FACULTAD DE CIENCIAS BIOLÓGICAS

PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE

DOCTORAL THESIS:

"Role of neutrophils during *Salmonella enterica* serovar Typhimurium infection"

BY:

BÁRBARA M. SCHULTZ LOMBARDIC

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Tesis entregada a la Pontificia Universidad Católica de Chile en cumplimiento parcial de los requisitos para optar al Grado de Doctor en Ciencias con Mención en Genética Molecular y Microbiología.

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"Role of neutrophils during *Salmonella enterica* serovar Typhimurium infection"

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Bárbara M. Schultz Lombardic

Ha sido aprobada por el Tribunal Examinador, constituido por los profesores abajo firmantes, calificando el trabajo realizado, el manuscrito sometido y la defensa oral con nota _____.

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"Esperanza. *Estado de ánimo que surge cuando se presenta como alcanzable lo que se desea."*

Estar y ser parte de este cambio, de este proceso de revolución social hace que mi corazón se llene de esperanza, porque veo como un futuro probable que seamos una mejor sociedad, un mejor Chile.

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ABREVIATIONS

S. enterica: *Salmonella enterica*

NTS: Non-typhoidal serovars

***S*. Typhimurium:** *Salmonella enterica* serovar Typhimurium

***S*. Enteritidis:** *Salmonella enterica* serovar Enteritidis

iNTS: Invasive non-typhoid salmonellosis

SPIs: *Salmonella* Pathogenicity Islands

T3SS: Type Three Secretion Systems

IECs: Intestinal epithelial cells

SCV: *Salmonella*-containing vacuole

MCs: Mast cells

Treg: Regulatory T cell

IL-10: Interleukin 10

WT: Wild type

IL-10$^{-/-}$: IL-10 knock out

ROS: Reactive oxygen species

NETs: Neutrophil extracellular traps

NO: Nitric oxide

MPO: Myeloperoxidase

NE: Neutrophil Elastase

PMA: Phorbol myristate acetate

PKC: Protein Kinase C

NOX2: NADPH oxidase 2

PAD4: Peptidyl arginine deiminase 4

TLR: Toll-Like Receptors

GSDM-D: Gasdermin-D

BCG: Bacillus Calmette-Guérin

PG: Prostaglandin

COX2: Cyclooxygenase 2

DAO: D-amino acid oxidase

H_2O_2: Hydrogen peroxide

HOCL: Hypochlorous acid

T3SS-2: Type Three Secretion Systems-2

APO: Apocynin

NAC: N-Acetyl Cysteine

Cl-A: Cl-Amidine

LY294: LY294002

PD980: PD98059

SB203: SB203580

Nec-1: Necrostatin-1

NSA: Necrosulfonamide

HK: Heat-killed

ΔSPI-2: *S.* Typhimurium 14028 *ΔspiA*::aph

ΔSPI-1: *S.* Typhimurium *ΔinvC*::aph

MOI: Multiplicity of infection

RESUMEN

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) es una bacteria Gram negativo y un patógeno gastrointestinal de importancia mundial, ya que es uno de los principales agentes causantes de intoxicación alimentaria y de enfermedades invasivas no tifoideas, principalmente en niños. *S*. Typhimurium presenta varios factores de virulencia, algunos de ellos le permiten colonizar la barrera epitelial y sobrevivir en el espacio intracelular de células fagocíticas, tales como macrófagos y células dendríticas. Una de las células inmunes más importantes en el control del crecimiento y la diseminación de las bacterias son los neutrófilos, los cuales presentan diferentes mecanismos antimicrobianos tales como la fagocitosis, producción de especies reactivas de oxígeno (ROS), degranulación de componentes citotóxicos, y la liberación trampas extracelulares derivadas de neutrófilos (NETs). Además de este rol inflamatorio, se les ha atribuido un rol modulador debido a la producción de interleuquina 10 (IL-10). Sin embargo, no se conoce bien cuál es el rol que cumplen estas células en una infección causada por esta bacteria y si alguno de sus factores de virulencia modula la respuesta de estas células y le permiten a la bacteria sobrevivir en el espacio intracelular y/o secretar IL-10, con el fin de favorecer la diseminación.

En este contexto, el presente trabajo evaluó el rol de los neutrófilos frente a una infección causada por *S.* Typhimurium. Para esto, se purificaron neutrófilos ($Ly6G^+$) derivados de médula ósea de ratón y se evaluó la respuesta efectora midiendo la producción de ROS, la liberación de NETs y la supervivencia bacteriana durante el proceso de infección de los neutrófilos. Como respuesta antiinflamatoria se cuantificó la liberación de IL-10 en el

sobrenadante de las células infectadas a diferentes tiempos. Nuestros resultados sugieren que:

1. *S.* Typhimurium infecta y sobrevive en el espacio intracelular de los neutrófilos a las 24h post infección.
2. La infección causada por *S.* Typhimurium induce la liberación de NETs 3h post infección.
3. La infección causada por *S.* Typhimurium induce la producción de IL-10 a las 24h post infección.
4. La respuesta inmune de los neutrófilos frente a una infección causada por *S.* Typhimurium depende del sexo del animal.

En resumen, nuestros resultados sugieren que *S.* Typhimurium induce una respuesta antimicrobiana considerando la liberación de NETs y la inducción de ROS intracelular. Sin embargo, es posible que la bacteria module la respuesta inmune de estas células debido a la producción temprana de IL-10, así como las diferencias observadas en la internalización de la bacteria, lo cual podría depender de los genes codificados en la Isla de patogenicidad 1 de *S.* Typhimurium (SPI-1).

ABSTRACT

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a Gram-negative bacterium and an important gastrointestinal pathogen. *S.* Typhimurium is a worldwide problem being an important cause of bacterial foodborne and the leading cause of invasive non-typhoid disease, mainly in children. *S.* Typhimurium has several genes that encode virulence factors that allow the colonization of the epithelial barrier and the survival inside phagocytic cells, such as macrophages and dendritic cells. During the first phase of infection, the inflammatory response generated by neutrophils ($Ly6G^+$ cells) plays a vital role, restricting the bacterial growth and dissemination, because these cells present different mechanisms to generate an efficient bacterial killing such as phagocytosis, NADPH oxidase-derived reactive oxygen species (ROS) production, degranulation of cytotoxic components, anti-microbial peptides, and the release of neutrophils extracellular traps (NETs). However, during *S.* Typhimurium infection, the role of NETs release remains unknown. As mentioned above, *S.* Typhimurium virulence factors allow the intracellular survival in phagocytic cells such as macrophages and dendritic cells. However, if the bacteria can survive inside neutrophils has not been entirely elucidated. Finally, published data from our laboratory demonstrate that the production of interleukin 10 (IL-10) during systemic *S.* Typhimurium infection could generate significant immunosuppression that favors the systemic dissemination of the bacteria (Salazar et al. 2017). In this sense, in the last years it has been observed that neutrophils are also able to secrete IL-10. However, it is not entirely known how neutrophils react during an *S.* Typhimurium infection. In this sense, the present work evaluates the role of neutrophils against *S.* Typhimurium infection. To evaluate this, neutrophils ($Ly6G^+$) were purified from mouse bone marrow and the inflammatory response measured as ROS production, NETs release, and survival of the

bacteria during the infection process of neutrophils. As an anti-inflammatory response, the release of IL-10 was quantified in the supernatant of infected cells at different times post-infection. Our results suggest that:

1. *S.* Typhimurium infect and survive inside neutrophils at least until 24h post-infection.
2. The infection caused by *S.* Typhimurium induce NETs release 3h post-infection.
3. *S.* Typhimurium infection induces the release of IL-10 24h post-infection.
4. The immune response of the neutrophil against *S.* Typhimurium depends on the sex of the animal.

In summary, our results suggest that *S.* Typhimurium induces an antimicrobial response considering the NETs release and the intracellular ROS induction. However, it is possible that the bacteria modulate the immune response of this cells due to the early IL-10 production, as well as the differences observed in the internalization of the bacteria, which could depend on genes encoded in SPI-1.

CHAPTER 1: Virulent *Salmonella enterica* serovar Typhimurium modulates the release of Neutrophil Extracellular Traps.

INTRODUCTION

Salmonella enterica (*S. enterica*) is a Gram-negative, non-spore-forming, facultative, anaerobic bacteria that belongs to the *Enterobacteriaceae* family (Fàbrega and Vila 2013). *S. enterica* present more than 2.500 serovars divided according to the host specificity. Most serovars infect a broad range of vertebrate animals but only a few of them are host-specific, being divided into human-restricted typhoidal serovars such as *S. enterica* serovar Typhi and Paratyphi, the causative agent of typhoid fever, and non-typhoidal *Salmonella* (NTS) (Wain et al. 2015). NTS have been linked to infection of a variety of hosts, frequently zoonotic, causing acute and self-limiting gastroenteritis that can commonly cause foodborne illness in humans (Scallan et al. 2011). *Salmonella* constitutes a common health problem accounting for about 93.7 million cases per year, including 155,000 deaths (Ao et al. 2015; Majowicz et al. 2010). Although NTS infections are generally self-limited, immunocompromised patients can present extra-intestinal complications and chronic carrier states, which have been implicated in invasive non-typhoid salmonellosis (iNTS) (Ao et al. 2015; Stanaway et al. 2019). *Salmonella enteric*a serovar Typhimurium (*S.* Typhimurium) is one of the two serovars commonly isolated of NTS and the leading serovar isolated of iNTS (Dekker et al. 2018; Mather et al. 2018; Williamson et al. 2018).

iNTS caused mainly by *S.* Typhimurium are generated due to the virulence factors encoded in at least 23 *Salmonella* Pathogenicity Islands (SPIs) (Blondel et al. 2009; Desai

et al. 2013; Hayward, Jansen, and Woodward 2013), being the most important to the infective cycle the SPI-1 and SPI-2. Both SPIs encode Type Three Secretion Systems (T3SS) that allow contact-dependent translocation of a set of effector proteins into the eukaryotic cytoplasm (Hansen-Wester and Hensel 2001; Moest and Méresse 2013; Pezoa et al. 2013). During *S.* Typhimurium infection, SPI-1 facilitates the entry to non-phagocytes cells, as intestinal epithelial cells (IECs) (Raffatellu et al. 2005), and the SPI-2 allow the intracellular survival of the bacteria inside a specialized compartment known as *Salmonella*-containing vacuole (SCV).

Neutrophils are the first immune cells to be recruited to the site of infection, as they display different mechanisms they display to kill microorganisms such as phagocytosis, NADPH oxidase-derived reactive oxygen species (ROS), degranulation of cytotoxic components (Segal 2005; Teng et al. 2017), and the release of neutrophil extracellular traps (NETs) (Brinkmann et al. 2004). In 2004, Brinkmann et al. (Brinkmann et al. 2004) observed for the first time that activated neutrophils were able to generate prominent extracellular structures composed by chromatin, anti-microbial proteins as histones, and granular proteins as Neutrophil Elastase (NE), myeloperoxidase (MPO), or cathepsin-G. These structures were able to capture, entrap and kill the microorganism (Brinkmann et al. 2004). The NETs process begins with the disruption of the nuclear membrane, continues with the mixture of nuclear, granular, and cytoplasmatic content, and ends with the disruption of the plasma membrane and the release of the lattice structure (Fuchs et al. 2007). In this sense, NETosis seems to be the innate immune mechanism that controls the pathogen's spreading by entrapping the microorganisms and placing them in direct contact with a high concentration of anti-microbial molecules derived from the cell.

NETs release can be induced by extracellular or intracellular stimulus activating different pathways (Brinkmann et al. 2004; Chen et al. 2018). The first pathway is activated by an extracellular stimulus generating an increase of intracellular calcium (Li et al. 2010) and the concomitant of NETs release as soon as 10 minutes post stimulation. The second pathway described is the classical or suicidal NETosis and induces the NETs release 3-4h post-stimulation, leading to cell death (lytic NETosis). Phorbol Myristate Acetate (PMA) generates the ROS production dependent of NADPH oxidase activation (Hakkim et al. 2011), which helps during the translocation of granular proteins as NE to the nucleus, promoting the decondensation of the chromatin (Metzler et al. 2014; Papayannopoulos et al. 2010), releasing NETs. The last pathway described is mediated by cytosolic LPS or intracellular Gram-negative pathogen, such as *S*. Typhimurium (Chen et al. 2018). This stimulus activates the non-canonical inflammasome (caspase-11 dependent) signaling and trigger the pore-forming gasdermin-D (GSDM-D) and the concomitant death of the neutrophil (Chen et al. 2018). In this case, the action of ROS or PAD4 is not necessary due to the formation of pores by GSDM-D in nuclear and plasma membranes (Chen et al. 2018) that allows the NETs releasing process.

It is well known from Brinkmann studies (Brinkmann et al. 2004) that *S.* Typhimurium induces NETs and, indeed, several reports have used this bacteria as an inductor of NETs (Aleyd et al. 2014). Even more, Chen et al. uses a mutant strain of *sifA,* which is a bacterium unable to survive inside de SCV and replicate in the cytosol, which imply the recognition of the bacterium and the activation of the GSDM-D pathway. However, little is known about the induction pathway of NETs caused by *S*. Typhimurium infection. In this sense, we do not know if the wild type strain of *S.* Typhimurium activates

the same signaling pathway that the mutant strain of *sifA* or if *S.* Typhimurium present some virulence factor encoded on its SPIs that can modulate this process.

HYPOTHESIS STATEMENT.

Hypothesis 1

"*Salmonella enterica* serovar Typhimurium infection induces the release of neutrophil extracellular traps".

OBJETIVES.

General aims:

To evaluate the immune response of neutrophils against Salmonella enterica serovar Typhimurium infection.

Specific aims 1:

1. To identify if *S.* Typhimurium infection induce the release of NETs in mice and human derived neutrophils.
2. To identify the pathway by which *S.* Typhimurium infection induces NETs release in mice and human derived neutrophils.
3. To identify whether virulence genes encoded in *Salmonella* pathogenicity island 2 are involved in the process of NETs release.

Submitted manuscript: Virulent *Salmonella enterica* serovar Typhimurium modulates the release of Neutrophil Extracellular Traps.

Abstract

Salmonella enterica serovar Typhimurium is an important cause of bacterial food borne poisoning and the leading cause of invasive non-typhoid disease, mainly in children and elderly. To successfully infect the host, *S.* Typhimurium produces virulence factors encoded in chromosome cluster denominated *Salmonella* pathogenicity island (SPI), which are involved in immune evasion. The most important SPIs are SPI-1 and SPI-2, which allow the invasion of intestinal epithelial cells and the survival inside phagocytic cells, respectively. One of the first responses against *S.* Typhimurium infection is the recruitment of neutrophils, which are key innate immune cells that control bacterial infection. These cells are important during the infection, due to several mechanisms that these cells use to restrict bacteria dissemination. However, during *S.* Typhimurium infection these mechanisms are not enough to avoid dissemination of the bacteria from the site of infection to deeper organs. One of the mechanisms used by neutrophils to prevent bacterial dissemination is neutrophil extracellular traps or NETs. Indeed, NETosis is a new type of cell death that occurs after neutrophil activation and it is essential to control extracellular bacteria. NETs are extracellular DNA associated with histones and granular proteins, which can trap and possible kill bacteria. Here, we report that *S.* Typhimurium induces the production of NETs in human and mouse-derived neutrophils by the classical pathway, which depends mainly on granular proteins. Importantly, this process is modulated by *Salmonella* Pathogenicity Islands and could be related to ROS production.

Key words: *Salmonella enterica* serovar Typhimurium, neutrophils, ROS production, NETs, virulence factors, SPI-2 mutant strain.

INTRODUCTION.

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a non-typhoid serovar that infects a wide range of hosts, causing several diseases, from self-limiting gastroenteritis to severe sepsis (Majowicz et al. 2010). Importantly, this is the most prevalent serovar of invasive non-typhoid disease worldwide, which can lead to death (Ao et al. 2015). *S.* Typhimurium has several virulence factors located in the chromosomal cluster known as *Salmonella* Pathogenicity Islands (SPI). An essential virulence factor allowing immune evasion to facilitate a systemic infection is the Type Three Secretion System encoded in SPI-2 (TTSS-2). Proteins secreted by this TTSS to infected cells prevent endosomal traffic and promote the survival of the bacteria inside immune cells, such as macrophages and dendritic cells, which are not able to activate the adaptive immune response properly (Tobar et al. 2006). Moreover, *S.* Typhimurium-infected cells secrete anti-inflammatory cytokines, promoting a tolerogenic environment that further prevents the proper function of innate immune cells (Salazar et al. 2017). During bacterial infections, neutrophils are rapidly recruited to the infection site and undergo a novel type of cell death characterized by chromatin decondensation and disintegration of the nuclear envelope, which allows the mixture of the nuclear and cytoplasmic components. This process culminates with the release of Neutrophil Extracellular Traps (NETs), which are DNA lattices associated with histones, cytoplasmic content and granular proteins. NETs are coated with several antimicrobial proteins, which aid degradation of some virulence elements, entrap and kill different types of microorganisms, such as parasites, fungi, viruses, and bacteria (Brinkmann et al. 2004). Previously, it has been shown that NETs fibers can entrap *S.* Typhimurium (Brinkmann et al. 2004), but whether *S.* Typhimurium

can trigger or prevent NETs formation, as well as the mechanism underlying this process are yet to be defined. Here we demonstrate that *S.* Typhimurium stimulates neutrophils to form NETs in a dose-dependent manner, with the involvement of granular proteins as neutrophil elastase and myeloperoxidase. Also, we observed that this process could be dependent on the TTSS-2 and it is independent of ROS burst.

MATERIALS AND METHODS

Mice.

Female C57BL/6 mice weighing 20-25 g were used. Animals were housed in institutional animal facilities with 12:12 h light/dark cycle, free access to water and food, and controlled temperature (21 ±1°C). All experimental protocols were reviewed and approved by the Ethics Committee on Animal Use at P. Universidade Católica do Rio Grande do Sul under protocol number 8259 and the Scientific Ethics Committee for Animal and Environmental Care of P. Universidad Católica de Chile under protocol number 170711024.

Bacterial strains and growth conditions.

Wild type (WT) *S.* Typhimurium strain ATCC 14028, and mutant strains *S.* Typhimurium-putA::gfp-pkk233.2 (*S.* Typhimurium-GFP) and *S.* Typhimurium Δ*spiA* (*S.* Typhimurium 14028 Δ*spiA*::aph- pkk233.2::egfp) (Tobar et al. 2006) were stored at -80ºC in Luria-Bertani (LB) medium supplemented with 20% glycerol and grown at 37°C in LB medium. Δ*spiA* strain was select using LB media supplemented with 50 mg/mL kanamycin.

Human neutrophil isolation and stimulation.

Whole blood (20 mL) was collected from healthy volunteer donors (with a mean age of 28 years, from both sexes) into heparin-treated tubes. Neutrophils were purified by density gradient centrifugation using Ficoll-Paque PLUS. Erythrocytes were removed by dextran sedimentation followed by two rounds of hypotonic lysis. Purified neutrophils were re-suspended at 2 X 10^6 cells/mL in RPMI 1640 medium. Neutrophil viability was assessed by the trypan blue exclusion assay and was always higher than 98%.

Human neutrophils (2x10^6 cells/mL) were stimulated with *S.* Typhimurium at a multiplicity of infection (MOI) of 1, 10, 25 and 50 (bacteria to neutrophils) for 180 min at 37ºC with 5% CO_2. Afterward, culture supernatant was collected, and extracellular DNA was measured using the Quant-iT dsDNA HS kit (Invitrogen), following manufacturer's instructions. To evaluate the role of specific signaling pathways on *S.* Typhimurium - induced NETs release, neutrophils were pretreated for 1 h with selective inhibitors: apocynin (APO; 10μM), N-Acetyl Cysteine (NAC; 1mM), Cl-Amidine (Cl-A; 12μM), LY294002 (LY294; 50μM), PD98059 (PD980; 30μM), SB203580 (SB203; 10μM), necrostatin-1 (Nec-1; 50 μM), or necrosulfonamide (NSA; 5μM). The Trypan Blue exclusion assay was used to evaluate the viability of cells treated with these inhibitors, and at the end of incubation, cell viability was always higher than 97%.

Mice-derived neutrophil isolation and stimulation.

Bone marrow-derived cells were isolated from femurs and tibias of C57BL/6 mice. Briefly, bones were removed aseptically, and bone marrow flushed out with RPMI 1640 medium supplemented with 5% fetal calf serum (FBS), 2mM glutamine, 1mM non-essential amino

acids, 1mM pyruvate and 2mM EDTA with a 10mL syringe. Bone marrow-derived cells were then pelleted at 1,400 rpm for 7 min and re-suspended in 2 mL of fresh medium for cell count. The cells were then prepared as described by the manufacturer´s protocol of MACs column and then passed through LS column for negative selection of $Ly6G^+$ cells (neutrophils).

Neutrophils freshly isolated were recovered from MACs column purification were re-suspended in new 2 mL of RPMI and counted in Neubauer chamber. Cells ($1x10^5$/300 µl) were stimulated with *S.* Typhimurium at a multiplicity of infection (MOI) of 1, 10, 25 and 50 (bacteria to neutrophils) for 180 min at 37ºC with 5% CO_2. Afterward, culture supernatant was collected, and extracellular DNA was measured using the Quant-iT dsDNA HS kit or Qubit dsDNA HS assay (Invitrogen), following manufacturer's instructions. To evaluate the role of specific signaling pathways on *S.* Typhimurium-induced NETs release, neutrophils were pretreated for 1 h with selective inhibitors: apocynin (APO; 10µM), N-Acetyl Cysteine (NAC; 1mM), Cl-Amidine (Cl-A; 12µM), necrostatin-1 (Nec-1; 50 µM), or necrosulfonamide (NSA; 5µM).

Immunofluorescence.

Human neutrophils ($1x10^5$/300µl) were seeded in 8-chamber culture slides and incubated with *S.* Typhimurium-GFP (MOI=25) for 180 min at 37°C under 5% CO_2. Afterwards, cells fixed with 4% paraformaldehyde (PFA) were stained with anti-neutrophil-elastase (NE, ab21595, 1:1000) or anti-myeloperoxidase antibody (MPO, BD341642, 1:1000), followed by anti-rabbit Cy3 antibody (Invitrogen, P21129, 1:500) and Hoechst 33342 (1:2000). Images were taken in a confocal Zeiss LSM 5 Exciter microscope.

Murine neutrophils (1x10^5/300µl) were seeded in 8-chamber culture slides and incubated with *S.* Typhimurium-GFP (MOI=25) for 180 min at 37°C under 5% CO_2. Afterwards, cells fixed with 4% PFA were stained with anti-neutrophil-elastase (NE, ab21595, 1/100) or anti-myeloperoxidase antibody (MPO, ab9535, 1/200), followed by anti-rabbit APC antibody and DAPI (564907 BD, 1/1000). Images were taken in a confocal Zeiss LSM 5 Exciter microscope**.**

RESULTS.

Infection of neutrophils with *S.* Typhimurium induces NETs formation.

It has been previously shown that *S.* Typhimurium is trapped by NETs (Brinkmann et al., 2004). However, whether *S.* Typhimurium can trigger NETs release is not fully elucidated. We observed that *S.* Typhimurium induce NETs release in human (**Fig. 1A**) and mice-derived neutrophils (**Fig. 1B**), in a dose-dependent manner, at 180 min post-infection. To determine whether NETs release required live bacteria, neutrophils were incubated with heat-killed (HK) *S.* Typhimurium for 180 min at a MOI 25. We found that HK *S.* Typhimurium did not induce NETs formation (**data not shown**), ruling out that the process is caused not by the only presence of PAMPs, but an active infection is required for neutrophils extrude NETs. Next, we performed confocal laser scanning microscopy to detect whether *S.* Typhimurium was bounded to the NETs. Consistently, our results revealed that NETs produced in response to *S.* Typhimurium infection entrapped bacteria, as visualized by the co-localization of *S.* Typhimurium-GFP with extracellular DNA (**Fig.**

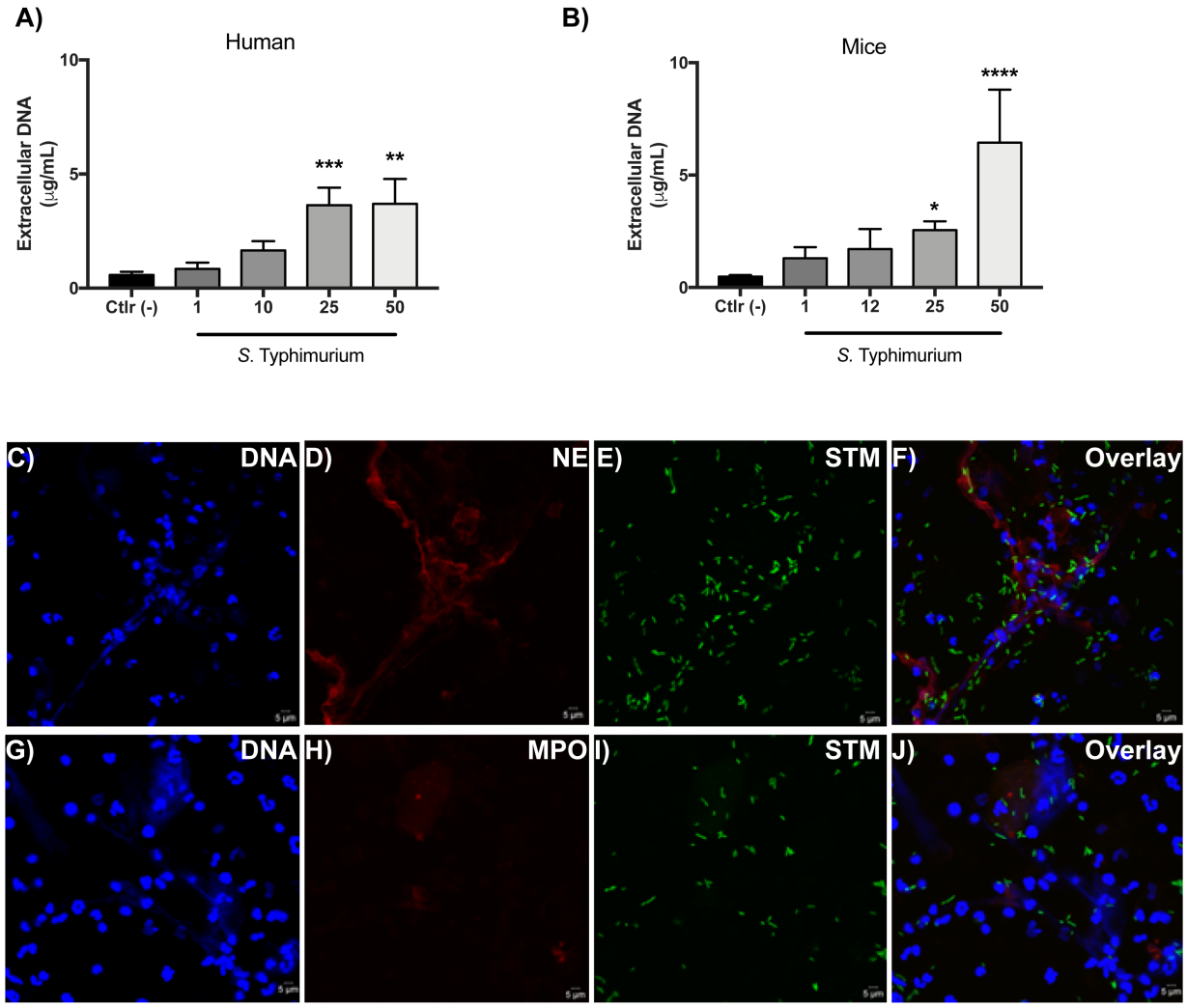

Figure 1: *S.* Typhimurium infection induces NETs formation. A) human derived neutrophils were incubated with different MOI (1, 12, 25, 50) of *S.* Typhimurium WT and the extracellular DNA was quantified at 180 minutes post-infection. P values were determined using Unpaired t Test to compare the different MOI of infection with the respective time Ctrl (-). **B)** Mouse bone marrow-derived neutrophils were incubated with different MOI (1, 12, 25, 50) of *S.* Typhimurium WT and the extracellular DNA was quantified at 180 minutes post-infection. P values were determined using Unpaired t Test to compare the different MOI of infection with the respective time Ctrl (-). **C)** The presence of MPO and NE was evaluated as part of the NETs formation in human blood derived neutrophils infected with *S.* Typhimurium-GFP at MOI 25- and 180-minutes post-infection.

1B-1I). Furthermore, *S.* Typhimurium induced the release of NETs coated with the granular proteins NE (**Fig. 1B-1E**) and MPO (**Fig. 1F-1I**), as visualized by immunostaining.

Signaling pathways activated by *S.* Typhimurium during NETosis.

Previous studies have shown that NETs release depends on different signaling pathways to occur (Desai et al. 2017; Douda et al. 2015; Hakkim et al. 2011; Keshari et al. 2013). Therefore, we took advantage of selective pharmacological inhibitors to characterize the signaling requirements of *S.* Typhimurium-induced NETs formation. As the majority of NETs-inducing stimuli relies on ROS production, we first investigated the role of ROS on NETs release elicited by *S.* Typhimurium. Interestingly, pretreatment of both human and mouse neutrophils with ROS scavenger N-acetyl cysteine (NAC) did not prevent NETs formation induced by the bacteria (**Fig. 2A**). Likewise, the inhibition of NADPH oxidase by apocynin (APO) did not significantly decrease *S.* Typhimurium-triggered NETosis (**Fig. 2A**). These results suggest that *S.* Typhimurium induces NETs release independently of NADPH oxidase-derived ROS.

Histone citrullination by the enzyme peptidyl arginine deiminase-4 (PAD-4) is necessary to chromatin decondensation and has been shown to be essential to NETs formation (Wang et al. 2009). Therefore, we sought to investigate the role of PAD-4 on *S.* Typhimurium-induced NETs production. Chloroamidine (PAD-4 inhibitor) treatment of human neutrophils did not interfere with NETs release elicited by the bacteria. However, treatment of mouse-derived neutrophils with PAD-4 inhibitor significantly impaired *S.* Typhimurium-induced NETs formation (**Fig. 2B**).

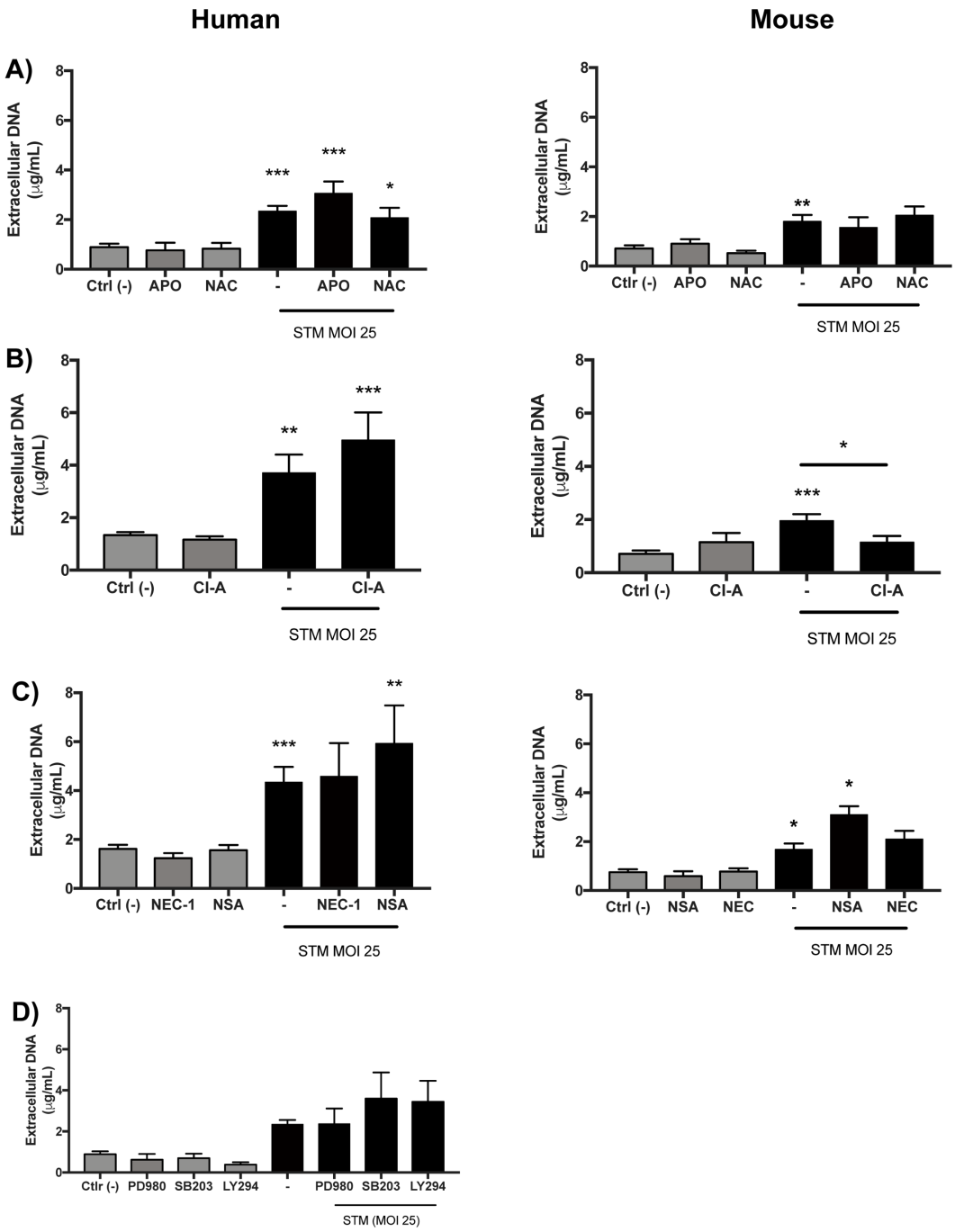

Figure 2: Typhimurium induce the classical pathways for the NETs production. Different NETs pathway as **A)** ROS production; **B)** PAD-4 activation and **C)** Necroptosis proteins were inhibited 1 h before the infection with *S.* Typhimurium WT at MOI 25 in **human blood and mouse-derived neutrophils**, at 180 min post-infection. **D)** ERK/Akt pathway were inhibited 1 h before the infection with *S.* Typhimurium WT at MOI 25 in **human blood-derived neutrophils**, at 180 min post-infection. P values were determined using

Unpaired t-test for parametric data or Mann-Whitney test for non-parametric data to compare different inhibitor treatment with untreated/infected. *P<0.0357; **P<0.0045; ***P<0.0007.

The role of necroptosis signaling pathways on NETosis has been controversial. The participation of RIPK1-RIPK3-MLKL on NETs release seems to depend on stimulus (Amini et al. 2016; Desai et al. 2016, 2017). We sought to elucidate the participation of RIPK1 and MLKL on NETs extrusion induced by *S.* Typhimurium, by incubating human and mouse neutrophils with selective inhibitors of these signaling pathways. Inhibiting RIPK1 kinase activity with NEC-1 or suppressing MLKL activation with NSA did not prevent NETs release induced by the bacteria, indicating that *S.* Typhimurium NETs release is a different process from necroptosis and that these proteins are dispensable to *S.* Typhimurium-triggered NETosis.

Previous studies have shown that MAPK and PI3K/AKT are essential to direct NETosis (Douda et al. 2015; Hakkim et al. 2011; Keshari et al. 2013). Therefore, we investigated the role of these signaling routes on *S.* Typhimurium-induced NETosis. Pre-treatment human neutrophils with PD98059, ERK inhibitor, SB203580 (p38 MAPK inhibitor), or with LY294002, (PI3K/AKT inhibitor), did not impair NETs formation triggered by *S.* Typhimurium (**Fig. 2D**).

TTSS-2-secreted proteins modulate the production of NETs in *S.* Typhimurium-infected neutrophils

We further evaluated the impact of the effector proteins secreted by the TTSS-2 in NETs formation, using the mutant strain of *S.* Typhimurium, Δ*spiA*, which lacks a functional TTSS-2. This strain is not able to survive inside phagocytic cells and therefore is

less virulent. The infection of human-derived neutrophils with Δ*spiA* strain induced the release of significantly higher amounts of extracellular DNA as compared to the WT strain (**Fig. 3A**). However, this effect does not occur in mouse-derived neutrophil, where we could not observe differences between these two strains (**Fig. 3B**). These results suggest that some virulence protein secreted by the TTSS-2 modulates the induction of NETs in human derived neutrophils, preventing excessive NETs formation due to virulent *S.* Typhimurium infection.

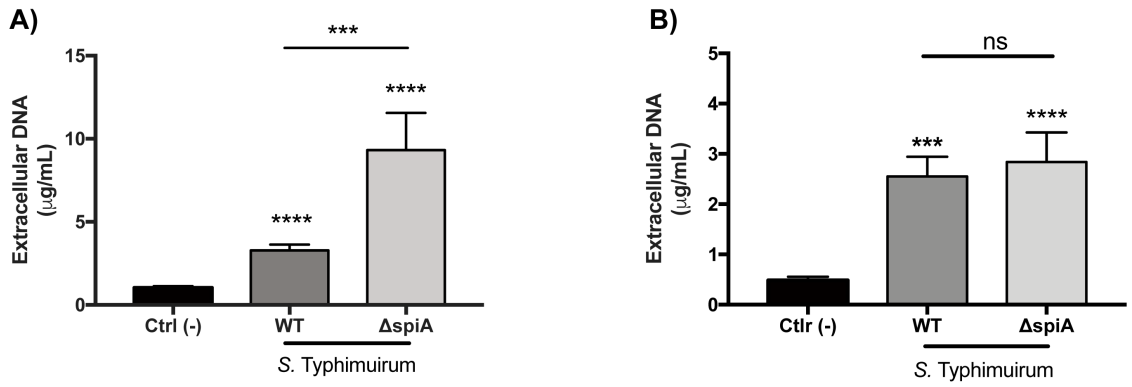

Figure 3*: S.* Typhimurium TTSS-2 modulates NETs production. The differentiated NETs production induced by the *S.* Typhimurium WT and the mutant strain of TTSS-2 (*ΔspiA*) of *S.* Typhimurium was evaluated in **A)** human blood and **B)** mouse-derived neutrophils, at MOI 25 and during 180 min. P values were determined using Mann-Whitney test for non-parametric data to compare different treatment against the Ctrl (-). ****P<0.0001.

DISCUSSION.

S. Typhimurium is an important gastrointestinal pathogen with several virulence factors that reduce immune response. As neutrophils are the first line of defense against this pathogen, we were interested in elucidating whether *S.* Typhimurium would be able to induce NETs release from human and mouse-derived neutrophils. We describe here that increasing concentrations of *S.* Typhimurium triggered NETs formation from human and mouse-derived neutrophils after 180 min of stimulation. Moreover, *S.* Typhimurium-induced NETs that trapped the bacteria, as shown by the co-localization of *S.* Typhimurium-GFP with extracellular DNA, which is in agreement with what had been previously demonstrated by IL-8-induced NETs in human neutrophils (Brinkmann et al. 2004). In this study, we used a physiological approach, demonstrating that the bacteria stimulate NETs release, and it is trapped by the DNA threads. In addition, *S*. Typhimurium triggered the formation of NETs coated with proteins from the azurophilic granules, NE and MPO. These proteins have been shown to modulate NETs release (Metzler et al. 2011; Papayannopoulos et al. 2010) and to exert potent antimicrobial activities. Whether NE and MPO can kill *S.* Typhimurium is yet to be demonstrated. Previous evidence has shown that the requirement for an active NADPH oxidase-derived ROS on NETs release depends on the stimulus (Parker and Winterbourn 2012). With the use of a potent NADPH oxidase inhibitor and a ROS scavenger, we show that *S.* Typhimurium-induced NETs formation from both human and mouse neutrophils occurs independently of ROS generation through an active NADPH oxidase. A previous study shows that *C. albicans* and the group B of *streptococcus* (GBS) can induce an oxidative burst in the neutrophils, and these two stimuli did not require the action of ROS during the NETs release (Kenny et al. 2017). This also

happen with *S.* Typhimurium, that induce a burst of intracellular ROS one-hour post-infection (**data not shown**).

Histone citrullination by peptidyl arginine deiminase-4 (PAD-4) is essential to chromatin decondensation and is a crucial step to NETs formation (Li et al. 2010). However, our results point out that *S.* Typhimurium-induced NETs release from mouse neutrophils is partially dependent on PAD-4 activity. Surprisingly, *S.* Typhimurium-induced NETosis in human neutrophils is independent of PAD-4. In a recent study, it was demonstrated that PAD-4 is dispensable to *C. albicans*-induced NETosis and is not required to anti-fungal immunity during mucosal and systemic infection (Guiducci et al. 2018). New studies have also shown that the decondensation of chromatin is dependent of the action of MPO and NE, a process that is mediated by the oxidative burst (Metzler et al. 2014; Papayannopoulos et al. 2010). One crucial point is that the infection of *S. enterica* can be quite different in human and mouse-derived neutrophils as was observed in dendritic cells (Bueno et al. 2008), and possibly this is the reason we observed differences in the inhibition of PAD-4 among human and mouse-derived neutrophils. Although we observed that ROS is not essential for the *S.* Typhimurium NETs release, the bacteria induce a significant oxidative burst in the neutrophils, which together with the action of the granular proteins can perform the decondensation process. This can be the reason why PAD-4 may not be universally required during NETosis.

AKT is fundamental to direct PMA-induced NADPH oxidase-dependent NETosis (Douda et al. 2015). In our conditions, the axis PI3K/AKT has shown to be dispensable to NETs release promoted by *S.* Typhimurium. As *S.* Typhimurium-induced NETosis is independent of NADPH oxidase-derived ROS, this might explain the lack of PI3K/AKT needs during NETosis incited by these bacteria. Similarly, *S.* Typhimurium promotes NETs

production independently of p38 MAPK activation and partially dependent on ERK activation. These results suggest that *S.* Typhimurium differentially turns on intracellular signaling cascades to induce NETosis.

NETosis has been postulated as a model of neutrophil death, during which cell lysis precedes NETs extrusion. This new type of neutrophil death would require the activation of necroptotic machinery (RIPK1-RIPK3-MLKL) to occur (Desai et al. 2016, 2017; Nakazawa et al. 2018; Schreiber et al. 2017). However, the requirement of these proteins for NETosis has been controversial (Amini et al. 2016; Desai et al. 2016). Our data point out that the necroptosis proteins are not essential to NETosis induced by *S.* Typhimurium.

S. Typhimurium WT is able to survive inside the *Salmonella* Containing Vacuole (SCV) in phagocytic cells, such as macrophages and dendritic cells. In these cells *S.* Typhimurium generates an altered state in ROS production and an incorrect assembly of the NADPH oxidase complex, which occurs in an SPI-2 dependent manner (Gallois et al. 2001; Vazquez-Torres et al. 2000). The mutant strain Δ*spiA* that lacks a functional TTSS encoded in SPI-2 (TTSS-2) is not able to survive inside phagocytic cells (Cano et al. 2001) and is sensitive to ROS action (Cano et al. 2001). The mutant strain induces higher NETs release compared with the WT strain in human derived neutrophils and this difference is statistically significant in human-derived neutrophils. *S.* Typhimurium can survive inside the SCV and also could do this in neutrophils, since it has been seen that *S*. Typhimurium WT is able to be hidden inside the neutrophils (Geddes, Cruz, and Heffron 2007). In concordance with this, the mutant strain could be more recognized by the neutrophils and induce, as we saw in this work, more NETs release than the WT strain. Finally, it is possible that the effector proteins secreted by the TTSS-2 regulate NETs release during *S.*

Typhimurium infection as an evasion mechanism, to avoid being trapped/killed by the antimicrobial proteins anchored in the NETs threads.

Acknowledgements

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Chapter 2: Effect of *S.* Typhimurium infection over neutrophils function

INTRODUCTION.

Neutrophils have different antimicrobial mechanisms. However, during *S.* Typhimurium infection it seems that these cells do not perform a proper clearance. In this regard, it has been demonstrated in a colitis mouse model that *S.* Typhimurium can use gut lumen neutrophils as a niche for a short-term period, and replicate inside them (Loetscher et al. 2012). In addition, Geddes et al. demonstrated that around 70% of bacteria in the spleen are found inside neutrophils, surviving, and replicating inside these cells in an SPI-2-dependent manner (Geddes et al. 2007). In addition, neutrophils have been traditionally described as inflammatory cells that highly contribute to tissue damage. However, in the last years several new properties of neutrophil have been discovered, one of them is the modulation of the immune response through the production of IL-10 (Mantovani et al. 2011). This modulatory function has been described during bacterial (Bouabe et al. 2011; Doz et al. 2013; Kasten, Muenzer, and Caldwell 2010; Ocuin et al. 2011; Zhang et al. 2009), fungal (Balderramas et al. 2014; Gresnigt et al. 2012) and parasitic (Boari et al. 2012) infection.

Interleukin 10 (IL-10) is an anti-inflammatory cytokine considered the master regulator of the inflammatory response. The effect of IL-10 production in infectious diseases depends on the infecting microorganism and the immune response associated (Peñaloza et al. 2016). In the case of *S.* Typhimurium infection, little is known about the role of IL-10. In this sense, data from our laboratory show that dendritic cells, macrophages, lymphocytes B and T secrete IL-10 24h post-infection with *S.* Typhimurium. In this sense, mice unable to produce IL-10 (IL-$10^{-/-}$) are resistant to systemic disease

caused by *S.* Typhimurium, which correlate with lower amounts of bacteria in internal organs, increased pro-inflammatory cytokines and better survival rate. Also, this observation correlates with an increased killing ability from bone marrow-derived macrophage from IL-$10^{-/-}$ as when compared to C57BL/6 wild type (WT) mice which are highly susceptible and all succumb to the infection after 20 days of infection, which is possibly related to increased secretion of IL-10 (Salazar et al. 2017).

Moreover, it is known that the immune response presents sexual dimorphism, which means that this response is different between males and females of the same species (Klein and Flanagan 2016). Pace et al. observed that neutrophils induce a differentiated response in two animal models of acute inflammation, where male neutrophils induced higher NF-κβ, cyclooxygenase 2 (COX2), and in consequence prostaglandins (PG) than female neutrophils, which can be one of the reasons of the differences in the immune response observed among both sexes (Pace et al. 2017).

After all this knowledge, some questions remain about the role of neutrophils during *S.* Typhimurium infection. We know that *S.* Typhimurium induces IL-10 in other immune cells and the production of IL-10 favor the dissemination and the survival of the bacteria. However, this mechanism is not described in neutrophils. It is well known that neutrophils have several antimicrobial mechanisms to contain the infection, but in the case of *S.* Typhimurium, they do not work adequately as the bacteria is still able to disseminate systemically in mice. In this sense, it is unclear if *S.* Typhimurium can survive inside neutrophils or if some antimicrobial mechanisms such as oxidative burst kill the bacteria. Also, it is unknown whether *S.* Typhimurium alters the cell lifetime to create a better niche to replicate and disseminate, and some virulence genes encoded in SPI-1 or SPI-2 are

involved to perform this process (Geddes et al. 2007), or whether the role of the neutrophils can be influenced by the secretion of IL-10. Finally, it is not known how sexual dimorphism of these cells can affect the immune response against the bacteria.

HYPOTHESIS STATEMENT.

Hypothesis 2

"The role of neutrophils against *Salmonella enterica* serovar Typhimurium is affected by sexual dimorphism "

General aim:

To evaluate the immune response of neutrophils from males and females against Salmonella enterica serovar Typhimurium infection.

Specific aims 2:

1. To identify the inflammatory response of neutrophils against *S.* Typhimurium infection.
2. To identify if virulence genes encoded in *Salmonella* pathogenicity island 1 or 2 are involved in the immune response generated by neutrophil against *S.* Typhimurium infection.
3. To identify the anti-inflammatory response of neutrophils against *S.* Typhimurium infection.
4. To evaluate if neutrophils present sexual dimorphism during *S.* Typhimurium infection

MATERIALS AND METHODS

Mice

Female and male C57BL/6 mice weighing 20 – 25 g were used. Animals were housed in institutional animal facilities with 12:12 h light/dark cycle, free access to water and food, and controlled temperature (21 ±1°C). The experimental protocols were reviewed and approved by the Scientific Ethics Committee for Animal and Environmental Care of Pontificia Universidad Católica de Chile under protocol number 170711024.

Bacterial strains and growth conditions.

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) wild type 14028 was originally obtained from the America Type Culture Collection and kindly provided by Dr. Carlos Santiviago, Universidad de Chile, Santiago, Chile. The WT or mutant strains were stored at −80°C in Luria-Bertani (LB) medium supplemented with 20% glycerol. To prepare infection doses, a small aliquot of the frozen bacteria was inoculated in LB medium and grown with agitation at 37°C, overnight. Then, bacteria were diluted 1/100 in LB medium and grown with agitation at 37°C until $OD_{600\ nm}$ equal to 0.6 was reached. Mutant strains *S.* Typhimurium-GFP (*S.* Typhimurium-putA::pkk233.2-*gfp*-Amp); *S.* Typhimurium ΔSPI-2 (*S.* Typhimurium 14028 Δ*spiA*::aph) (Tobar et al. 2006); *S.* Typhimurium ΔSPI-2-GFP (*S.* Typhimurium 14028 Δ*spiA*::aph-pkk233.2::egfp); *S.* Typhimurium ΔSPI-1 (*S.* Typhimurium 14028Δ*invC*::aph*)* (Eichelberg, Ginocchio, and Galan 1994); *S.* Typhimurium ΔSPI-1-GFP (*S.* Typhimurium Δ*invC*::aph-pkk233.2::egfp*)*. The mutant strains were selected using LB media supplemented with 50 mg/ml kanamycin and/or 100 mg/mL ampicillin in the case of the GFP mutant strains.

Mouse-derived neutrophil isolation and stimulation.

Bone marrow-derived cells were isolated from femurs and tibias of C57BL/6 mice. Briefly, bones were removed aseptically, and bone marrow flushed out with RPMI 1640 medium supplemented with 5% fetal calf serum (FBS), 2mM glutamine, 1mM non-essential amino acids, 1mM pyruvate and 2mM EDTA with a 10mL syringe. Bone marrow-derived cells were then pelleted at 400g for 7 min and re-suspended in 2 mL of fresh medium for cell count. The cells were then prepared as described by the manufacturer´s protocol of MACs column and then passed through LS column for negative selection of $Ly6G^+$ cells (neutrophils).

Quantification of NETs induction.

Neutrophils freshly isolated were recovered from MACs column purification and were re-suspended in 2 mL of fresh RPMI and counted in Neubauer chamber. Cells ($1x10^5$/300 µl) were stimulated with *S.* Typhimurium or with different mutant strains of *S.* Typhimurium at a multiplicity of infection (MOI) 25 (bacteria to neutrophils) for 180 min at 37ºC with 5% CO_2. Afterward, culture supernatant was collected, and extracellular DNA was measured using Quant-iT PicoGreen dsDNA (Invitrogen), following manufacturer's instructions.

Intracellular ROS quantification.

Mouse-derived neutrophils ($1x10^5$/200ul) were collected in HBSS and treated with the probe CM-H_2DCFDA (1µM, Invitrogen, cat number: C6827) that react with intracellular ROS production, for 30 min at 37°C under 5% CO_2 in darkness. Then the cells

were treated with H_2O_2 (0.003%), by different strains of *S.* Typhimurium (MOI: 25) or left untreated for 1 h at 37°C under 5% CO_2 in darkness. The data were obtained in LSR Fortessa X-20 Flow cytometry (BD Biosciences) and analyzed in Flow Jo software V7.0.

Neutrophils survival and IL-10 secretion after *S.* Typhimurium infection.

Mouse-derived neutrophils ($1x10^5$/200µl) were infected with different strains of *S.* Typhimurium-GFP for 1 h in rotation at 37ºC under 5% CO_2 with fish gelatin 0.1%. After the infection the culture were treated with gentamicin (100 µg/mL) and evaluated after 2, 6, 12 and 24 h post gentamicin treatment. Then, cells were stained with fixable viability stain Bv510 (BD, cat number: 564406), to evaluate the percentage of cell viability during *S.* Typhimurium infection. The data of cell survival and infection ($FITC^+$ cells) were obtained in LSR Fortessa X-20 Flow cytometry (BD Biosciences) and analyzed in Flow Jo software V7.0. The supernatant of the samples was used to quantify the neutrophils production of IL-10 by ELISA.

RESULTS

1. *S.* Typhimurium *wild type* differentially infects neutrophils from males and females.

It has been previously shown that *S*. Typhimurium *wild type* (WT) is able to infect dendritic cells (Bueno et al. 2012) and macrophages (Monack, Bouley, and Falkow 2004). However, whether *S.* Typhimurium is able to infect neutrophils has not been fully elucidated. In this sense, as it has been shown that the immune response between male and female can be different, we evaluated the infection and all the parameters in neutrophils derived from both male and female mice. Neutrophils derived from male or female mice were infected with *S*. Typhimurium *wild type* (WT) for 1h and then treated for 2, 6 or 24h with gentamicin. As shown in **Fig. 4A**, *S.* Typhimurium infection does not change the viability of neutrophils up to 24h post treatment as compared to the control in each time measured. Further, *S*. Typhimurium can infect bone marrow-derived neutrophils purified from male and female mice at 2h post-infection and survive up to 24 h **Fig. 4B**. The higher rate of infected cells are at 24h post-infection and as shown in **Fig. 4,** neutrophils derived from male mice are infected in higher proportion than the neutrophils derived from female mice, observed as percentage of infection (**Fig. 4B**), mean fluorescence intensity MFI (**Fig. 4C**), and the fold change (FI) over the control (**Fig. 4D**) between both sex, at 24 h post gentamicin treatment. Thus, differential neutrophil responses depending on the sex of the donor occur during *S.* Typhimurium infection at 24h post-infection.

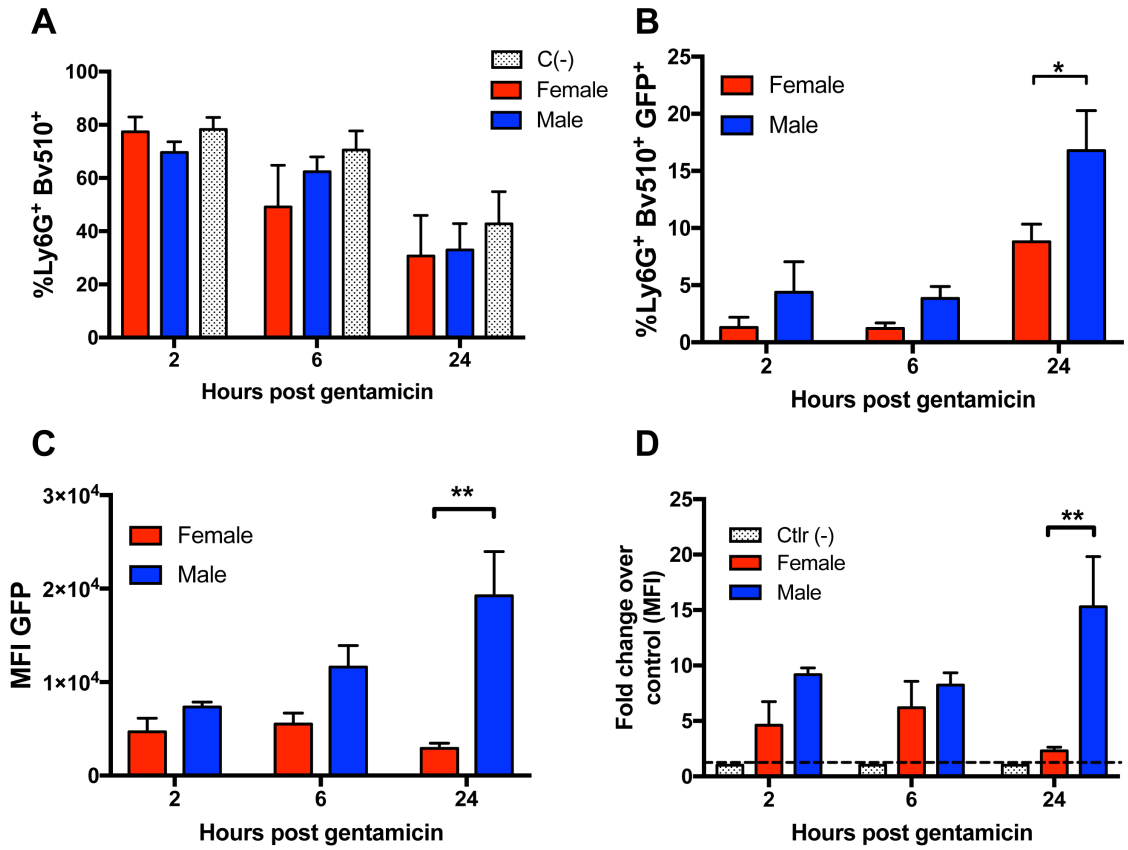

Figure 4: Neutrophil derived from male mice are infected in higher proportion than neutrophils derived from female mice. Neutrophils derived from male mice (blue bars) and female mice (red bars) were infected with *S.* Typhimurium, for one hour and then treated with gentamicin and neutrophils survival, and infection of the cell was evaluated after 2, 6 and 24h, using flow cytometry. **A**. percentage of neutrophil survival after *S.* Typhimurium infection. **B**. percentage of infected cells. **C.** MFI of neutrophils infected (GFP^+ cells). **D**. Fold increase of MFI shown in C respect to each Ctlr(-). P values were determined using 2way ANOVA comparing female versus male in each hour post-infection, *P< 0.0458; **P= 0.0042. Three independent experiment were performed with neutrophils derived from male (n=7) and two independent experiment were performed with neutrophils derived from female mice (n=5).

2. Neutrophils are infected differentially by mutant strains of *S.* Typhimurium.

In order to evaluate the impact of the effector proteins encoded secreted by TTSS-1 and TTSS-2, we infected neutrophils from female and male mice with the mutant strain *invC* (ΔSPI-1) and *spiA* (ΔSPI-2), which lack a functional TTSS-1 and 2, respectively. The mutant strain of the ΔSPI-1 is not able to infect non-phagocytic cells and ΔSPI-2 is not able to survive inside dendritic cells (Bueno et al. 2012) and therefore is less virulent (Schultz et al. 2018).

Neutrophils derived from male or female mice were infected with the three strains, *S.* Typhimurium WT, ΔSPI-1 and ΔSPI-2 for 1h and then treated for 2, 6 or 24h with gentamicin. As mentioned before, *S.* Typhimurium WT infects in higher percentage the male derived neutrophils from 2h post treatment, which is statistically different at 24h post treatment (**Fig. 5A**). In contrast, the ΔSPI-2 strain shows an increased percentage of infection of the female derived-neutrophils at 2 and 6h post gentamicin treatment, although this difference disappear at 24h post treatment (**Fig. 5A**). Neutrophils derived from female mice also present higher percentage of intracellular bacteria at 6 and 24h post treatment with the ΔSPI-1 strain (**Fig. 5A**), compared to what is observed in neutrophils infected with *S.* Typhimurium WT. However, when the MFI (**Fig. 5B**) and FI (**Fig. 5C**) are analyzed this difference is lost. In addition, it is important to mention that the mutant strain ΔSPI-1 infect in less proportion each time measured compared to the infection with the WT or ΔSPI-2 mutant strain of *S.* Typhimurium and, although there was an increased percentage of female derived-neutrophils at 6h and 24h post-infection, this difference was not significant compared to male-derived neutrophils. Importantly, the proportion of infected cells derived from male mice did not increase at any time point (**Fig. 5C**), suggesting that SPI-1 is

required for male-derived neutrophil infection. These results indicate that both SPI-1 and SPI-2 are responsible to some extent to the differential responses observed in male and female-derived neutrophils.

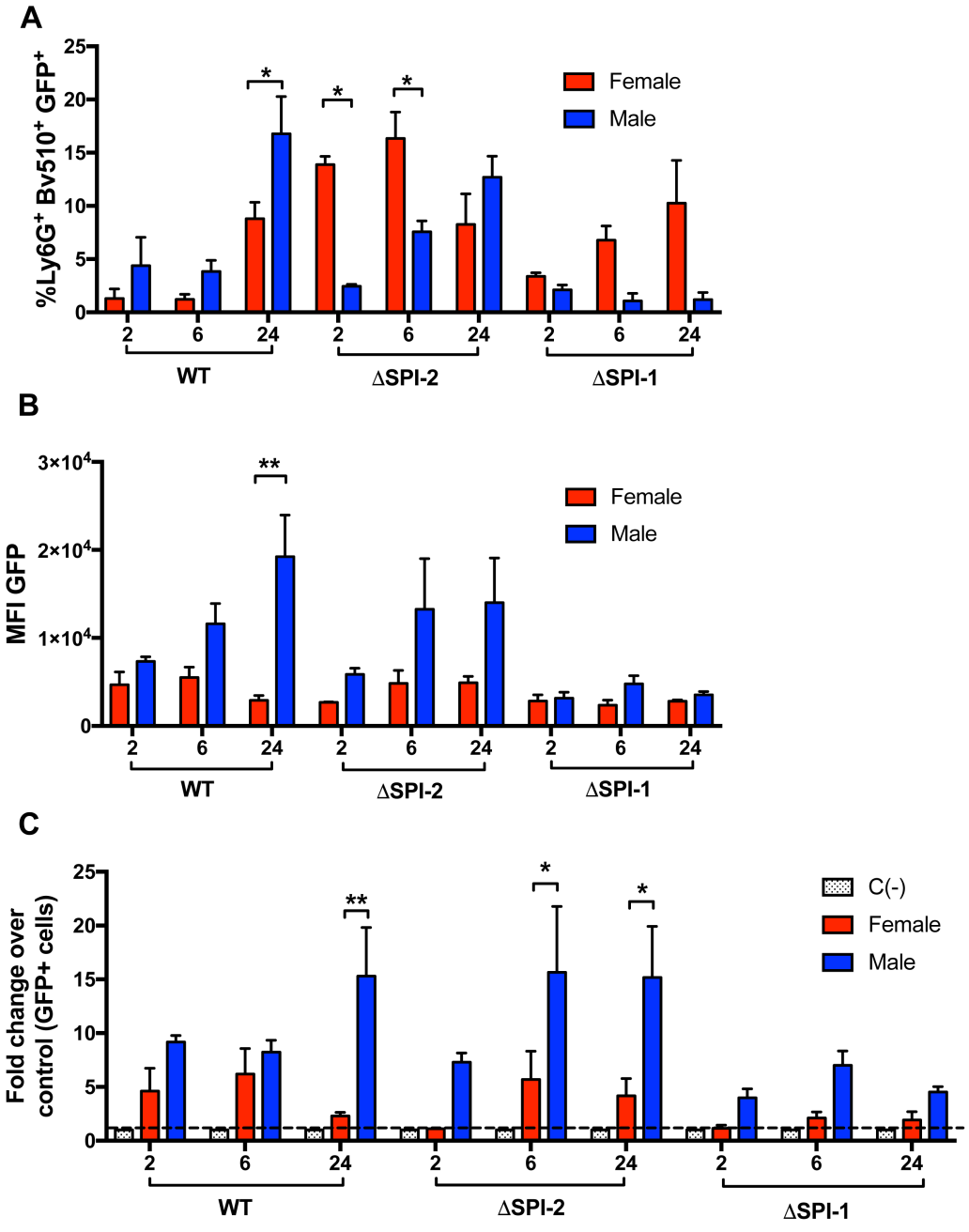

Figure 5: Neutrophils are infected differentially by mutant strains of *S.* Typhimurium. Neutrophils were infected with different strains of S. Typhimurium. Neutrophils derived from male mice (blue bars)

and female mice (red bars) were infected with *S.* Typhimurium WT, ΔSPI-2 and ΔSPI-2, for one hour and then treated with gentamicin. After this, using flow cytometry the infection of the cell was evaluated after 2, 6 and 24h. **A.** percentage of neutrophils derived from male and female mice infected with different strains of *S.* Typhimurium. **B.** MFI of neutrophils infected (GFP^+ cells). **C.** Fold increase of MFI shown in B with respect to each C(-). P values were determined using 2way ANOVA comparing female versus male in each hour post-infection. *P<0.049; **P<0.004. Three independent experiment were performed with neutrophils derived from male (n=6) and two independent experiment were performed with neutrophils derived from female mice (n=4).

3. Infection by *S.* Typhimurium differentially induces NETs release according to sex.

It has been previously shown that *S.* Typhimurium is trapped by NETs (Brinkmann et al., 2004). However, whether *S.* Typhimurium is able to trigger NET release is not fully elucidated. As described in the Submitted paper of this thesis*, S.* Typhimurium induces NETs release from neutrophils derived from female mice in a dose-dependent manner at 3 h post-infection. According to this result, we wanted to evaluate if, as in the case of infection, there are differences between the NETs release among neutrophils derived from male and female mice. We observed that neutrophils derived from male mice release higher concentration of NETs than cells derived from female mice (**Fig. 6A**). Next, we performed an infection with the mutant strains and observed that infection with the ΔSPI-2 strain induces NETs in a similar pattern as the WT strain does (**Fig. 6B**). However, the ΔSPI-1 strain showed increase NETs release in neutrophils derived from female mice (**Fig. 6B**). Also, we performed confocal laser scanning microscopy to detect whether *S.* Typhimurium WT was bound to the NETs. Consistently, our results revealed that NETs produced in response to *S.* Typhimurium infection entrapped bacteria, as visualized by the co-localization of *S.* Typhimurium-GFP with extracellular DNA (**data not shown**). However,

if the NETs entrap and kill the bacteria is still unknown, and also if there some differences in the extracellular killing mediated by the NETs with the different strains used in these experiments.

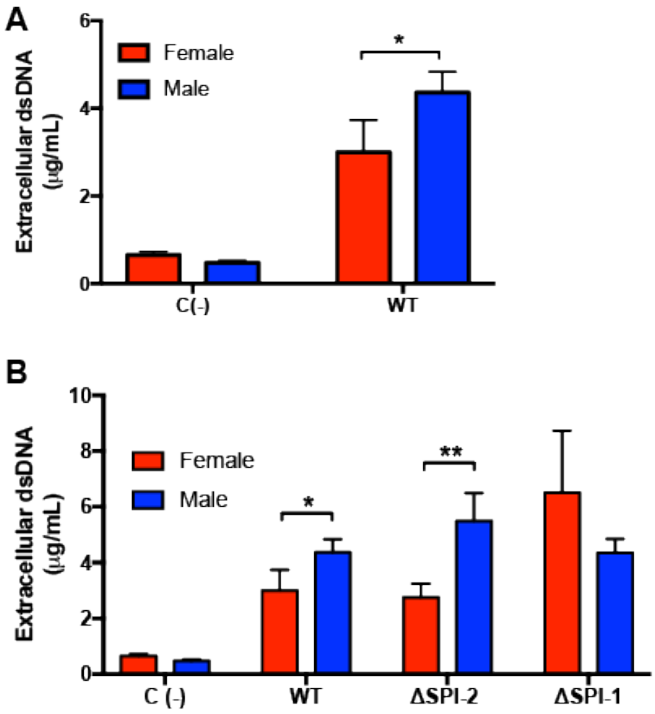

Figure 6. Mutant strains of *S.* Typhimurium induce differentially NETs release. Differentiated NETs release due the infection with *S.* Typhimurium WT **(A)** and the mutant strains of *S.* Typhimurium, SPI-1 and SPI2 **(B)** was evaluated by quantifying extracellular dsDNA. **A.** comparison of NETs release between neutrophils derived from male and female mice infected with *S.* Typhimurium WT, evaluated by detection of extracellular dsDNA. **B.** comparison of NETs release, evaluated by detection of extracellular dsDNA, between neutrophils derived from male and female mice infected with *S.* Typhimurium WT, ΔSPI-1 and ΔSPI-2. P values were determined using 2way ANOVA comparing female versus male. *P=0.0423; **P=0.0047. Eight independent experiments were performed with neutrophils derived from male (n=16) and six independent experiments were performed with neutrophils derived from female mice (n=12).

4. *S.* Typhimurium infection induces intracellular ROS in a sex-dependent manner.

After evaluating the infection and NETs release, we wanted to evaluate the intracellular ROS production in neutrophils derived from male and female mice infected with *S.* Typhimurium WT, ΔSPI-1 and ΔSPI-2 (**Fig 7A**). After one-hour post-infection we could observe that neutrophils derived from female mice induced higher intracellular ROS than neutrophils derived from male mice. Furthermore, the induction among the three different strains presented a similar pattern. However, the production of ROS by female neutrophils infected with the mutant strain ΔSPI-2, which is unable to survive inside the phagocytic cells, is significantly higher. In contrast, the mutant strain ΔSPI-1 did not induce increased ROS production in either female or male-derived neutrophils. These results suggest that SPI-2 but not SPI-1 is able to modulate ROS production in female-derived neutrophils.

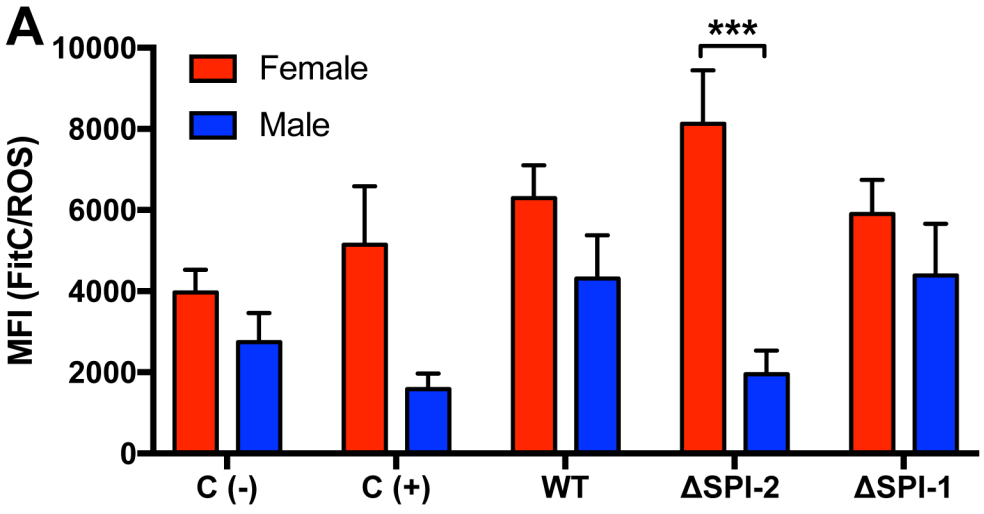

Figure 7. *S.* Typhimurium induces intracellular ROS in a sex-dependent manner. Neutrophils derived from male mice (blue bars) and female mice (red bars) were infected with *S.* Typhimurium WT, ΔSPI-2 and

ΔSPI-2, for one hour and then treated with CM-H_2DCFDA for 30 minutes and then evaluated by flow cytometry the intracellular ROS expressed as MFI. C (-) are neutrophils treated with the probe, but without and stimulus; C (+) are neutrophils treated with the probe and 0,003% of H2O2. P values were determined using 2way ANOVA comparing neutrophils derived from female versus male mice. * ***P=0.0008. Three independent experiment were performed with neutrophils derived from male (n=7) and three independent experiment were performed with neutrophils derived from female mice (n=9).

5. *S*. Typhimurium infection induces the secretion of IL-10.

As different immune cells infected with *S.* Typhimurium secrete IL-10 (Salazar et al. 2017), we wanted to evaluate if neutrophils infected with *S.* Typhimurium also secrete this anti-inflammatory cytokine and if there is some differences between male and female responses. As in the experiments performed above, the production of IL-10 in the supernatant was quantified at 2, 6, and 24h post treatment. We could observe that neutrophils derived from male mice begin to secrete IL-10 at 24h post treatment (**Fig. 8A**) and when we compared the concentration of this protein between male and female-derived neutrophils we observed that male neutrophils secrete higher amounts of this immunomodulatory cytokine compared to female-derived neutrophils (**Fig. 8B and C**). Thus, these results suggest that *S.* Typhimurium is able to induce IL-10 expression in male-derived neutrophils but not female-derived neutrophils in a SPI-1 and SPI-2 independent manner.

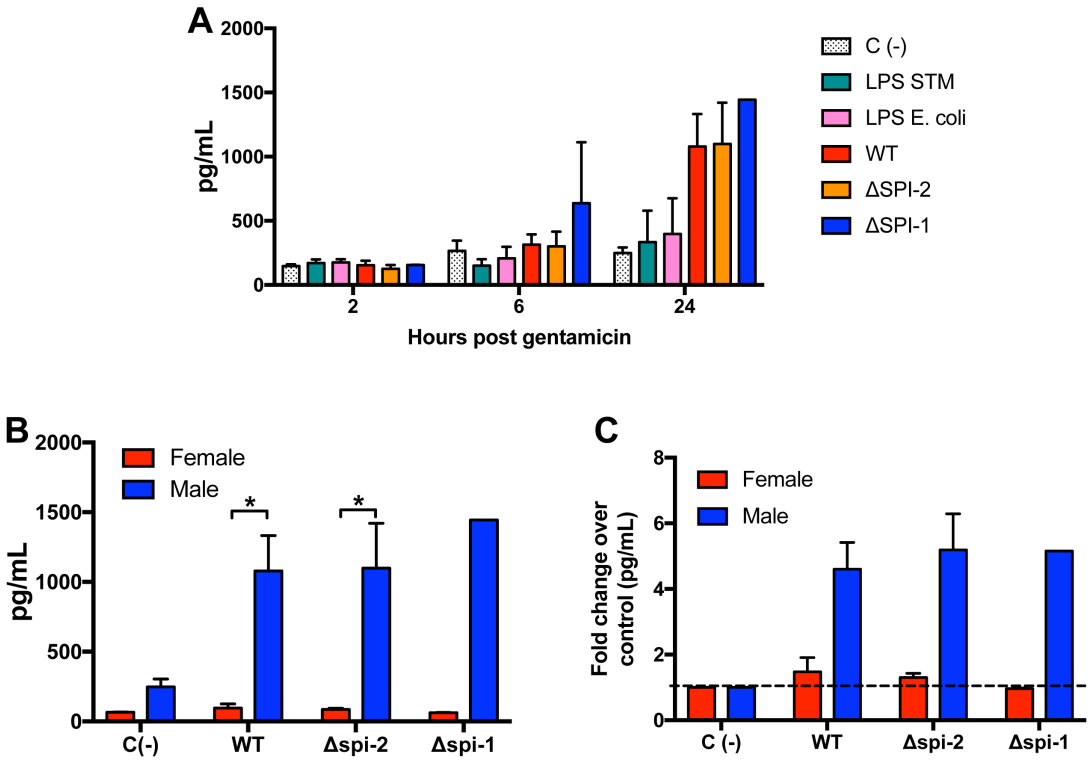

Figure 8: *S.* Typhimurium infection induces the secretion of IL-10. Neutrophils derived from male mice (blue bars) and female mice (red bars) were infected with *S.* Typhimurium WT, ΔSPI-2 and ΔSPI-2, for one hour and then treated with gentamicin. After this, the production of IL-10 was evaluated by WLISA in the supernatant after 2, 6 and 24h **A**. neutrophils derived from male mice secrete IL-10 at 24h post-infection with the different strains of *S.* Typhimurium. **B.** differences between neutrophils derived from male and female mice are observed at 24h post-infection in pg/mL (B) and fold increase with respect to the C (-). P values were determined using 2way ANOVA comparing neutrophils derived from female versus male. *P<0.0220. Two independent experiment were performed with neutrophils derived from male infected with WT and the ΔSPI-2 (n=4) and for the infection with the ΔSPI-1 n=1. One independent experiment was performed with neutrophils derived from female mice infected with WT and ΔSPI-2 (n=2) and for the infection with ΔSPI-1 n=1

GENERAL DISCUSSION

Neutrophils are the first immune cell to respond against bacterial infection and are essential in the immune response against *S.* Typhimurium, and its depletion allows rapid dissemination and colonization of systemic organs (Conlan 1997). *S.* Typhimurium takes advantage of the immune response generated by neutrophils, inducing its recruitment to the intestine, because of the inflammatory environment generated by these cells that is favorable to the development of the disease (Silva et al. 2004). In fact, in streptomycin treated mice, approximately 70% of gut neutrophils are infected with *S.* Typhimurium WT at 1-day post-infection. However, less than 1% of the bacteria are recovered in a gentamycin assay, implying that some neutrophils phagocytose the bacteria and clear them, some of them are maybe dying by the infection, and others are possibly suffering NETosis. Also, after four days of infection, the bacteria are replicating only in the cecum, and the action of neutrophils is not working (Loetscher et al. 2012).

Little is known about the exact interaction between neutrophils and *Salmonella*. In this sense, we could observe that *S.* Typhimurium WT can infect neutrophils from 2h post-infection, not affecting the survival of the cells as compared with uninfected neutrophils (**Fig. 4A**). This was observed in a study with primed human polymorphonuclear cells, where the cells could decrease the number of *S.* Typhimurium infection at 2h post-infection. However, at 24h post-infection, the bacteria increase in number and can lyse the cell (**Fig. 4D**) (Chiu and Ou 1999). It is essential to mention that in our experiments, the neutrophils are infected for 1h, then the cells are treated with gentamycin up to 24h, to eliminate the extracellular bacteria. For this reason, and based on previous studies, the bacteria probably replicate inside the neutrophil and increase in number at 24h post-

infection when was performed the measurement (**Fig 4B-D**) (Chiu and Ou 1999; Dunlap et al. 1992; Geddes et al. 2007). Along this line, a study performed in the early phase of infection in mice showed that at 24h post-infection, most of the bacteria are intracellular, at least in neutrophils derived from the spleen (Dunlap et al. 1992). This data is in agreement with Geddes et al., which show that *S.* Typhimurium can infect different immune cells such as neutrophils, monocytes, and B and T lymphocytes, but spleen neutrophils were infected in higher proportion than the other cells and also can replicate inside them (Geddes et al. 2007).

The infection of neutrophils with a mutant strain shows that the bacteria can induce the phagocytic process in neutrophils, as the mutant strain of SPI-1 (ΔSPI-1) is found to a lesser degree than the WT strain and the SPI-2 mutant strain (ΔSPI-2) (**Fig. 5**). This is because the proteins encoded in SPI-1 genes are related to non-phagocytic cell infection and can mediate this internalization in neutrophils. However, when Geddes et al. infected lymphocytes with ΔSPI-1, they did not observe differences in the percentage of infection. In addition, they did not observe differences in the colonization of these cells when performed a competition assay, a difference that can be due to the type of immune cell evaluated (Geddes et al. 2007). On the other hand, a study performed with the same mutant strain (*ΔinvC*) shows that the TTSS-1 reduce the number of bacteria that the dendritic cell can engulf, increasing when this gene is absent. In the case of neutrophils, we observed the opposite pattern, decreasing the internalization of the bacteria when we infected the cells with ΔSPI-1, which can imply a different mechanism used in both cells (Bueno et al. 2010). In the case of ΔSPI-2, this strain does not show a different infection pattern as compared to the WT strain (**Fig. 5**). However, during murine infection with ΔSPI-2, neutrophils

recruitment and the immune response activation is diminished (Cheminay, Chakravortty, and Hensel 2004). Even more, this strain does not generate a systemic infection in mice (Schultz et al. 2018). Of note, the action of intestinal epithelial cells is detrimental in the activation of neutrophils (Diebel et al. 2005). It has been evaluated that these cells can modulate the immune response, reduce neutrophil apoptosis, and activate them. There are different signals that can activate neutrophils as PAMPs (such as LPS) and cytokines such as CXCL8, TNF-α, and GM-CSF (Brazil and Parkos 2016; Diebel et al. 2005). This activation was not evaluated in this work, and maybe is necessary the priming or activation of neutrophils to exhibit a different pattern of infection or immune response.

S. Typhimurium triggered NETs release from human and mouse-derived neutrophils after 3h post-infection (**Fig 1**). Moreover, as we can observe in chapter two, neutrophils derived from female and male mice infected with *S.* Typhimurium WT induced NETs release 3h post-stimulation (**Fig. 6A**) (Brinkmann et al. 2004). We replicated NETs release experiments performed in neutrophils derived from female mice due to the infection of *S.* Typhimurium WT, and ΔSPI-2 performed in Brazil (**Fig. 1-3 compared to Fig. 6**). Furthermore, when we stimulated neutrophils derived from female mice with the ΔSPI-1, the NETs release is higher than the induced by the WT and ΔSPI-2. However, this difference was not significant (**Fig. 6B**). The infection with different strains of *S.* Typhimurium did not present significant differences between males and female-derived neutrophils in mice, suggesting that genes encoded in SPI-1 and SPI-2 do not modulate NET release. Furthermore, as shown in the results of chapter 1 (**Fig. 1**), *S.* Typhimurium induces NETs release accompanied by granular proteins MPO and NE in human derived-neutrophils. However, we could not corroborate this pattern in mouse-derived neutrophils.

An essential point of these results is that we observed differences between female human and mouse-derived neutrophils (**Fig. 3**), which can be due to the bacteria's host-specific disease. In this sense, further studies need to be done to identify if these differences can also be observed in the pattern of granular proteins that accompanied the NETs release and evaluate also if this sex-dependent immune response is observed in human-derived neutrophils.

We also studied whether ROS production in neutrophils during *S.* Typhimurium was affected in a sex-dependent-manner using mouse-derived neutrophils. In human-derived neutrophils infected with *S.* Typhimurium, the induction of intracellular ROS begins at 1h post-infection, but the peak of this intracellular ROS was at 2h post-infection, even more in GM-CSF-primed human neutrophils (Westerman et al. 2018). In another study also performed in human neutrophils, the internalization of the bacteria and NADPH oxidase activation depends on the opsonization of the bacteria and the activation of TLR4 (Van Bruggen et al. 2007). In our experiments, intracellular ROS induction by *S.* Typhimurium was evaluated in non-primed neutrophils and we could observe higher production intracellular ROS in neutrophils derived from female mice, with a significant difference in neutrophils infected with ΔSPI-2 (**Fig. 7**), which is expected since neutrophils do not have the genes necessary for the inactivation of NADPH oxidase (Vazquez-Torres et al. 2000). We did not observe significant differences between the different strains in ROS production, suggesting that neither SPI-1 nor SPI-2 modulate ROS production in neutrophils. However, there is a tendency in the ROS induction by ΔSPI-2 in female mice, which is contrary in the case of male mice. In this line, the probe CM-H_2DCFDA used in this study is not specific for a type of oxidative agent and is more a measure of cellular

oxidant level, which imply that our measure is not precisely a measure of ROS level. We have a mixture of these reactive species that not necessarily depends on NADPH oxidase (Oparka et al. 2016). In this sense, first, it is possible that primed neutrophils generate a more robust oxidative burst, and second, we are measuring all types of oxidative agents, which could explain why we did not observe any difference, as other studies have reported.

Our results highlight an important role of SPI-2 in the regulation of female-derived neutrophil responses against *S.* Typhimurium. However, we observed some discrepancies between the percentage of infected cells and the mean fluorescence intensity (MFI) analyses. During the infection of female-derived neutrophils with ΔSPI-2, a higher percentage of GFP^+ cells was observed (**Fig. 4A**), which is lost when we analyzed the MFI and FI (**Fig. 4B-C**). This effect can be due to the percentage of GFP^+ cells is not indicative of the fluorescence intensity. The MFI evaluates the fluorescence of each individual, which can be high or low for some cells. In this case, an important GFP^+ (and infected) population may present a low fluorescence that decreases the MFI. In this sense, most of the bacteria inside the neutrophils derived from female mice infected with ΔSPI-2 could be dead. This could reduce the GFP^+ signal and it is consistent with the effect in the neutrophil oxidative stress, observed in **Fig. 7**, which can affect the survival of the bacteria and, in consequence, the GFP fluorescence. Therefore, these results suggest that the lack of essential genes encoded in TTSS-2 make the mutant strain of SPI-2 to be more susceptible to the intracellular response of the phagocytic cells, inhibiting intracellular survival and replication of the bacteria. Importantly, male-derived neutrophils did not exhibit increased ROS production in response to ΔSPI-2, although they still exhibited increased yet

insignificant neutrophil infection. These results suggest that intrinsic sex-dependent mechanisms may regulate neutrophils responses during *S.* Typhimurium infection.

One of the molecules involved with sex-dependent differences in immune cells are PG, which are important mediators of the immune response (Tilley, Coffman, and Koller 2001). In a model of acute inflammation neutrophils respond differently depending on the sex of the animal: male neutrophils present higher expression of NF-κβ, COX-2, and PGE2 than female neutrophils (Pace et al. 2017). *S.* Typhimurium induces, in an SPI-2-dependent manner, the production of PG in murine macrophages, which could enhance the capacity of the bacteria to survive inside the cell (Uchiya and Nikai 2004). The induction of PGE2 decreases the killing abilities of alveolar macrophages and NADPH oxidase localization and, consequently, ROS production (Serezani et al. 2007). Also, the induction of PGE2 by *S.* Typhimurium activates the protein kinase A and upregulate the production of IL-10 by this cell, promoting an immunosuppressive phenotype (Uchiya, Groisman, and Nikai 2004). This induction is not just related to *S.* Typhimurium infection, in fact *Escherichia coli*, *Shigella*, different strains of *Mycobacterium spp*., and *Streptococcus pneumoniae*, induce the production of PGE2 (Agard et al. 2013), to take advantage of the anti-inflammatory environment that is induced. In the case of neutrophils, it has been seen that the induction of PGE2 affects the recruitment, uptake, and respiratory burst of these cells during *Listeria monocytogenes* infection (Pitts and D'Orazio 2019). All this evidence is in line with our observations: increased percentages of bacteria inside the neutrophils derived from male compared to female-derived neutrophils (**Fig. 3 and Fig 4**), less intracellular killing, which can be related to less oxidative burst (**Fig 7**), and higher IL-10 production (**Fig. 8**).

In the case of NETs release, it has been seen that PGE2 inhibits the NETs release induced by PMA (Shishikura et al. 2016). However, in the case of *S.* Typhimurium infection, the pathway of NETs release is not related to ROS or PAD4 (**Fig. 2**). In this sense, it is possible that the NETs release is related to an intracellular signal and the activation of the pore-forming gasdermin-D (Chen et al. 2018), which may be the reason why we do not observe a decrease in NETs release of male neutrophils (**Fig. 6**). Further work needs to be done in this mechanism, in order to identify the specific pathway activated during *S.* Typhimurium infection.

S. Typhimurium may generate a different immune response in *in vivo* infection between male and female mice, which must be evaluated and observe if there are different neutrophils recruitment to the intestine or if the specific response of these cells changes between both sexes in the liver or spleen. Grainger *et al.* show that during the infection with the intracellular parasite *Toxoplasma gondii* monocytes modulate the immune response of neutrophils by the induction of PGE2, decreasing the immune response and improving mouse survival. In contrast, if this immune regulation mediated by PGE2 did not occur, mice died exhibiting an excessive infiltrate of neutrophils and inflammation (Grainger et al. 2013). *In vivo* experiments performed with *Campylobacter jejuni,* another gastrointestinal pathogen, shows that male mice were more susceptible to be heavily colonized in 100% of the animal versus only 25% of the female mice. Female mice show a better clearance of the bacteria four weeks after infection and it has been reviewed that gastrointestinal infection presents a successful outcome in the females. However, it depends on the type of infection and the pathogen (Vázquez-Martínez et al. 2018). In the case of *S.* Typhimurium infection, it is not well known if male mice are more susceptible to the infection.

It is also essential to evaluate neutrophils immune response in *in vivo* infection because different signals present in the intestine can activate them. The differences between both sexes begin with the colonization of the intestine and the effects in the microbiota. Fachi *et al.* show that the microbiota-derived short chain fatty acid acetate affects the activation, recruitment, and IL-1β secretion by the neutrophils (Fachi et al. 2020). However, the acetate also can act as an inductor of virulence genes of *S.* Typhimurium necessary to a proper colonization of the ileum (Lawhon et al. 2002). Moreover, it is also essential to evaluate the immune response generated by human neutrophils against *S.* Typhimurium because the immune response between humans and mice is different due to the host restriction, as we could observe in chapter 1 of this work (**Fig. 1 and 3**). Previous work in our laboratory corroborates this, showing that the infection and the immune modulation of human or mice-derived dendritic cells depend on what serovar of *Salmonella enterica* is used (Bueno et al. 2008). In this sense, *S.* Typhi and *S.* Enteritidis failed to avoid the antigen presentation mediated by murine dendritic cells, which did not happen when dendritic cells are infected with *S.* Typhimurium. In contrast, only *S.* Typhi can replicate inside human dendritic cells, and *S.* Typhimurium and *S.* Enteritidis are degraded and a proper immune response is generated (Bueno et al. 2008).

Finally, further work is required to corroborate these differences between male and female human-derived neutrophils infected with *S.* Typhimurium. In that case, these data could be necessary for a proper treatment for salmonellosis or even in invasive non-typhoidal salmonellosis. As an example of this sexual dimorphism, *S.* Typhi infections are more likely to generate chronic diseases or carriage in women than men (Gal-Mor 2019;

Gunn et al. 2014). However, as mentioned above, this can be due to the host restriction and can be different in *S.* Typhimurium infection in humans.

CONCLUSIONS

Here, we analyze different antimicrobial mechanisms generated by neutrophils at different times post infection with *S.* Typhimurium (**Fig. 9**). The work performed in this thesis is the first evaluating the neutrophils function against *S.* Typhimurium infection in animals of both sexes. In this sense, the immune mechanisms evaluated, shown in **Fig. 9A,** present a different pattern depending on the sex of mice-derived cells. *S.* Typhimurium can infect and survive inside both sexes' neutrophils and possibly replicate inside them, inducing NETs release and oxidative burst. In male and female derived neutrophils, the engulfment process was inhibited by genes encoded in SPI-1. In addition, neutrophils derived from male mice present higher intracellular bacteria and IL-10 production at 24h post infection, which is consistent with the increase bacterial burden, since a higher production of IL-10 inhibits the bacterial killing and ROS production (**Fig. 9B**). On the other hand, female-derived neutrophils (**Fig. 9C**) present less IL-10 production which correlates to a better intracellular killing due to higher intracellular ROS production and in consequence less proportion of intracellular bacteria at 24h post-infection. This effect on male neutrophils seems to be mediated by genes encoded in SPI-2, as seen in our assays using an SPI-2 mutant strain.

All these results are in agreement with the previous results observed in macrophages infected with *S.* Typhimurium (Uchiya et al. 2004; Uchiya and Nikai 2004). These results show that *S.* Typhimurium induces PGE2 in a SPI-2-dependent manner, which induce the

secretion of IL-10 and subsequently this cytokine inhibited intracellular ROS production, the killing of intracellular bacteria and the NETs release induced by the classical pathway. These results are consistent with the data presented here in neutrophils derived from male mice. A limitation of these study is that all these results are evaluated independently, however during the infection all of them are occurring at a certain time and is necessary to evaluate this during *S.* Typhimurium infection in order to identify which of them are induced first or which is the best mechanism to kill the bacteria.

Finally, we can conclude that *S.* Typhimurium induces NETs release at 3h post infection, in human and in mice, although we were not able to identify the pathway of induction. Additionally, *S.* Typhimurium can live in the intracellular space of neutrophils, modulating some of the response of these cells by genes encoded in SPI-1 and SPI-2. However, the exact mechanisms used to modulate the phagocytic process, or the induction of ROS was not evaluated. Also, *S.* Typhimurium induce the immunomodulatory function in neutrophils at 24h post-infection, which is in line with previous reports. All the immune responses evaluated here depends on the sex of the animal and it is possible that this response and the overall response of the animal to infection with *S.* Typhimurium will depend on this, which must be evaluated.

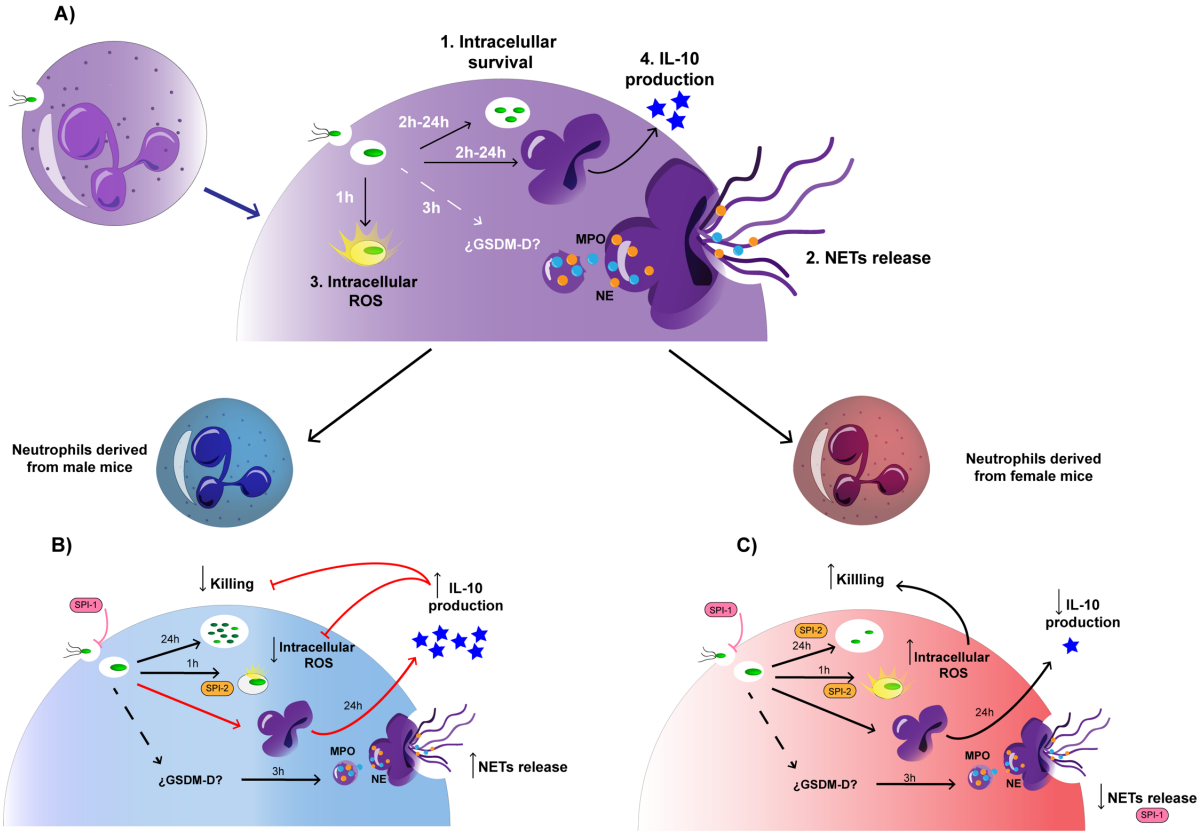

Figure 9: Differences between the immune response of neutrophils derived from male and female mice. **A)** different responses evaluated in neutrophils at different times: 1. Intracellular survival evaluated at 2 to 24h; 2. NETs release evaluated at 3h post infection; 3. Intracellular ROS, evaluated at 1h post-infection; 4. IL-10 production evaluated at 2 to 24h. **B)** Immune response evaluated in male derived neutrophils. **C)** Immune response evaluated in female derived neutrophils

FUTURE PERSPECTIVES

Some experiments need to be performed to complete the results observed in this thesis.

1. Experiments related to the infection are necessary to evaluate if female neutrophils can kill more efficiently the bacteria during the experiment, and if the bacteria can replicate inside the cell. These experiments have to be performed with the three strains to evaluate the differences observed with the mutant strain of SPI-2. It is also necessary to evaluate the cytokine production in the supernatant of the neutrophils infected and measure the production of PGE2 by the immune cells. All these in order to confirm the results observed here and delineate a pathway.
2. Related to the NETs induction, it is necessary to corroborate MPO and NE's colocalization with the extracellular DNA and the pathway by which this structure is release. It is also important to evaluate if the NETs entrap the bacteria, or the bacteria can replicate independently of this antimicrobial mechanism. All these experiments have to be performed with the three strains and using female and male-derived neutrophils. In the case of NETs release is possible to perform this experiment with primed neutrophils.
3. To evaluate ROS proper induction is necessary to utilize another probe, as HyPer, which is specific from H_2O_2 and perform the experiments with primed neutrophils (with PMA or GM-CSF).
4. Complete the experiment related to the induction of IL-10 by female neutrophils, as the experiment replicates were not enough.
5. In regard to the results observed in human neutrophils, as the experiments were performed just in women-derived neutrophils, it is necessary to perform new

experiment to observed differences between women and men-derived neutrophils and human versus mice derived neutrophils.

6. The pending experiments should be carried out in animal of both sexes to elucidate if PGE2 has a role in the differences observed.

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APPENDIX

Scientific meetings attended during this thesis

1) B. M. Schultz, V. P. Sebastian, P. H. Silva, C. P. Saavedra, C. Santiviago, A. M. Kalergis, S. M. Bueno. *Salmonella enterica* serovar Typhimurium Infection Affects Ly6G$^+$ Cells. **Association Society for Microbiology Microbe**, Online 2020, ePoster
2) Schultz Bárbara M**.**, Valentina P. Sebastian, Fernanda Balmaceda, Pedro H. Silva, Liliana Gonzalez, Alexis M. Kalergis, Susan M. Bueno. **XLI Congreso Chileno de Microbiología (SOMICH)**. Puerto Varas. Role of Ly6G$^+$ cells during *Salmonella enterica* serovar Typhimurium infection. November 5-8, 2019. Poster presentation.
3) Schultz Bárbara M., Fernanda Balmaceda, Valentina P. Sebastian, Susan M. Bueno. **8th Congreso Europeo de Microbiología**. Glasgow. Role of Ly6G$^+$ cells during *Salmonella enterica* serovar Typhimurium infection. July 7-11, 2019. Poster presentation.
4) **Schultz Bárbara M.,** Balmaceda Fernanda, Murano Stefanie, Fabiano De Souza Gabriela, Dias De Oliveira Silvia, Porto Bárbara N., Bueno Susan M. Virulent *Salmonella enterica* serovar Typhimurium modulates the production of neutrophils extracellular traps. **XXIV Congreso Latinoamericano de Microbiologia**, Santiago, November 13-16, 2018. Poster presentation.
5) Schultz, Bárbara M., Primon Murano, Stéfanie, Souza Fabiano, Gabriela, Silveira Gregis, Marina, Landvoight Schmitt, Brenda, Dias de Oliveira, Silvia, Porto, Bárbara N. and Bueno, Susan M. Virulent *Salmonella enterica* serovar Typhimurium modulates the production of neutrophils extracellular traps. **5th**

European congress of Immunology, Amsterdam, September 2-5, 2018. Poster presentation.

6) Bárbara M. Schultz, Geraldyne A. Salazar, Carolina A. Paduro, Catalina Pardo-Roa, Daniela P. Pizarro, Francisco J. Salazar-Echegarai, María J. Altamirano, Javiera Torres, Claudia A. Riedel, Alexis M. Kalergis, Manuel M. Álvarez-Lobos, and Susan M. Bueno. Persistent *Salmonella enterica* serovar Typhimurium infection promotes chronic intestinal inflammation in susceptible mice. **XXXIX Congreso Chileno de Microbiología (SOMICH)**, La Serena, November 17-17, 2017. Poster presentation.
7) Schultz, Bárbara M., Salazar, Geraldyne A., Paduro, Carolina A., Pardo-Roa, Catalina, Salazar-Echegarai, Francisco J., Altamirano, María J., Torres, Javiera, Riedel Claudia A., Kalergis, Alexis M., Alvarez-Lobos, Manuel M. and Bueno, Susan M. Persistent *Salmonella enterica* serovar Typhimurium infection promotes chronic intestinal inflammation in susceptible mice. **18th International congress of mucosal immunology**. Washington D.C., July 19-22, 2017. Poster presentation.
8) Bárbara M. Schultz**,** Geraldyne Salazar, Carolina paduro, Catalina Pardo, Daniela Pizarro, Francisco Salazar-Echegarai, Maria Jose Altamirano, Javiera Torres, Claudia Riedel, Alexis Kalergis, Manuel Alvares-Lobos, Susan Bueno, *Salmonella enterica* serovar Typhimurium infection promotes persistent intestinal inflammation in susceptible mice. **XXXVIII Congreso Chileno de Microbiología (SOMICH)**, Chile, Valdivia, 2016. Oral presentation

Scientific publications generated in this thesis

1) Hernan Peñaloza, **Bárbara M. Schultz**, Pamela Nieto, Geraldyne Salazar, Isidora Suazo, Pablo Gonzalez, Claudia Riedel, Manuel Alvarez-Lobos, Alexis Kalergis, Opposing roles of IL-10 in acute bacterial infection, Cytokine Growth Factor Review, 2016.
2) **Bárbara M. Schultz**, Carolina Paduro, Geraldyne Salazar, Francisco Salazar-Echegarai, Valentina Sebastian, Claudia Riedel, Alexis Kalergis, Manuel Alvares-Lobos, A potential role of *Salmonella* infection in the onset of Inflammatory Bowel Diseases., Frontiers in immunology - Mucosal Immunity, Aug 2017.
3) Geraldyne A. Salazar, Hernán F. Peñaloza, Catalina Pardo-Roa, **Bárbara M. Schultz**, Natalia Muñoz-Durango, Roberto S. Gómez-Johnson, Francisco J. Salazar, Daniela P. Pizarro, Claudia A. Riedel, Pablo A. González, Manuel Alvarez-Lobos, Alexis M. Kalergis, Susan M. Bueno. Interleukin-10 Production by T and B Cells Is a Key Factor to Promote Systemic *Salmonella enterica* Serovar Typhimurium Infection in Mice. Frontiers in immunology, No. 8, Aug 2017.
4) **Bárbara M. Schultz**, Geraldyne A. Salazar, Carolina A. Paduro, Catalina Pardo-Roa, Daniela P. Pizarro, Francisco J. Salazar-Echegarai, Javiera Torres, Claudia A. Riedel, Alexis M. Kalergis, Manuel M. Álvarez-Lobos and Susan M. Bueno. Persistent *Salmonella enterica* serovar Typhimurium infection increases the susceptibility of Mice to Develop intestinal inflammation. Frontiers in Immunology, 2018.
5) Valentina P. Sebastián, Geraldyne A. Salazar , Irenice Coronado-Arrázola, **Bárbara M. Schultz**, Omar P. Vallejos, Loni Berkowitz, Manuel M. Álvarez-Lobos, Claudia

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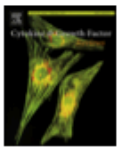

Mini review

Opposing roles of IL-10 in acute bacterial infection☆

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ABSTRACT

Interleukin-10 (IL-10) is recognized as an anti-inflammatory cytokine that downmodulates inflammatory immune responses at multiple levels. In innate cells, production of this cytokine is usually triggered after pathogen recognition receptor (PRR) engagement by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patters (DAMPs), as well as by other soluble factors. Importantly, IL-10 is frequently secreted during acute bacterial infections and has been described to play a key role in infection resolution, although its effects can significantly vary depending on the infecting bacterium. While the production of IL-10 might favor host survival in some cases, it may also result harmful for the host in other circumstances, as it can prevent appropriate bacterial clearance. In this review we discuss the role of IL-10 in bacterial clearance and propose that this cytokine is required to recover from infection caused by extracellular or highly pro-inflammatory bacteria. Altogether, we propose that IL-10 drives excessive suppression of the immune response upon infection with intracellular bacteria or in non-inflammatory bacterial infections, which ultimately favors bacterial persistence and dissemination within the host. Thus, the nature of the bacterium causing infection is an important factor that needs to be taken into account when considering new immunotherapies that consist on the modulation of inflammation, such as IL-10. Indeed, induction of this cytokine may significantly improve the host's immune response to certain bacteria when antibiotics are not completely effective.

1. Introduction

Vaccines and antibiotics have dramatically reduced the mortality rate caused by bacterial infections worldwide [1]. However, bacterial infections remain a major death cause in children, the elderly and immunocompromised patients, especially in low-income countries [1].

Upon bacterial infection, the host initiates an immediate and unspecific immune response intended to eliminate the pathogen as quick as possible, which usually is driven by neutrophils, macrophages and dendritic cells (DCs) [2]. To further promote pathogen clearance, the immune system develops an adaptive and antigen-specific immune response that is mainly driven by T and B cells, which work synergistically with innate cells to limit pathogen dissemination. Effective clearance of microbial infections also depends on the concerted action of cytokines, secreted both by the immune cells and by the infected tissues [3]. Cytokines and chemokines are soluble proteins produced after diverse stimuli, and may derive from immune [4,5] and non-immune cells [6,7]. Importantly, these proteins can attract immune cells to the site of

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A Potential Role of *Salmonella* Infection in the Onset of Inflammatory Bowel Diseases

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Inflammatory bowel disease (IBD) includes a set of pathologies that result from a deregulated immune response that may affect any portion of the gastrointestinal tract. The most prevalent and defined forms of IBD are Crohn's disease and ulcerative colitis. Although the etiology of IBD is not well defined, it has been suggested that environmental and genetic factors contribute to disease development and that the interaction between these two factors can trigger the pathology. Diet, medication use, vitamin D status, smoking, and bacterial infections have been proposed to influence or contribute to the onset or development of the disease in susceptible individuals. The infection with pathogenic bacteria is a key factor that can influence the development and severity of this disease. Here, we present a comprehensive review of studies performed in human and mice susceptible to IBD, which supports the notion that infection with bacterial pathogens, such as *Salmonella*, could promote the onset of IBD due to permanent changes in the intestinal microbiota, disruption of the epithelial barrier and alterations of the intestinal immune response after infection.

Keywords: inflammatory bowel disease, Crohn's disease, ulcerative colitis, gut microbiota, *Salmonella enterica* serovar Typhimurium, innate immune response, virulence factors

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INTRODUCTION

Inflammatory bowel disease (IBD) is defined as a set of pathologies that exhibit a progressive and chronic phenotype, where the intestinal immune response and the normal gut microbiota are altered (1). IBD usually begins in adolescence and persists lifelong (2). The symptoms of these inflammatory disease are not only limited to the gastrointestinal level but also produces systemic complications such as fever, weight loss, delayed sexual maturation and growth, among others. Further, extraintestinal diseases can be associated with IBD, including arthritis (3). The most common clinical manifestations of IBD are Crohn's disease (CD) and ulcerative colitis (UC) (2). CD, a manifestation that affects females in a greater proportion, is characterized by a chronic and transmural inflammation, specifically at the colon and small intestine. However, inflammatory lesion during CD can be found at any section of the gastrointestinal tract, from the mouth to the anus (4). These lesions can affect

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Interleukin-10 Production by T and B Cells Is a Key Factor to Promote Systemic *Salmonella enterica* Serovar Typhimurium Infection in Mice

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Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a Gram-negative bacterium that produces disease in numerous hosts. In mice, oral inoculation is followed by intestinal colonization and subsequent systemic dissemination, which leads to severe pathogenesis without the activation of an efficient anti-*Salmonella* immune response. This feature suggests that the infection caused by *S.* Typhimurium may promote the production of anti-inflammatory molecules by the host that prevent efficient T cell activation and bacterial clearance. In this study, we describe the contribution of immune cells producing the anti-inflammatory cytokine interleukin-10 (IL-10) to the systemic infection caused by *S.* Typhimurium in mice. We observed that the production of IL-10 was required by *S.* Typhimurium to cause a systemic disease, since mice lacking IL-10 (IL-10$^{-/-}$) were significantly more resistant to die after an infection as compared to wild-type (WT) mice. IL-10$^{-/-}$ mice had reduced bacterial loads in internal organs and increased levels of pro-inflammatory cytokines in serum at 5 days of infection. Importantly, WT mice showed high bacterial loads in tissues and no increase of cytokines in serum after 5 days of *S.* Typhimurium infection, except for IL-10. In WT mice, we observed a peak of *il-10* messenger RNA production in ileum, spleen, and liver after 5 days of infection. Importantly, the adoptive transfer of T or B cells from WT mice restored the susceptibility of IL-10$^{-/-}$ mice to systemic *S.* Typhimurium infection, suggesting that the generation of regulatory cells *in vivo* is required to sustain a systemic infection by *S.* Typhimurium. These findings support the notion that IL-10 production

Abbreviations: *S.* Typhimurium, *Salmonella enterica* serovar Typhimurium; IL-10, interleukin-10; DCs, dendritic cells; BMM, bone marrow-derived macrophages; EGFP, enhanced green fluorescent protein; MOI, multiplicity of infection; Treg, regulatory T cells.

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ORIGINAL RESEARCH
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Persistent *Salmonella enterica* serovar Typhimurium Infection Increases the Susceptibility of Mice to Develop Intestinal Inflammation

*Bárbara M. Schultz[1], Geraldyne A. Salazar[1], Carolina A. Paduro[1], Catalina Pardo-Roa[1], Daniela P. Pizarro[1], Francisco J. Salazar-Echegarai[1], Javiera Torres[2], Claudia A. Riedel[3], Alexis M. Kalergis[1,4], Manuel M. Álvarez-Lobos[5] and Susan M. Bueno[1]**

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Chronic intestinal inflammations are triggered by genetic and environmental components. However, it remains unclear how specific changes in the microbiota, host immunity, or pathogen exposure could promote the onset and exacerbation of these diseases. Here, we evaluated whether *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) infection increases the susceptibility to develop intestinal inflammation in mice. Two mouse models were used to evaluate the impact of *S.* Typhimurium infection: the chemical induction of colitis by dextran sulfate sodium (DSS) and interleukin (IL)-10$^{-/-}$ mice, which develop spontaneous intestinal inflammation. We observed that *S.* Typhimurium infection makes DSS-treated and IL-10$^{-/-}$ mice more susceptible to develop intestinal inflammation. Importantly, this increased susceptibility is associated to the ability of *S.* Typhimurium to persist in liver and spleen of infected mice, which depends on the virulence proteins secreted by *Salmonella* Pathogenicity Island 2-encoded type three secretion system (TTSS-2). Although immunization with a live attenuated vaccine resulted in a moderate reduction of the IL-10$^{-/-}$ mice susceptibility to develop intestinal inflammation due to previous *S.* Typhimurium infection, it did not prevent bacterial persistence. Our results suggest that persistent *S.* Typhimurium infection may increase the susceptibility of mice to develop inflammation in the intestine, which could be associated with virulence proteins secreted by TTSS-2.

Keywords: ***Salmonella enterica*** **serovar Typhimurium, inflammatory bowel disease, colitis, interleukin-10, persistence, dextran sulfate sodium**

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INTRODUCTION

Inflammatory bowel diseases (IBDs) are chronic intestinal immune disorders that include Crohn's disease (CD) and ulcerative colitis (UC) (1). Both have increased their incidence worldwide in the last decades and are becoming an important social and economic burden (2, 3). Although the etiology of IBD remains unknown, the development of the disease requires a genetic component

Abbreviations: *S.* Typhimurium, *Salmonella enterica* serovar Typhimurium; SPI, *Salmonella* pathogenicity island; IBD, inflammatory bowel disease; DSS, dextran sulfate sodium; IL-10, interleukin-10; TTSS, type three secretion system; WT, wild type; p.i., post-infection.

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REVIEW
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Heme Oxygenase-1 as a Modulator of Intestinal Inflammation Development and Progression

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Heme Oxygenase 1 (HMOX1) is an enzyme that catalyzes the reaction that degrades the heme group contained in several important proteins, such as hemoglobin, myoglobin, and cytochrome p450. The enzymatic reaction catalyzed by HMOX1 generates Fe^{2+}, biliverdin and CO. It has been shown that HMOX1 activity and the by-product CO can downmodulate the damaging immune response in several models of intestinal inflammation as a result of pharmacological induction of HMOX1 expression and the administration of non-toxic amounts of CO. Inflammatory Bowel Diseases, which includes Crohn's Disease (CD) and Ulcerative Colitis (UC), are one of the most studied ailments associated to HMOX1 effects. However, microbiota imbalances and infections are also important factors influencing the occurrence of acute and chronic intestinal inflammation, where HMOX1 activity may play a major role. As part of this article we discuss the immune modulatory capacity of HMOX1 during IBD, as well during the infections and interactions with the microbiota that contribute to this inflammatory disease.

Keywords: heme oxygenase-1, inflammatory bowel disease, carbon monoxide, infection, inflammation, nuclear factor erythroid 2-related factor 2, colorectal cancer, microbiota

INTRODUCTION

Heme oxygenase 1 (HMOX1) is an enzyme that catalyzes the first step of the oxidative degradation of the heme group, which is a rate-limiting reaction that releases the following molecules as by-products: carbon monoxide (CO), free iron, and biliverdin (which is lately reduced to bilirubin) (1). HMOX1 is composed of 288 amino acid residues with an active site located between the first two alpha-helixes (2). The exact reactions leading to the conversion of hemoglobin, hemin, and myoglobin into bilirubin were identified in 1969, when Tenhunen, Marver, and Schmid described that this reaction was catalyzed by a heme oxygenase enzyme, based on two observations. First, the reaction required a metal chelate and the formation of only one isomer of bile pigment, suggesting the participation of an enzyme in this reaction. Second, the insertion of two hydroxyl groups that indicated that the cleavage of the porphyrin ring was an oxidative reaction. Based on these observations, authors described that heme oxygenase stoichiometrically required NADPH and

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Impact of Cigarette Smoking on the Gastrointestinal Tract Inflammation: Opposing Effects in Crohn's Disease and Ulcerative Colitis

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Cigarette smoking is a major risk factor for gastrointestinal disorders, such as peptic ulcer, Crohn's disease (CD), and several cancers. The mechanisms proposed to explain the role of smoking in these disorders include mucosal damage, changes in gut irrigation, and impaired mucosal immune response. Paradoxically, cigarette smoking is a protective factor for the development and progression of ulcerative colitis (UC). UC and CD represent the two most important conditions of inflammatory bowel diseases, and share several clinical features. The opposite effects of smoking on these two conditions have been a topic of great interest in the last 30 years, and has not yet been clarified. In this review, we summarize the most important and well-understood effects of smoking in the gastrointestinal tract; and particularly, in intestinal inflammation, discussing available studies that have addressed the causes that would explain the opposite effects of smoking in CD and UC.

Keywords: cigarette smoking, gastrointestinal inflammation, inflammatory bowel disease, ulcerative colitis, Crohn's disease

INTRODUCTION

Cigarette smoking is a major risk factor for the development of vascular diseases, such as atherosclerosis and pulmonary hypertension (1). Moreover, smoking-related diseases are the leading cause of preventable deaths and respiratory dysfunctions worldwide. Not only respiratory and cardiovascular diseases contribute to the morbidity and mortality associated with smoking but also other conditions such as multiple inflammatory and carcinogenic disorders (2). The underlying cause of these diseases is the presence of a large number of toxic components in cigarette smoke, whose impact at the respiratory and vascular level has been widely studied (1). Nevertheless, the pathways that are affected by cigarette smoke within the gastrointestinal tract, especially in inflammatory bowel disease (IBD), have not been clarified.

This review will focus on the impact of cigarette smoking on the gastrointestinal system, focusing on recent studies that have addressed its opposite effects on the two major forms of IBD: Crohn's disease (CD) and ulcerative colitis (UC).

PLOS PATHOGENS

RESEARCH ARTICLE

Pathogenicity island excision during an infection by *Salmonella enterica* serovar Enteritidis is required for crossing the intestinal epithelial barrier in mice to cause systemic infection

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Abstract

Pathogenicity island excision is a phenomenon that occurs in several *Salmonella enterica* serovars and other members of the family *Enterobacteriaceae*. ROD21 is an excisable pathogenicity island found in the chromosome of *S.* Enteritidis, *S.* Dublin and *S.* Typhi among others, which contain several genes encoding virulence-associated proteins. Excision of ROD21 may play a role in the ability of *S.* Enteritidis to cause a systemic infection in mice. Our previous studies have shown that *Salmonella* strains unable to excise ROD21 display a reduced ability to colonize the liver and spleen. In this work, we determined the kinetics of ROD21 excision *in vivo* in C57BL/6 mice and its effect on virulence. We quantified bacterial burden and excision frequency in different portions of the digestive tract and internal organs throughout the infection. We observed that the frequency of ROD21 excision was significantly increased in the bacterial population colonizing mesenteric lymph nodes at early stages of the infective cycle, before 48 hours post-infection. In contrast, excision frequency remained very low in the liver and spleen at these stages. Interestingly, excision increased drastically after 48 h post infection, when intestinal re-infection and mortality begun. Moreover, we observed that the inability to excise ROD21 had a negative effect on *S.* Enteritidis capacity to translocate from the intestine to deeper organs, which correlates with an abnormal transcription of *invA* in the *S.* Enteritidis strain unable to excise ROD21. These results suggest that excision of ROD21 is a genetic mechanism required by *S.* Enteritidis to produce a successful invasion of the intestinal epithelium, a step required to generate systemic infection in mice.

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L-Arginine Enhances Intracellular Killing of Carbapenem-Resistant *Klebsiella pneumoniae* ST258 by Murine Neutrophils

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Carbapenem-resistant *Klebsiella pneumoniae* ST258 (CRKP-ST258) are a global concern due to their rapid dissemination, high lethality, antibiotic resistance and resistance to components of the immune response, such as neutrophils. Neutrophils are major host mediators, able to kill well-studied and antibiotic-sensitive laboratory reference strains of *K. pneumoniae*. However, CRKP-ST258 are able to evade neutrophil phagocytic killing, persisting longer in the host despite robust neutrophil recruitment. Here, we show that neutrophils are unable to clear a CRKP-ST258 isolate (KP35). Compared to the response elicited by a prototypic *K. pneumoniae* ATCC 43816 (KPPR1), the neutrophil intracellular response against KP35 is characterized by equivalent production of reactive oxygen species (ROS) and myeloperoxidase content, but impaired phagosomal acidification. Our results ruled out that this phenomenon is due to a phagocytosis defect, as we observed similar efficiency of phagocytosis by neutrophils infected with KP35 or KPPR1. Genomic analysis of the *cps* loci of KPPR1 and KP35 suggest that the capsule composition of KP35 explain the high phagocytosis efficiency by neutrophils. Consistent with other reports, we show that KP35 did not induce DNA release by neutrophils and KPPR1 only induced it at 3 h, when most of the bacteria have already been cleared. L-arginine metabolism has been identified as an important modulator of the host immune response and positively regulate T cells, macrophages and neutrophils in response to microbes. Our data show that L-arginine supplementation improved phagosome acidification, increased ROS production and enhanced nitric oxide consumption by neutrophils in response to KP35. The enhanced intracellular response observed after L-arginine supplementation ultimately improved KP35 clearance *in vitro*. KP35 was able to dysregulate the intracellular microbicidal machinery of neutrophils to survive in the intracellular environment. This process, however, can be reversed after L-arginine supplementation.

Keywords: *Klebsiella pneumoniae* ST258, neutrophils, phagocytosis, phagosome acidification, L-arginine

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Hemoxigenase 1 Contribution to Modulating the Severity of Salmonella enterica serovar Typhimurium Infection in Mice

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