In order to facilitate the visualization of the bacteria, mice were inoculated, after three weeks of treatment with saline or paraoxon, with high dose of SL1344::lux (1x10⁵ CFUs/mouse). Mice were then imaged at different time points until they succumbed to the infection, usually between day 0 and day 16 post-infection (Fig.49).

Initially, all the mice in both groups showed a localized distribution of the bacteria in the intestinal region. At day 7 post-infection some of the animals from the control group showed a rapid and massive dissemination of the bacteria to the systemic organs and all of them succumbed to the infection by day 9 after infection. On the contrary, paraoxon-treated mice showed a delayed dissemination of the bacteria to the systemic organs that started being apparent at day 8 (not shown). Moreover, some of the mice survived up to day 16 after infection.

These data suggested that a delay on the bacterial translocation at the level of the intestinal epithelium could be the key for the enhanced survival of infected mice pre-treated with the AChE inhibitor, paraoxon.
Figure 49: In-vivo bioluminescent imaging of *Salmonella* infected mice.

Following 3 weeks exposure to saline or paraoxon, mice were infected orally with $10^5$ *SL1344::lux*. Control (A) and paraoxon pre-treated (B) mice were imaged ventrally starting at 3h postinfection (D.0). Data are representative of 2 independent experiments (5 mice/group). Color scale ranges from 1,450 photons/sec/cm$^3$/sr (purple) to 176,000 photons/sec/cm$^3$/sr (red).
5.10 Summary of the effect of AChE inhibition on the immune response following oral infection

- At early time points following oral infection no differences were observed between paraoxon- and saline-treated mice in the bacterial load and serum IL-12.

- At day 7 post infection with SL1344:
  - Significantly lower bacterial load in MLN, spleen and liver in paraoxon-treated mice
  - Lower state of activation of splenocytes and MLN cells in paraoxon-treated mice
  - Lower production of inflammatory cytokines in paraoxon-treated mice

- BLI results showed a delay in systemic dissemination of the bacteria in paraoxon-treated mice.
5.11 Anti-microbial peptides in the intestinal epithelium

Next, it was decided to investigate whether paraoxon treatment has any effect on the intestinal mucosa as it is the first barrier that bacteria will encounter following an oral infection. Therefore, the expression of the anti-microbial peptides (AMPs) produced by Paneth cells in the small intestine was studied. The last part of the small intestine (ileum) was aseptically removed from mice treated for three weeks with either saline or paraoxon. Small intestine was also obtained from mice treated with saline or paraoxon and then infected (by oral inoculation) with SL1344 Salmonella strain (10⁴ CFUs/mouse), at day 4 post-infection.

The results showed that relative mRNA expression of AMPs like defensin 1, defensin 4, CR51C, CR54C, Ang-4 and the enzyme MMP-7 were almost identical in the intestinal cells from control and paraoxon-treated mice (Fig.50A-C), meaning that paraoxon treatment did not induce an up-regulation of this peptides. However, the expression of RegIII-γ was 3-folds higher in paraoxon-treated mice than in control animals, even though, the relative expression of this gene showed to be not significant (Fig.50C).

Figure 50D shows the fold increase in the expression of AMPs in the paraoxon-treated group in respect to their expression in the saline treated group.
Figure 50: Gene expression of anti-microbial peptides (AMPs) in epithelial cells of un-infected mice.

Mice were treated with paraoxon or saline for 3 weeks. Mice were sacrificed and intestinal epithelial cells were isolated. RNA was extracted from the isolated epithelial cells, converted to cDNA and ran for real-time PCR. Graphs depicted the mRNA relative expression each gene in both control and paraoxon groups (A-C) and fold change in the expression of AMPs of paraoxon in comparison to control (D). Depicted are the mean values ±SD of data pooled from 2 independent experiments for fold change and a representative experiment for mRNA relative expression graphs.

The expression of these genes was also analyzed in intestinal epithelial cells of infected mice after 4 days of infection. In control mice, the expression of defensin 1, defensin 4, MMP-7, RegIII-γ and Ang-4 were up-regulated by 6-folds, 2-folds, 3-folds, 3-folds and 6-folds respectively, but no major changes were observed in CRS1C and CRS4C expression after 4 days of oral infection in comparison to saline un-infected mice (Fig.51A). In figures 51B-C were represented the relative expression of each of these
peptides, before and after infection. Interestingly, paraoxon pre-treated mice showed
same pattern but with lower levels of induction as the expression of defensin 1, MMP-7,
and Ang-4 as they were up-regulated by 3-folds, 1.5-folds, and 3-folds, respectively.
However, no changes were observed in defensin-4, CRS1C, CRS4C and RegIII-γ
eexpression after 4 days of oral infection in comparison to paraoxon un-infected mice
(Fig.52A). In figures 52B-C were represented the relative expression of each of these
peptides, before and after infection.

Figure 51: Gene expression of AMPs in small intestine epithelial cells of infected
saline-treated mice.

Mice were treated with paraoxon or saline for 3 weeks. Intestinal epithelial cells
were isolated from control un-infected mice and after 4 days of oral infection. RNA
was extracted from the isolated epithelial cells, converted to cDNA and ran for real-
time PCR. Graphs depicted fold change in the expression of AMPs of control
infected in comparison to control un-infected (A) and the mRNA relative expression
each gene in both control infected and un-infected groups (B-D). Depicted are the
mean values ±SD of data pooled from 2 independent experiments for fold change
and a representative experiment for mRNA relative expression graphs. Asterisks
denote statistically significant differences between infected and un-infected groups* p<0.05.
Figure 52: Gene expression of AMPs in small intestine epithelial cells of infected paraoxon-treated mice.

Mice were treated with paraoxon or saline for 3 weeks. Intestinal epithelial cells were isolated from paraoxon un-infected mice and after 4 days of oral infection. RNA was extracted from the isolated epithelial cells, converted to cDNA and ran for real-time PCR. Graphs depicted fold change in the expression of AMPs of paraoxon infected in comparison to paraoxon un-infected (A) and the mRNA relative expression each gene in both paraoxon infected and un-infected groups (B-D). Depicted are the mean values ±SD of data pooled from 2 independent experiments for fold change and a representative experiment for mRNA relative expression graphs.
5.12 Morphological analysis of the intestinal mucosa

Finally, it was investigated whether paraoxon treatment induces any morphological changes at the level of the intestinal mucosa that could affect the translocation of bacteria from the intestinal lumen to the submucosa. Small intestine (ileum) was collected from saline and paraoxon-treated mice, and prepared for microscopic observation.

5.12.1 Light microscopy

Light microscopy examination of the small intestine (ileum) revealed the intestinal mucosa with villi and crypts. Villi were covered by columnar absorbing cells and goblet cells. At the end of the crypts of Lieberkühn, groups of Paneth cells were observed with their cytoplasm filled of abundant cytoplasmic granules. In figures 53A-B are shown two intestinal sections from two different saline-treated mice.

Tissue sections from paraoxon-treated mice revealed the same structure, with no obvious morphological changes. However, in some areas we could observe less number of granules in the cytoplasm of Paneth cells located in the crypts (Fig. 53C). Sometimes, some of these granules looked to be outside the cells. Moreover, the goblet cells seemed to also have their apical part opened to the lumen (Fig. 53D).
Figure 53: Ileum sections stained with Hematoxilin and Eosin.

Ileum sections stained with Hematoxilin and Eosin from saline (A-B) or paraoxon (C-D)-treated mice. Goblet cells (black arrows) can be seen between the columnar absorbing cells. Paneth cells (white arrows) at the bottom of the intestinal crypts contain a cytoplasm full of acidophylic granules. Areas with degraulated goblet cells and Paneth cells are seen in sections from the experimental group. Images were taken at x 400 magnification. L: intestinal lumen, MM: muscularis mucosa.
5.12.2 Electron microscopy

Mice treated for three weeks with saline or paraoxon were sacrificed and their small intestine aseptically removed. The terminal part of the small intestine (ileum) was processed for electron microscopy. Ultrastructural examination of the saline-treated mice intestinal epithelium showed the presence of columnar absorbing cells with apical microvilli and goblet cells. Goblet cells presented an apical part distended with large rounded mucous globules (Fig. 54A and B). However, in the paraoxon-treated group, intestinal goblet cells appeared almost depleted of the mucous globules that were released to the lumen of the intestine (Fig. 54C and D). This release appeared to be massive, leading to the disruption of the apical cytoplasmic membrane, the release of cytoplasmic fragments and cell death.
Figure 54: Transmission electron micrographs of small intestine (ileum) epithelium showing goblet cells.

Goblet cells in ileum sections from saline treated mice (A-B) and paraoxon treated animals (C-D). Goblet cells (arrow) in saline treated animals presented a distended apical part that contains large rounded mucous globules of moderate electron density. In contrast, goblet cells from paraoxon treated animals showed either an active degranulation (C) of their granules to the intestinal lumen (L) or complete degranulated cells (D). Original magnification of the micrographs is x4200.
Paneth cells, in the intestine of the saline group, were seen in groups at the bottom of the crypts of Lieberhuen. These cells were filled with large spherical electron dense granules (Fig. 55A) that in some cases were surrounded with clear halos (Fig. 55B). The basal part of these Paneth cells contained abundant layered endoplasmic reticulum, mitochondria and a nucleus with a prominent nucleolus (Fig. 55C). In the paraoxon group, Paneth cells were found less abundant and to have fewer electron dense granules of various sizes (Fig. 56A) compare to the saline group. Enteroendocrine cells were often seen in close proximity to Paneth cells (Fig. 56A). Additionally, numerous cells with enlarged endoplasmic reticulum and small granules were seen (Fig 56B). Moreover, cells with dilated endoplasmic reticulum and with no granules were observed (Fig 56B), these cells could be Paneth cells that had lost the electron dense secretory granules and the endoplasmic reticulum which was replaced by cytoplasmic vesicles. Additionally, mitotic figures were observed in the close vicinity of the Paneth cells (Fig. 56C) which could indicate the arrival of new cells that will differentiate into new Paneth cells or goblet cells.
Figure 55: Transmission electron micrographs of small intestine (ileum) epithelium showing goblet cells.

Electron micrograph of Paneth cells in the crypt base of the small intestine of saline treated mice. A. Their cytoplasm is filled with large spherical granules (white arrows) B. some of them surrounded by clear halos (red arrows). C. Higher magnification shows a Paneth cell with abundant endoplasmic reticulum (ER) in the proximity of the nucleus and
Figure 56: Transmission electron micrograph of the ileum epithelium of paraoxon-treated mice.

(A) Paneth cell (PC) containing electron dense spherical granules of different sizes (white arrows), next to a enteroendocrine cell (E) containing small electron dense granules. (B) group of cells in the intestinal cripts with enlarged endoplasmic reticulum (ER) and lack of granules. (C) Mitotic cells (M) can be observed approaching the intestinal epithelium. (L) Intestinal lumen, (N) nucleus, (GC) goblet cell.

Micrographs original magnification: (A) x8200, (B) x4800 and (C) x2900.
Chapter 6: Discussion

Previous studies in our laboratory have shown that inhibition of AChE acts on the immune system in an immune-regulatory fashion, and confers protection against lethal Gram-negative bacterial infection (43). The aim of this study is to elucidate the mechanism by which AChE inhibition and, therefore, an increase in the levels of ACh, enhanced the survival of infected mice.

In all our experiments, the effectiveness of the paraoxon was monitored by measuring the activity of AChE in blood during the administration period. In every experiment, the AChE activity was inhibited by 45-50% which correlated with a slower growth of the mice as we have previously reported (43, 98).

First we investigated the effect that subchronic administration of paraoxon could have on the main lymphoid organs involved in the immune response and that could influence the body response to a bacterial infection. So, we started by analyzing the spleen, as an essential secondary lymphoid organ, and the mesenteric lymphoid node (MLN) important for the immune response to gastro-intestinal infections. Additionally, PECs were also investigated as the daily administration of paraoxon took place through the intraperitoneal route.

Our data showed no major influence of AChE inhibition pre-treatment on the spleen weights or viable cell counts although weights and counts tended to be lower in paraoxon pre-treated mice. This observation was more significant in the PECs cell counts as they were significantly reduced by one third in paraoxon pre-treated mice. These observations were accompanied with no change neither in spleen nor PECs
phenotypic analysis. In-vitro studies on murine EL.4 T cell line had shown that paraoxon, induced toxic effects characterized by disruption of the mitochondria transmembrane potential, release of cytochrome C and activation of caspases (93,94). Therefore, the reduction in viable cell counts in PECs, and to lesser extend in spleen, could be a result of the induced apoptosis by paraoxon. The fact that cells phenotype was not affected in paraoxon pre-treated mice could indicate that the apoptosis is not selective and does not target any specific cell type.

In addition, AChE inhibition did not affect the spleen cells phenotype or their activation status which correlated with no changes on pro-inflammatory cytokines production, at protein levels as well as gene expression, from un-stimulated spleen cells. Moreover, splenic macrophages and T cells from paraoxon-treated animals did not showed any delay or inhibition on the production of pro-inflammatory cytokines after in vitro stimulation. However, we observed a moderate but not significant increase in the production of IL-12 after LPS stimulation and IFN-γ in ConA stimulated cells. Several studies had addressed the effect of AChE inhibitors on the immune system using different inflammatory models and different treatments, and not always with the same outcome which makes difficult to extract a definitive conclusion. Our results were in agreement with a study in which the AChE inhibitor pyridostigmine bromide, administered for 28 days, reported no effect on the humoral immune response but it had a moderate effect on splenic T cells response to ConA stimulation, experimenting a moderate increase in the production of IL-2 and IFN-γ (91). In another study, the i.p. administration (3 times a week for five weeks) of the AChE inhibitor dimethoate showed no effect on the normal response of immune cells of the brain toward LPS
stimulation, although the compound on its own resulted in an increase of TNF-α and IL-6 mRNA expression (92).

As mentioned earlier, in our model, AChE inhibition did not have an inhibitory effect on the immune response to mitogens. However, we found that it does have an effect on the CD11b⁺ splenic cells showed by a significant enhancement in their phagocytic activity. Similar finding was demonstrated in a study on peritoneal and intestinal macrophages which showed an increase in their phagocytic activity after being stimulated with nicotine and ACh in-vitro (117).

Furthermore, our results showed that paraoxon pre-treatment did not have any effect on the MLNs weights, cell viability or cells phenotype. However, we found an increase in the number of Sca-1⁺ among the CD8 cells as well as an increase in the expression of CD25 following AChE inhibition treatment. It is known that IL-2 receptor (CD25) respond in a autocrine pattern to IL-2 produced by activated T cells and induce cell proliferation (118) and that Sca-1 (Ly-6A/E) expression is up-regulated in response to IFN-γ and TNF (119). Therefore, both markers are involved in the activation of T cells, thus our data suggests that AChE inhibition resulted in an increase in the activation status of MLN T cells.

Phenotypic analysis of the peritoneal cavity cells revealed no changes after treatment with AChE inhibitor. However, following in vitro LPS stimulation, B1 and B2 cell populations showed to have a partially impaired induction of CD40 expression. In B cells, CD40 stimulation is involved in activation, proliferation, isotype switching, germinal center formation and memory cell generation (120). It has been demonstrated
that, in B cells, \( \alpha 7 \) and \( \alpha 9(\alpha 10) \) nAChRs are coupled to CD40, and stimulating \( \alpha 7 \) nAChR had an inhibitory effect on CD40-induced proliferation (24). Thus, the reduced induction in the CD40 expression on B cells could be related to the fact that ACh is activating nicotinic receptors on B cells and resulting in less activation of these cells mediated by a reduction in the percentage of CD40\(^+\) B cells. Like the spleen, PECs of paraoxon pre-treated mice showed a moderate increase in the production of IL-12 after LPS stimulation but not in un-stimulated cells.

It is well known that ACh, the main parasympathetic neurotransmitter, stimulates gut functions as acid production and intestine motility (121). Thus in our study, an increase in the intestine motility could be a mechanism by which bacteria is eliminated from the intestinal lumen, not allowing the bacteria to attach to the epithelium and, consequently resulting in the improved survival. Our results showed that after the three weeks of treatment with the AChE inhibitor paraoxon, there was no enhancement of the intestinal motility and, therefore, ruled out the possibility of decreased contact time between bacteria and intestinal epithelium and increased bacterial elimination from the lumen. Several studies showed that AChE inhibitors causes and increase in the gastrointestinal motility. This effect was proven in an \textit{in-vitro} study on rat forestomach (122) and others \textit{in-vivo} in conscious dogs (123) and anesthetized rabbits (124). The difference between our study and those studies is that the measurements were taken within minutes or few hours of the administration of the AChE inhibitor, while in our study we measured the motility after 48 hours of the last injection of paraoxon. This could indicate that paraoxon if it has any effect on the intestinal motility, by 48 hours post injection that influence can’t be detected.
Next, we wanted to evaluate the effect of AChE inhibition pre-treatment on the immune response induced by a systemic infection. First, we assessed the immune response to a systemic infection with virulent bacteria SL1344. SL1344 is a very virulent bacterium that will kill the host within few days, so in these experiments we checked for early immune responses along with the bacterial burden in the host body. Our data showed that AChE inhibition did not have a significant impact on the host survival which was correlated with the fact that bacterial burden in peritoneal cavity, spleen and liver at 2 and 20 hours post-infection were similar in pre-treated and non-treated groups. These observations were accompanied by no influence of paraoxon on the cell viability in PECs and spleen and IL-12 production in serum and spleen cells. So, we thought that this systemic infection with virulent SL1344 was masking any influence of paraoxon pre-treatment on the host immunity and decided to pursue a systemic infection using an the attenuated strain *AroA'*/AroD' mutant *Salmonella* BRD509E.

BRD509E *Salmonella* strain lacks the ability to biosynthesize aromatic compound that are necessary for the bacterium ability to multiply and, therefore, has a slower multiplication rate (115). This is a well established model, where bacteria load in the host increases and reach its peak by 7-10 days after infection and then the host is able to contain the infection and clear the bacteria within 20-30 days after infection (115). Our results showed that paraoxon pre-treatment had a mild influence over the host bacterial burden at early time point (day 2) only, as paraoxon pre-treated group had a lower bacterial burden, in general, and more significantly in the spleen which was correlated with lower spleen weights in comparison to control group. This difference was not observed in the later time point (day 7) when both groups had comparable bacterial
burden and experimented an increase with respect to early time point (day 2). Despite the lower bacterial burden at day 2, our treatment did not seem to have any effect on the PECs and spleen cells: cell viability, cytokine production, cell phenotype or activation markers expression. However, we could explain the lower bacterial burden in the spleen at early time point, by the enhanced phagocytic activity of splenic macrophages and the moderate increase in IL-12 and IFN-γ production induced by the AChE inhibition. This way, bacteria entering the peritoneal cavity of paraoxon-treated mice could encounter stronger macrophages that would limit, initially, their systemic dissemination. Additionally, bacteria arriving to the spleen would also find a more hostile environment that would limit their growth. This early modest control can’t influence the immunity through the whole course of infection, thus lose its effect at later time point (day 7). We can speculate that this initial control of the bacterial infection is, probably, lost due to the lack of ACh stimulation during the infection.

Few studies had demonstrated that stimulation of the inflammatory reflex had protective effects in sterile sepsis model and different inflammatory diseases models by inhibiting the production of pro-inflammatory cytokines thus reducing inflammation (28,35,39,40,125). However, the effect of this pathway in live bacterial infection is controversial as in some cases appears to fail to protect the host and to clear the bacteria (36). Pro-inflammatory cytokines and neutrophils recruitment are of importance to protect the host and clear the bacterium in an infection model; therefore, inhibition of the inflammatory response would cause a retarded immune response and result in increased bacterial load in the organs, failure to clear the bacteria and subsequently worsen the survival of mice. In a mouse model where peritonitis was induced by the
injection of *E. coli*, nicotine administration induced the stimulation of the inflammatory reflex that caused a reduction in the production of inflammatory cytokines such as IL-6, TNF-α and IL-1β but, these animals had increased their bacterial load in the organs and consequently worse host survival in comparison to control (36). However, when α7 nAChRs knock out mice were used in this model, nicotine effect was reversed and bacterial load in these mice was reduced (41). These results are in agreement with our own data where we had shown that cholinergic stimulation did not improved the immune response to a systemic infection. However, we have to point out the fact that paraoxon is an AChE inhibitor and, therefore, increases the Ach levels that, in turn, will stimulate both, nicotinic and muscarinic ACh receptors. A recent study had shown that the stimulation of the M3 muscarinic AChR (mAChR) can enhance Th1 responses by increasing the production of IFN-γ from CD4 T cells and improve mice survival in a systemic attenuated *Salmonella* infection (44).

Moreover, it has been shown that ACh and norepinephrine have a stimulatory effect on the secretion of secretory IgA in the colonic mucosa (72) but, we were not able to detect any changes in the production of anti-*Salmonella* fecal IgA or total fecal IgA following neither systemic nor oral infection in mice previously treated with paraoxon.

Our and other, results indicated that AChE inhibition did not have any effect on the immune response following a systemic infection. All together the data suggested that the effects of our treatment are related to the route of infection as it clearly had shown a marvelous impact on oral infection model by reducing the bacterial burden in the organs and subsequently improved host survival (43).
We decided to investigate more in detail the events that followed the oral inoculation of SL1344. Early in the infection our results suggested that paraoxon pre-treatment did not have any effect either the bacterial burden or serum IL-12 production. Surprisingly, after 7 days of infection the bacterial growth in the paraoxon-treated group, compared to the saline-treated group, was significantly retarded which was accompanied by lower spleen weights, lower cytokine production by spleen cells, and lower expression of activation markers in spleen and MLNs cells. In addition, serum IL-12 levels remained constant throughout the course of infection from day 2 to 7 in paraoxon group. These data suggested that the abridged immune responses in paraoxon pre-treated group is related to the lower bacterial burden in the different organs in these mice, thus our treatment somehow caused a retardation in the bacterial dissemination or growth. In the same contest, bioluminescence in-vivo imaging suggested that bacteria was held in the abdominal area, most probably the intestine, and took longer time to disseminate to the systemic organs. This delay could be due to a lower number of bacteria able to invade the intestinal epithelium or/and to a more effective localized immune response able to delay the bacterial growth and dissemination. We had mentioned earlier that AChE inhibition induced an increase in the activation markers in MLN T cells which could indicate a better immune response to the invading bacteria that gets to the MLN from the intestine and consequently their elimination. However, other mechanisms could be involved in the retention of bacteria at the mucosal and sub-mucosal levels.

Consequently, we concentrated on studying the effect of AChE inhibition on the gastrointestinal tract, particularly the small intestine as it is considered to be the first line of defense against oral pathogens.
Gene expression of different anti-microbial peptides in the ileum epithelial cells, revealed that paraoxon treatment did not enhance the expression of AMPs except a mild up-regulation of RegIII-γ which has been shown to have an important role in the defense against Gram positive and Gram negative pathogens and the distribution of mucus in the ilium (126). This supports our hypothesis that AChE inhibition has a direct effect on the intestine immune defense.

It has been shown that in Salmonella infection, defensins are involved in the clearance of the pathogen from the intestinal lumen. In the intestinal epithelial cells, defensins are synthesized as pro-defensins that need to be cleaved by the enzyme MMP-7 to become active. In a study using MMP-7 knockout mice infected with Salmonella has been reported an impaired clearance of the pathogen, showing the important role of defensins in defense against Salmonella (56). Furthermore, Angiogenin 4 (Ang4) was found to also have bactericidal ability against Salmonella (104). Our study showed an up-regulation in Defa 1 and 4, RegIIIγ, Ang-4 and MMP-7 enzyme following oral infection with Salmonella in normal mice. Thus this up-regulation is a response to the interaction of the Paneth cells with the pathogen in order to avoid the invasion of the pathogen. However, this up-regulation was much lower in paraoxon-treated mice. Our data also showed that paraoxon treatment induced the degranulation of Paneth cells that release anti-microbial peptides in response to bacterial stimuli. It has been shown that atropine, a muscarinic receptor antagonist, was able to inhibit the secretion of AMPs (81). Moreover, it was also demonstrated that bethanechol, a muscarinic agonist, induced Paneth cell secretion in mouse intestine by direct interaction with the muscarinic receptors expressed on Paneth cells, indicating that ACh has a regulatory role on the
degranulation of AMPs containing granules (80). In our model the degranulation of Paneth cells and the release of their AMPs could kill some of the *Salmonella* bacteria in the intestine, leaving only few that could possibly interact directly with the Paneth cells and induce a further release of AMPs. This could explain the lower expression of AMPs in infected mice pre-treated with paraoxon. Furthermore, some studies had demonstrated that degranulation and secretion of the content of Paneth cells induced their death (127) which support our observations of disintegrated Paneth cells together with young ones. A recent study in primary epithelial organoid cultures have showed that Paneth cells degranulation is induced by IFNγ treatment (127). A question that remains to be answer is whether AChE inhibition induces the secretion of IFNγ by intraepithelial lymphocytes.

Cholinergic stimulation is also known to induce mucus secretion in the intestine (79) and it was shown to be blocked by atropine indicating that this effect of ACh on mucus secretion is derived by stimulation of muscarinic receptors (128). Mucus is an important first line of defense to oral pathogen like *Salmonella* and the more mucus secreted the more difficult for the bacteria to penetrate and invade the intestinal epithelium. Therefore, the degranulation of goblet cell and, therefore, the increased mucus release in paraoxon pre-treated mice could be part of the mechanism by which paraoxon protects mice from lethal oral infection. Degranulation of goblet cells and Paneth cells and, therefore, the release of mucins and AMPs could be responsible of killing the majority of the pathogenic bacteria in the intestinal lumen before they are able to interact and penetrate the intestinal epithelium. This mechanism could explain the hindered bacteria
in the gut, the slower dissemination to the systemic organs and low bacterial burden in the organs which lead to improved host survival in mice treated with AChE inhibitor.

One future approach will be to study the immune cells present in the intestinal epithelium and lamina propria as AChE inhibition might have some influence on these cells as it showed to have on spleen and MLN cells.

Lastly, our study could open a new perspective about the relationship between nervous and immune system and how cholinergic pathway could influence the immunity especially innate immunity against oral pathogens.
Chapter 7: Conclusions

7.1 Conclusions

- AChE inhibition enhanced immunity after oral but not systemic infection with virulent Salmonella.

- The observed effect is due to enhanced innate immune mucosal defense mechanisms as shown by the increased degranulation of immuno-competent cells in the GI tract.

- This could potentially explain the reduction in systemic spread of oral Salmonella leading to enhanced survival.

In conclusion, our data suggest a possible neuro-immune mechanism that leads to enhanced host survival after a lethal oral infection model, by enhancing the gastrointestinal barrier defense mechanisms, including the secretion of AMPs and mucins, as well as increasing macrophages phagocytic activity and T cells activation in the spleen and MLN.
7.2 Proposed mechanism

The proposed mechanism by which AChE inhibition modulates the immune response to lethal oral bacterial infection can be summarized in the below diagram.
Bibliography


120. Vogel LA, Noelle RJ. CD40 and its crucial role as a member of the TNFR family. Seminars in Immunology. 1998 Dec;10(6):435–42.


