

Applications of acute inflammation in livestock immunity

by

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Abstract

Higher vertebrates share multiple mechanisms of immune defences; however, evolutionarily divergent species display unique characteristics to achieve immunity. Although significant knowledge regarding acute inflammation comes from studies in classical models, understanding of distinct immune features in livestock is limited. Hence, my overall goal was to provide a deeper understanding of cellular processes during acute inflammation in livestock animals, and subsequently applying this knowledge to evaluate the effects of drinking water contaminants on early and long-term immune responses.

My findings demonstrated that the peritoneal (abdominal in poultry) challenge model represents a reliable tool for assessing pro-inflammatory and pro-resolving phases of acute inflammation in swine and poultry. This model allows analysis of resident leukocytes to evaluate antimicrobial responses under *ex vivo* exposure and the study of cellular functions following *in vivo* challenge. Taking advantage of high-resolution imaging and cell-sorting flow cytometry along with antibody-based and conventional staining, I established an approach for characterization of acute inflammation and the transition to adaptive immunity in livestock. This self-resolving challenge model in poultry based on zymosan, displayed different dynamics of antimicrobial responses compared to other vertebrates. This showcased that chickens mount distinct immune responses against pathogens compared to mammals, while heat-killed *Salmonella enterica* serovar Typhimurium (HKST) induced a more conserved self-resolving model in swine compared to other vertebrates. Nevertheless, major differences were observed

in the antimicrobial and functional level at resolving phases of inflammation. For instance, peritoneal leukocytes displayed higher levels of reactive oxygen species (ROS) at 4 h post challenge and gradual decrease up to 48 h in swine. In contrast, poultry showed maximum ROS proportions at 12 h and decreased up to 48 h following zymosan challenge. Additionally, apoptotic leukocytes promoted distinct inflammatory downregulation in poultry compared to mammals and lower vertebrates. Altogether, this demonstrates the distinct mechanisms in avian and swine immune function during acute inflammation.

In non-industrialized farms, use of untreated underground water is linked to the occurrence of several problems in exposed animals. I wanted to address, the extent to which, water contaminants diminish the immune capacity of livestock to battle infections. To this purpose, I used raw well water from small farms in areas of potential environmental pollution in Alberta, Canada. The objective was to evaluate whether drinking water contaminants altered immune responses following an *in vivo* challenge in poultry and swine. My results showed that water contaminants altered the homeostatic immunological status and the acute inflammation in both poultry and swine. In chickens, short-term exposure to raw well water increased KUL01+ cells and their ROS production capacity under zymosan (fungal) exposure, but not with GFP-*Salmonella enterica* serovar Typhimurium (GFP-ST). Additionally, it downregulated nitric oxide production in heterophils when challenged with zymosan, whilst altered dynamics of leukocyte subsets with HKST.

In swine experiments, in addition to raw well water, I included a spiked water treatment. This contained a combination of raw water, sulfolene and diesel oil to evaluate their

combinatorial effect on immune responses. Homeostatic (prior to HKST challenge) levels of ROS production were downregulated while NF- κ B translocation were higher in spiked water. Following HKST challenge, the neutrophil pool remained higher even at 48 h post HKST challenge in this spiked group, while monocyte/macrophage and lymphocytes pools remained lower at 12 and 48 h compared to control group. Levels of ROS production were unaffected by water contaminants. However, NF- κ B activation was downregulated as early as 12 h post challenge. Taken together, this reveals that water contaminants induced a prolonged inflammatory leukocytes migration in poultry and swine. This suggested alterations in the timely return to homeostasis and effects in the development of the long-term immunity. Indeed, following 2 and 4 weeks, water contaminants differentially altered development and antimicrobial function of circulating monocytes and lymphocytes. This demonstrates that water contaminants have impacts the development of adaptive immunity in swine and poultry.

Altogether this thesis provides additional understanding of the mechanistic processes during acute inflammation in poultry and swine establishing divergent mechanisms to battle pathogens. Furthermore, it describes combinatorial effects of water contaminants in the progression of acute inflammation into long-term immunity. Lastly, this thesis offers a platform of immunoassays that can be useful for assessment of immune performance in livestock.

PREFACE

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Chapter III:

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Chapter V:

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Table of Contents

Chapter I: Introduction and literature review	1
1.1. Introduction	2
1.2. Thesis objectives	4
1.3. Thesis outline	4
1.4. Literature review: Acute inflammation in higher vertebrates	5
1.4.1. Introduction	5
1.4.2. The inflammatory milieu	7
1.4.2.1. PAMPs and DAMPs	9
1.4.2.2. Pattern recognition receptors (PRRs)	10
1.4.2.3. Cellular components	11
1.4.2.3.1. Resident macrophages	12
1.4.2.3.2. Polymorphonuclear leukocytes	14
1.4.2.3.3. Lymphocytes	16
1.4.2.4. Biochemical mediators of inflammation	17
1.4.2.4.1. Chemokines	17
1.4.2.4.1.1. CXCL-8	18
1.4.2.4.1.2. Leukotriene B4 (LTB4)	19
1.4.2.4.2. Pro-inflammatory cytokines	20
1.4.2.4.2.1. Interferon gamma	20
1.4.2.4.2.2. Tumor necrosis factor alpha (TNF- α)	22
1.4.2.4.2.3. Interleukin 1 beta (IL-1 β)	24
1.4.2.4.3. Anti-inflammatory cytokines	26
1.4.2.4.3.1. Transforming growth factor beta (TGF- β)	26
1.4.2.4.3.2. Interleukin 10 (IL-10)	27
1.4.2.4.3.3. Interleukin 2 (IL-2)	29
1.4.2.5. Antimicrobial responses	31
1.4.2.5.1. NF- κ B activation	31
1.4.2.5.2. Reactive oxygen species (ROS) production	33
1.4.2.5.3. Nitric oxide (NO) production	35

1.4.2.5.4. Phagocytosis	37
1.4.3. Inflammation models	39
1.4.3.1. <i>S. enterica</i> ser. Typhimurium	39
1.4.3.1.1. Classification	40
1.4.3.1.2. Entrance of <i>S. enterica</i> ser. Typhimurium into the host	41
1.4.3.1.3. Salmonella-cell interactions	42
1.4.3.1.4. Development of adaptive responses to ST	44
1.4.3.1.5. <i>S. enterica</i> ser. Typhimurium in livestock	45
1.4.3.2. Zymosan	46
1.4.3.2.1. Origins	46
1.4.3.2.2. Stimulating properties	47
1.4.3.2.3. Zymosan-induced inflammation	48
1.4.4. Water and health	49
1.4.4.1. Water in biological processes	49
1.4.4.2. Drinking water quality	50
1.4.5. Comparative immunobiology of livestock	52
1.4.5.1. Comparative immunology of swine	52
1.4.5.2. Comparative immunology of poultry	56
Chapter II: Materials and methods	61
2.1. Animals	62
2.2. Treatments	63
2.3. Intraperitoneal / Intrabdominal challenges	64
2.3.1. Zymosan	64
2.3.2. <i>S. enterica</i> ser. Typhimurium (ST)	65
2.3.3. Intrabdominal injections in chickens	67
2.3.4. Intraperitoneal injection in pigs	67
2.4. Abdominal lavage	67
2.5. Blood samples	68
2.6. Samples for gene expression analysis	69
2.7. Definition of immune cell populations	69
2.7.1. Modified Wright-Giemsa staining (Hema3)	69

2.7.2.	Flow cytometry cells sorting (FACS).....	70
2.7.3.	Imaging Flow cytometry	70
2.7.4.	Antibody staining	71
2.8.	Effect of apoptotic leukocytes on chicken immunity.....	71
2.8.1.	Induction of apoptotic leukocytes	71
2.8.2.	<i>Ex vivo</i> effect of apoptotic leukocytes.....	72
2.8.3.	<i>In vivo</i> effect of apoptotic leukocytes.....	72
2.9.	Analysis of leukocyte function	72
2.9.1.	Reactive oxygen species.....	73
2.9.2.	Nitric oxide (NO) production	74
2.9.3.	Cell viability	74
2.9.4.	NF- κ B nuclear translocation.....	75
2.10.	Gene expression	75
2.11.	Statistical analysis.....	76
Chapter III: Characterization of acute inflammation in poultry.....		79
3.1.	Introduction.....	80
3.2.	Results.....	82
3.2.1.	Intra-abdominal injection of zymosan induces leukocyte infiltration in chickens.	82
3.2.2.	Maximum capacity to mount a respiratory burst closely mirrored the peak of leukocyte infiltration into the challenge site.	83
3.2.3.	Higher levels of apoptotic leukocytes are detected in later phases of avian acute inflammation	84
3.2.4.	Multi-parametric analysis via imaging flow cytometry highlights divergent responses to pathogen-derived and homeostatic particles in chicken phagocytes.....	85
3.2.5.	Impact of exogenous apoptotic leukocytes on chicken leukocyte antimicrobial mechanisms at the inflammatory site.....	89
3.2.6.	Chicken apoptotic leukocytes do not affect phagocytic capacity in inflammatory leukocytes	90
3.3.	Discussion	91
Chapter IV: Characterization of acute inflammation in swine.....		105
4.1.	Introduction.....	106

4.2.	Results.....	107
4.2.1.	Intraperitoneal injection of HKST induce a self-resolving inflammatory process lead by neutrophils	107
4.2.2.	HKST inflammatory process resolves within 48 h post challenge.....	108
4.2.3.	<i>In vivo</i> HKST challenge induces active ROS production during pro-inflammatory state and down-regulation during pro-resolving phase...	109
4.3.	Discussion.....	111
Chapter V: Contribution of drinking water quality to livestock immune parameters ..		121
5.1.	Introduction.....	122
5.2.	Results.....	123
5.2.1.	The poultry model	123
5.2.1.1.	Raw water source selection and analysis	123
5.2.1.2.	Raw water increases abdominal macrophage numbers in broiler chickens.....	124
5.2.1.3.	Drinking water treatment induced changes in leukocyte proportion dynamics during acute inflammation.....	125
5.2.1.4.	Acute raw drinking water exposure alters innate antimicrobial responses in chickens	126
5.2.1.5.	Raw drinking water induced changes in NO production during chicken acute inflammation	127
5.2.1.6.	Raw water induces changes in the expression of inflammatory mediators during acute inflammation	128
5.2.1.7.	Short-term exposure to raw water induces changes in the chicken early adaptive immune responses	129
5.2.2.	The swine model.....	130
5.2.2.1.	Raw and spiked water	130
5.2.2.2.	Water contaminants modulated leukocyte activation in basal resident swine leukocytes.....	131
5.2.2.3.	Water contaminants upregulated basal NF- κ B nuclear translocation in swine resident leukocytes	133
5.2.2.4.	Water contaminants altered leukocyte dynamics following <i>in vivo</i> <i>S. enterica</i> ser. Typhimurium challenge	134
5.2.2.5.	Water treatment did not alter ROS production during <i>S. enterica</i> ser. Typhimurium <i>in vivo</i> challenge	135

5.2.2.6.	Spiked water altered leukocyte activation through NF- κ B nuclear translocation following <i>in vivo</i> <i>S. enterica</i> ser. Typhimurium challenge	136
5.2.2.7.	Raw water induced changes in early development of long-term immunity.	138
5.3.	Discussion	139
Chapter VI:	Discussion	161
6.1.	Introduction	162
6.2.	Summary of findings.....	164
6.2.1.	Characterization of immunological capacity of poultry and swine in the abdominal/peritoneal compartment.....	164
6.2.2.	Self-resolving acute inflammatory process displays differential mechanisms in poultry and porcine.....	167
6.2.2.1.	Characterization of self-resolving inflammatory process in poultry	169
6.2.2.2.	Characterization of self-resolving inflammatory process in porcine	172
6.2.3.	Drinking water contaminants induce divergent immune effects in poultry and porcine	173
6.3.	Future directions	183
References	188

List of tables

Table 1. Primers used for qPCR analysis

Table 2. Relevant xenobiotics found in raw water for chicken experiments.

Table 3. Relevant xenobiotics found in raw and spiked water for pig experiments.

List of Figures

- Fig. 2.1. pAT113-GFP inserted into *S. enterica* ser. Typhimurium
- Fig. 3.1. Zymosan intra-peritoneal challenge induces recruitment of heterophils, lymphocytes and monocyte/macrophages in chickens
- Fig. 3.2. Zymosan intraperitoneal challenge induces ROS production that correlates with the peak of leukocyte infiltration.
- Fig. 3.3. Kinetics of ROS production during the chicken acute inflammatory response.
- Fig. 3.4. Chicken leukocytes display equivalent capacity for uptake of pro-inflammatory and homeostatic particles at early and late phases of the acute inflammatory response.
- Fig. 3.5. Imaging flow cytometry shows selective decrease in zymosan phagocytosis among heterophils infiltrating the acute inflammatory site.
- Fig. 3.6. Chicken phagocytes display divergent functional responses to pathogen-derived and homeostatic particles.
- Fig. 3.7. Introduction of apoptotic leukocytes differentially inhibit leukocyte recruitment into the avian challenge site.
- Fig. 3.8. Chicken apoptotic leukocytes downregulate ROS production but do not affect the phagocytic capacity of inflammatory leukocytes.
- Fig. 4.1. HKST intra-peritoneal challenge induces recruitment of neutrophils, lymphocytes and monocyte/macrophages in swine.
- Fig. 4.2. HKST intraperitoneal challenge induces self-resolving inflammatory process.
- Fig. 4.3. HKST intraperitoneal challenge induces ROS production that correlates with the peak of leukocyte infiltration.

- Fig. 4.4. Porcine peritoneal leukocytes are a reliable tool for assessment of antimicrobial responses against *S. enterica* ser. Typhimurium.
- Fig. 4.5. Assessment of antimicrobial responses against *S. enterica* ser. Typhimurium in different leukocytes during inflammation.
- Fig. 5.1. Short-term exposure to raw water increases numbers of abdominal macrophages and increases their antimicrobial responses in chickens
- Fig. 5.2. Raw water differentially induces changes in leukocyte migration during acute inflammation
- Fig. 5.3. Raw water treatment downregulates antimicrobial responses post fungal challenge.
- Fig. 5.4. Raw water treatment induces changes in proinflammatory mediators during acute inflammation
- Fig. 5.5. Short-term exposure to raw water induces long-term effect on macrophage performance.
- Fig. 5.6. Raw water treatment induces long-term effects in the early adaptive immune responses
- Fig. 5.7. Water contaminants decrease the basal levels of activation state in porcine peritoneal leukocytes, but it is restored following *S. enterica* ser. Typhimurium.
- Fig. 5.8. Water contaminants increases basal levels of NF- κ B nuclear translocation in porcine peritoneal leukocytes.
- Fig. 5.9. Water contaminants alter the dynamics proportion of porcine peritoneal leukocytes during acute inflammation against HKST.

Fig. 5.10. Water contaminants do not induce changes in ROS production levels during acute inflammation against HKST.

Fig. 5.11. Water contaminants down-regulate NF- κ B nuclear translocation during acute inflammation against HKST.

Fig. 5.12. Short-term exposure to water contaminants induces alterations in the peripheral blood mononuclear cells proportions.

Fig. 6.1. Differential effect of raw water in chicken acute inflammation against fungi and bacterial challenges

Fig. 6.2. Differential effect of raw and spiked water in porcine acute inflammation against HKST.

Fig. 6.3. Long-term effect of raw water contaminants on poultry and porcine immunity against *S. enterica* ser. Typhimurium.

List of Abbreviations

AC	apoptotic cells
ACTB	actin B
AhR	Aryl hydrocarbon receptor
ANOVA	analysis of variance
AP	activator protein
APC	antigen presenting cell
ATPase	adenosine triphosphatase
B cell	B lymphocyte
BF	bursa of Fabricius
BLT	leukotriene B receptor
C3a	complement component 3a
C5a	complement component 5a
CARD	caspase activation and recruitment domain
CD	cluster differentiation
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
Ch-IL-2	chicken interleukin 2
CNS	central neuro system
CR	complement receptor
CSF	colony stimulating factor
CSIF	cytokine synthesis inhibitory factor
Cy5	cyanine 5
DAF-FM	4-amino-5-methylamino-2',7'-difluorofluorescein
DAMP	danger-associated molecular pattern
DHR	dihydrorhodamine
DI	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DP	double positive
dsRNA	double-stranded ribonucleic acid
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
EP	envelop protein
ERK	extracellular-signal-regulated kinase
ETC	electron transport chain
FACS	fluorescent-activated cell sorter
FITC	fluorescein isothiocyanate
FliC	flagellin
fMLP	N-formyl-L-methionyl-L-leucyl-phenylalanine
FOXp3	forkhead box P3
FP	fusion protein
FSC	forward scatter
Fw	forward
GALT	gut-associated lymphoid tissue

GATA3	GATA binding protein 3
GFP	green fluorescent protein
GFP-ST	green fluorescent protein <i>S. enterica</i> ser. Typhimurium
GM-CSF	granulocytic/macrophage colony stimulating factor
gp	glycoprotein
GPCR	G protein-coupled receptor
H ₂ O ₂	hydrogen peroxide
HA	hemagglutinin
HD-11	chicken macrophage cell line
HET	heterophil extracellular traps
HIF-1alpha	hypoxia-inducible factor-1 alpha
HIV	human immunodeficiency virus
HKST	heat-killed <i>S. enterica</i> ser. Typhimurium
HMGB1	high-mobility group box-1
HO-	hydroxide
HOCl	hypochlorous acid
HSV	herpes simplex virus
HTC	hepatoma tissue culture cell line
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IgM	immunoglobulin M
IL	interleukin
IL-2R	interleukin 2 receptor
ILC	innate lymphoid cell
iNOS	inducible nitric oxide synthase
IPP	ileal Peyer's patches
IκB	I kappa B protein
Iκκ	I kappa kinase
Jak	janus kinase
K60	multifunctional fusion protein
LAM	lipoarabinomannan
LB	Luria-Bertani
LBR	lamin B receptor
LCMV	lymphocytic choriomeningitis virus
LPM	large peritoneal macrophages
LPS	lipopolysaccharide
LTA	lipoteichoic acid
LTB	leukotriene B
LTi	lymphoid tissue inducer
mab	monoclonal antibody
MAMP	molecular-associated molecular pattern
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
M-CSF	macrophage colony stimulating factor
MHC	major histocompatibility complex
MHV	mouse hepatitis virus

MOE	Minister of Environment
MQ-NCSU	Muquarrab Qureshi-North Carolina State University
MRC	mannose receptor C-type 1
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
NET	neutrophil extracellular trap
NFAT	nuclear factor of activated T-cell
NF- κ B	nuclear factor kappa beta
NK	natural killer cell
NKT	natural killer T cell
NLR	Nod-like receptor
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
O ₂ ⁻	superoxide anion
OCT-1	octamer binding transcription factor
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS ^{-/-}	phosphate buffer solution (no calcium, no magnesium)
PE	phycoerythrin
PerC	peritoneal cells
PI	propidium iodide
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear cells
POPs	persistent organic pollutants
PPCPs	pharmaceutical and personal care products
ppm	parts per million
PRR	pattern recognition receptor
qPCR	quantitative polymerase chain reaction
r28S	ribosomal 28 subunit
RAF	rapid accelerated fibrosarcoma
RelA	transcription factor p65
RelB	transcription factor RelB
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute – 1640
Rv	reverse
SB	surface bound
SCV	salmonella-containing vacuole
SEM	standard error of media
SPI-1	Salmonella pathogenicity island 1
SPM	small peritoneal macrophages
SSC	side scatter
ssRNA	single-stranded ribonucleic acid
Stat	signal transducer and activator of transcription

T cell	T lymphocyte
T3SS	type 3 secretion system
T-bet	T-box transcription factor
TCDD	tetrachlorodibenzo-p-dioxin
TCR	T cell receptor
TGF	transforming growth factor
TGFR	transforming growth factor receptor
Th	T helper cell
TLR	Toll-like receptor
TMEV	Theiler's murine encephalomyelitis virus
TNFR	tumoral necrosis factor receptor
TNFSF	tumoral necrosis factor superfamily
TNF- α	tumoral necrosis factor alpha
TRADD	tumoral necrosis factor receptor type 1-associated death domain protein
TRAF	tumoral necrosis factor associated factor
Treg	T regulatory cells
UTR	untranslated region
VSV	vesicular stomatitis virus
WGA	wheat germ agglutinin
WHO	World Health Organization
Zym	zymosan
$\gamma\lambda$ T cell	gamma delta T cell

Chapter I

Introduction and literature review

1.1. Introduction

As species continuously emerge throughout evolution, the immune system has dynamically been driven, in addition to other factors, by selection pressure from surrounding pathogens (Buchmann, 2014; Kasahara, 2011). Hence, higher species have established a highly specialized and sophisticated system that ultimately provides them protection against infections (Boehm, 2012). To achieve immunity, defense mechanisms are mounted in an integrated fashion that enable them to battle infections and result in a timely return to homeostasis (Freire and Van Dyke, 2013; Sansbury and Spite, 2016; Soehnlein and Lindbom, 2010). Even though the majority of processes are highly conserved across species, remarkable differences exist between species that highlight the acquisition of unique mechanisms to achieve immunity, while exhibiting differential susceptibility to infections. Within immune responses, innate immunity is the first barrier to control infections (Kohchi et al., 2009). Rapid, robust and highly-regulated antimicrobial responses are displayed immediately after infections begin. Later, this innate immunity must be able to promote a strong and specific response known as long-term immunity, also referred to as adaptive immunity (Moticka, 2016; Pillay, 2015). Acute inflammation, as part of innate immunity, represents a highly conserved set of molecular and cellular processes during early responses following infections (Kumar et al., 2004). Studies in classical models such as mice and teleost fish have revealed that pro-inflammatory and pro-resolving phases of acute inflammation display similar patterns, despite remarkable differential mechanisms among species (Havixbeck et al., 2016; Rieger et al., 2012). Conversely, despite numerous advances in studies in non-

classical models, several immunobiological processes remain unclear in species such as livestock animals.

Livestock animals are primarily raised in conditions with the primary objective to achieve maximum performance. For instance, unselected chicken strains (Alberta Meat Control, AMC) AMC-1957 and AMC-1978 showed lower body weight compared to commercial Ross 308 chicken strains measured weekly for 8 weeks after hatching.

Authors revealed body weight increase of 400% in commercial strains compared to unselected chicken strains (Zuidhof et al., 2014). In pigs, a third of the daily energy is invested only in physiological maintenance and this proportion increases under immunological disorders (Patience et al., 2015). Economically, livestock animals must maximize growth and efficiently convert feed into meat, milk or eggs, while specific farm conditions might become potential stressors. Under these conditions, appearance of changes in immunity, even in the absence of clinical disease (Carroll and Forsberg, 2007; Ingvarsten and Moyes, 2013) can not be assessed by current methodologies.

In this thesis, I developed a reliable *in vivo* approach that allows the study of acute inflammation in livestock animals such as poultry and swine. Using fungal and bacterial pathogen mimics, I used self-resolving peritoneal challenge models to obtain an improved understanding in pro-inflammatory and pro-resolving stages of acute inflammation. Furthermore, I used this knowledge to evaluate the combinatorial effect of drinking water contaminants on the normal development from early to long-term immunity. As a result, this thesis provides a platform of bioassays that allow the evaluation of immune function in livestock animals.

1.2. Thesis objectives

My thesis objectives were to characterize the acute immune responses in livestock animals and assess effects of drinking water quality on the development of immune defences in poultry and swine systems. Drinking water is one of the vehicles used by pathogens to invade a host, therefore drinking water quality is of major importance in farm management. Even in the absence of pathogens, other water contaminants (such as chemical and toxic agents) have been shown to diminish performance in livestock, such as delaying growth and normal development (do Amaral, 2004). However, it is yet unclear, to what extent these factors have effects on the efficiency of livestock animals to battle infections. Hence, I hypothesized that even though major processes underlying immune responses are highly conserved between poultry and swine, they displayed key differences, and therefore, are differentially affected by water contaminants. Specific objectives of this thesis were characterization of (1) acute inflammation in poultry and swine systems; (2) the effect of drinking water quality on early immune responses; and (3) the effect of drinking water quality on early development of long-term immunity. These aims allowed me to establish a reliable platform to study effects of some on-farm conditions on immune responses that will enable the assessment of livestock animal health.

1.3. Thesis outline

This thesis encompasses six chapters. The first chapter consists of an introduction and literature review of acute inflammation in classical and non-classical models and the transition to early development of adaptive immunity describing molecular and cellular

components of immunity. Additionally, it describes the importance of water in biological systems and the proper assessment of drinking water quality. Finally, it highlights key points of the comparative immunobiology of poultry and swine. Chapter 2 provides a description about methodologies, materials and protocols used throughout this thesis. In Chapter 3, I characterized the acute inflammation processes in poultry using a combination of *ex vivo* and *in vivo* approaches. In Chapter 4, I characterized the acute inflammatory process in porcine using *ex vivo* and *in vivo* approaches. Chapter 5 describes the assessment of effects of drinking water quality on acute inflammation and the early adaptive immune responses. Chapter 6 offers an integrative view of overall results and discusses their fundamental and applied significance while proposing further studies.

1.4. Literature review: Acute inflammation in higher vertebrates

1.4.1. Introduction

Acute inflammation is the first defence response against pathogens and also sterile injury. Within minutes following pathogen invasion or injury occurrence, the immune system mounts a highly sophisticated and controlled molecular and cellular response with the purpose of controlling the inflammatory trigger (Kumar et al., 2004). During active pathogenic infections, this immune response needs to be displayed in a highly regulated fashion to promote an active return to homeostasis after pathogen clearance (Fujieda et al., 2013). Later, this immune response must be capable of inducing a robust and specific memory response led by adaptive immunity providing protection for a

secondary exposure (Gilroy and De Maeyer, 2015; Lan et al., 2008; Maderna and Godson, 2003).

Following pathogen entrance during acute inflammation, a large host cellular component including stromal, epithelial cells and resident leukocytes in the inflammatory site contribute to the first step of immune responses (Soehnlein and Lindbom, 2010). In addition to antimicrobial responses, these cells are responsible for triggering specific signals and release of biochemical mediators for pathogen restriction and further leukocyte migration (Bianchi and Manfredi, 2014; Davies et al., 2013; Dunster, 2016). This leukocyte migration is a coordinated process allowing the arrival of considerable numbers of activated leukocytes from systemic circulation and hematopoietic compartments (Mukaida, 2003; Pillay, 2015). The primary leukocyte type arriving at the inflammatory site is the polymorphonuclear (PMN) cell prototype, neutrophil (or heterophil, its homolog in avian). These neutrophils mainly migrate from the hematopoietic site (bone marrow in avian and mammal species) to the systemic blood where they circulate throughout the body. Along with PMNs, another cell type that contribute to this process is the monocyte lineage that migrate to the inflammatory site (Dal Secco et al., 2004; Rankin, 2010; Summers et al., 2010).

Following their arrival at an infection site, monocytes and neutrophils, along with other resident cells play a pivotal role in fighting and controlling infections. Following pathogen control, the inflammatory milieu is driven to a resolving state, led by the release of pro-resolving mediators such as chemokines and lymphokines, cell-death markers and others (Esmann et al., 2010; Maderna and Godson, 2003). Hence, the inflammatory process returns to a homeostatic state following pathogen clearance. At

this state, lymphocyte development displays a range of processes to induce a strong and robust memory immunity.

1.4.2. The inflammatory milieu

As previously mentioned, following pathogen entrance, a diverse range of cell types are involved in the development of inflammatory responses. These cells interact with specific conserved molecular structures on pathogenic organisms (known as pathogen-associated molecular patterns, PAMPs) or endogenous signals (danger-associated molecular patterns, DAMPs) that will activate intracellular pathways through genetically encoded receptors called pattern recognition receptors (PRRs) (Ito, 2014; Tang et al., 2012) such as Toll like receptors (TLRs) (Akira, 2003), Nod like receptors (NLRs) (Franchi et al., 2009; Kim et al., 2016), C-type lectin receptors and others (Aderem, 2003; Freeman and Grinstein, 2014; Roach et al., 2005). These broad families of molecular receptors are constitutively expressed among multiple cell types such as macrophages, monocytes, neutrophils, lymphocytes, epithelial and mesenchymal cells (Gordon, 2002; Kestra et al., 2013). After ligand-receptor interaction, intracellular activation pathways promote gene expression that ultimately leads to release of a vast array of pro-inflammatory cytokines and other chemical mediators to sustain the inflammatory process (Freeman and Grinstein, 2014; Geijtenbeek and Gringhuis, 2009). Within the pro-inflammatory cytokine profile are tumoral necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1 β), interleukin 6 (IL-6), interleukin 8 (IL-8) and interferon gamma (IFN- γ) among others (Boehm, 2012; Gough et al., 2008; Kumar et al., 2004; Sims and Smith, 2010). As the acute inflammation progresses, this dynamic pro-

inflammatory milieu shifts to a distinct niche of biochemical mediators such as transforming growth factor beta (TGF- β), interleukin 10 (IL-10) and interleukin 4 (IL-4) that plays a role in shutting down the inflammatory process (Fadok et al., 2001; Huynh et al., 2002; Johnston et al., 2016; Lan et al., 2008).

In terms of leukocyte dynamics, neutrophil migration to the inflammatory site is driven mainly by *CXCL-8 (IL-8)* expression that induces an increased vascular permeability in initial phases of acute inflammation. This IL-8 expression promotes a massive neutrophil migration that starts within minutes following pathogen entrance remaining high for hours and days (De Oliveira et al., 2013; Kunkel et al., 1991; Mukaida, 2003). Leukocyte migration to the inflammatory site also promotes the arrival of monocytes to contribute to the pathogen restriction (Soehnlein and Lindbom, 2010). This monocyte pool, in the presence of pro-inflammatory mediators will turn into the specialized, macrophage type that in concert with resident macrophages and activated neutrophils will display a massive pathogen engulfment to avoid dissemination (Crockett-Torabi and Ward, 1996). This process, called phagocytosis is the paramount response during acute inflammation against pathogens, because it will restrict and clear pathogens, while promoting further antimicrobial mechanisms through release of soluble mediators (Botelho and Grinstein, 2011; Henneke and Golenbock, 2004; May and Machesky, 2001). Following pathogen internalization, cells will present processed antigens to the other arm of the immune response, the adaptive immunity, in a process called antigen presentation (Flannagan et al., 2012). This antigen presentation process will further induce activation of T and B lymphocyte lineages, that participate in the development of

memory responses (Gordon, 2016; Rosales and Uribe-Querol, 2017; Uribe-Querol and Rosales, 2017).

1.4.2.1. PAMPs and DAMPs

Complex molecules present on pathogens, are highly stimulatory to immune system and are called pathogen-associated molecular patterns. These molecules are highly immunogenic and comprises: microbial DNA/RNA (such as unmethylated CpGs, single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), etc), as well as lipoproteins, glycoproteins and surface components (LPS, peptidoglycans, lipoteichoic acid and others) (Ito, 2014; Miyaji et al., 2011). Within bacterial PAMPs, LPS is a major cell wall component of most gram-negative bacteria involved in triggering cellular activation. With respect to gram-positive bacteria, peptidoglycan is considered one of the most important PAMPs to induce cellular responses. For some intracellular bacteria, lipoarabinomannan (LAM) plays a significant role as PAMP for leukocyte activation (Aderem, 2003). Bacterial DNA, richer in unmethylated CpG compared to host genome, is a potent PAMP for cellular activation (Rothfuchs et al., 2004). Viral PAMPs mainly comprise a wide array of components such as nucleic acid (single-stranded DNA, double-stranded DNA, etc.). Additionally, haemagglutinin (HA) is a potent cellular activation protein for Measles virus (Griffin, 2010). Fusion protein (FP) and envelope proteins (EP) are pivotal for triggering cellular activation in Respiratory Syncytial virus and Murine Leukemia virus infections, respectively (Pillay, 2015; Thornburg et al., 2010). Fungal infections induce the immune response through cell wall components

such as β -glucans and glucuronoxylomannans for *Candida albicans* and *Aspergillus fumigatus* (Fukazawa et al., 2009; Herwald and Egesten, 2016; Romani, 2011).

DAMPs are a multi-array of signals produced following tissue damage. High-mobility group box 1 (HMGB1) is a DNA binding protein that participates in transcriptional process and is a late-phase pro-inflammatory protein that is released and highly immune active (Miyaji et al., 2011; Rock et al., 2010). The S100 protein family is a group of calcium-binding proteins that are released from phagocytes in initial steps of pro-inflammatory process, for instance, S100A8 and S100A9 are involved in endotoxic shock (Bianchi, 2007; Ibi et al., 2008). Heat-shock proteins are a group of intracellular chaperones that intracellularly contribute to protein trafficking and misfolding, but they are pro-inflammatory modulators when released during the initial stages of immune responses (Beere, 2004; Miyaji et al., 2011; Rock et al., 2010). Alarmins, mainly produced by polymorphonuclear cells, are a large group of cationic proteins that contribute to pathogen targeting and leukocyte activation (Chan et al., 2012; Said-Sadier and Ojcius, 2012). Within this group α - and β - defensins, cathelicidins and lactoferrins are potent alarmins produced by neutrophils and some epithelial cells. Extracellular matrix such as cleaved fibronectin and fibrinogen are potent immune mediators during acute inflammation (Pestka et al., 2004; Poon et al., 2014).

1.4.2.2. Pattern recognition receptors (PRRs)

Due to antigen diversity, organisms have successfully evolved a vast array of surface receptors to recognize pathogens (Lipsitch and Hagan, 2007). Danger signals and pathogen-derived molecules interact directly with genetically encoded surface receptors,

expressed in multiple cell types, that participate in intracellular signalling. These homodimer or heterodimer complex molecules encompass a wide group of receptors such as toll like receptors (TLR) (Gordon, 2002; Roach et al., 2005; Tipping, 2006). TLR were first described in drosophila as regulator proteins in embryogenesis. However, they have been shown to be involved in immune responses (Lemaitre et al., 1996). TLR family is a highly conserved group of receptors described from lower vertebrates to higher organisms. However, different genetic background in divergent species and expression has been reported. TLRs have different specificity to different PAMPs, for instance, TLR1-9 are conserved and expressed in mice and humans. Conversely, TLR-10 is just expressed in humans but not in mice, whereas in humans TLR-11 is not expressed but it is in mice (Oliveira-Nascimento et al., 2012; Plato et al., 2013; Wu and Kaiser, 2011).

1.4.2.3. Cellular components

During the inflammatory process, different cell types are involved in maintenance and infiltration of pro-inflammatory leukocytes. Epithelial and stromal cells, along with resident leukocytes, such as macrophages and lymphocytes, contribute to the initial cellular response to avoid spreading of infections (Hogenkamp, 2007; Mukaida, 2003). Following initial steps, soluble and chemical mediators promote the arrival of polymorphonuclear cells (such as neutrophils or heterophils) that further contribute to infection control. Later in the inflammatory response, polymorphonuclear cell types along with other biochemical mediators promote the shut down of the inflammation. This mechanism is driven, among others, by the expression of death signals produced by

dying neutrophils or heterophils (Crockett-Torabi and Ward, 1996; Nourshargh and Alon, 2014; Poon et al., 2014).

1.4.2.3.1. Resident macrophages

Tissues need the presence of resident leukocytes to protect them from pathogen invasion (Davies et al., 2013). The main cell type that offers protection in these tissues are the professional phagocytic leukocytes known as macrophages (Hoeffel and Ginhoux, 2015; Roberts et al., 2017). In addition to their role in protection against foreign threats, these macrophages are also involved in the maintenance of homeostasis through dead cell and debris clearance (Humphrey and Klasing, 2004; Okabe, 2018). Hence, we can describe a wide range of functional activities for macrophages such as phagocytosis, as the capacity of engulfing, processing and destruction of pathogenic and non-pathogenic particles; pathogen recognition through PPRs; and cellular differentiation and survival (Freeman and Grinstein, 2014; Okabe, 2018; Richards and Endres, 2014). Depending upon tissue specificity, higher organisms have developed different macrophage phenotypes that will provide them with a differential response against pathogens (Davies et al., 2013; Hoeffel and Ginhoux, 2015; Munro and Hughes, 2017). Even though early concepts in macrophage development described the capacity of monocytes to differentiate into mature macrophages with soluble and chemical factors such as granulocyte/macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) and others (Barreda, 2004; Barreda and Belosevic, 2001; Davison et al., 2014), it is also described that tissue-resident macrophages are equipped with mechanisms for self-proliferation (Hoeffel and Ginhoux, 2015; Munro and Hughes,

2017). In alignment with these concepts, monocytes/macrophages display differential mechanisms to promote and support initial immune responses (Crockett-Torabi and Ward, 1996; Richards and Endres, 2014).

Within the group of tissue-resident macrophages are: microglia, which provide central neuro system (CNS) protection and contribute to neural development (Nakajima and Kohsaka, 2001); alveolar macrophages, which are responsible for surfactant and pathogen clearance in lungs (Cheung et al., 2000; Pribul et al., 2008); osteoclasts, which are responsible for bone remodeling (Teitelbaum, 2007, 2000); peritoneal macrophages, which confer pathogen protection to the peritoneal cavity in higher organisms (Liao et al., 2017; Rao et al., 1994); and others.

Peritoneal macrophages are first phagocytic leukocytes that contributes to initial response to invading micro-organisms into the peritoneal cavity (Soehnlein and Lindbom, 2010). Mice peritoneal cells (PerC), as other classical macrophages, are mainly characterized by the expression of F4/80 glycoprotein, CD11b, CSF-1 receptor and others (Zhang et al., 2010). However, it has been revealed that peritoneal macrophages comprise two phenotypically distinct populations, known as small peritoneal macrophages (SPMs) and large peritoneal macrophages (LPMs) (dos Anjos Cassado et al., 2015; Eid et al., 2010). Both phenotypes express F4/80 and CD11b markers and display a strong phagocytic capacity, however, they differ in their Gr-1, MHC-II expression and other surface markers; interestingly these two subsets of peritoneal macrophages display a differential response following activation (dos Anjos Cassado et al., 2015). These differential features and functionality have been recently explained due to these two distinct peritoneal macrophages which arise from two

different sources, while LPMs are bone marrow-derived macrophages, SPMs are monocyte-derived macrophages (Bain and Jenkins, 2018; dos Anjos Cassado et al., 2015; Eid et al., 2010).

1.4.2.3.2. Polymorphonuclear leukocytes

Polymorphonuclear cells (PMNs) are the most numerous leukocytes in systemic circulation in mammalian species ranging within 40-75% of the total circulating leukocytes (Veda, 2011). However, species variation has been described. Thus; in cattle, neutrophil percentage ranges from 15-33%; in pigs, from 30-50%; and in chickens, heterophils range within 20-50% (Maxwell and Robertson, 1998) throughout their lifetime. This group comprises three leukocyte types including: neutrophils (heterophils in avian systems); eosinophils and basophils, with the later two being significantly less numerous (Veda, 2011). Due to its importance in acute inflammation, neutrophil is the most important polymorphonuclear cell that contributes to pathogen control following microbial invasion and plays a significant role in down-regulation of the inflammatory process after pathogen clearance (Jones et al., 1998; Kobayashi et al., 2018; Pillay, 2015; Summers et al., 2010). Neutrophils are derived from precursor cells in the hematopoietic compartment, in a process called granulopoiesis with participation of granulocytic colony-stimulating factor (G-CSF), prior to their release to the bloodstream (Nicolas-Avila et al., 2017; Summers et al., 2010). Hence, mature neutrophils migrate to the inflammatory site through a process called chemotaxis, where neutrophils are attracted via chemokines produced by epithelial, endothelial and other resident leukocytes (Rosales et al., 2016) to the inflammatory site.

Following arrival, as other professional phagocytic leukocytes, neutrophils possess a broad repertoire of antimicrobial mechanisms to battle micro-organisms. Hence, neutrophils have the capacity to engulf, process and destroy pathogens; release of a wide-array of soluble mediators (such as cathelicidins and defensins); display antimicrobial responses such as ROS production, nitric oxide, extracellular traps production (NETs, through a process called netosis) and others (Mocsai, 2013; Nathan, 2006). In contrast to macrophages, neutrophils are short-lived leukocytes (within 6-8 h) in human, but neutrophil lifetime varies in other species (around 11 h in mice). These cells migrate to the site of infection within minutes and hours of the initial trigger and cooperate with other leukocyte types to maintain the antimicrobial responses throughout inflammation (Esmann et al., 2010).

From an evolutionary perspective, neutrophils or neutrophil-like cells have been described in several animal classes. In higher invertebrates, these neutrophil-like cells are called hemocytes since they exhibit features of neutrophils and platelets. In lower vertebrates such as cnidarians they are called amoebocytes; while in higher vertebrates, they evolved to classical neutrophils in mammals and fish, and heterophils in avian systems (Jenne et al., 2018; Styrud, 1989). During evolution, these phagocytic leukocytes have evolved in such a way that have gained some specific features, but also have lost primordial characteristics of the typical neutrophil. Although various successful phagocytic cells are found across animal classes, neutrophils (and their counterparts in other species) play a pivotal and unique importance in immune response (Havixbeck et al., 2016, 2014; Rieger et al., 2012; Summers et al., 2010).

1.4.2.3.3. Lymphocytes

The third group of leukocytes involved in immune responses are lymphocytes (Mason, 1998). Even though lymphocytes are historically related to long-term immunity processes, recent evidence has shown that they also play a role in the initial stages of immune responses, due to their capacity to interact with other leukocytes, contributing to the mounting of an inflammatory response (Chang et al., 2013; Kolaczowska et al., 2008). For instance, innate lymphoid cells (ILCs) comprise a group of lymphocytes similar to prototypical adaptive immune cells which possess divergent features (Eberl et al., 2015). Natural killer cells (NK cells) are considered the counterpart of CD8⁺ T lymphocytes, while ILC1, ILC2 and ILC3 are the equivalent to the CD4⁺ T helper 1 (Th1), Th2 and Th17 lymphocytes (Eberl et al., 2015; Vivier et al., 2016). In contrast to adaptive immune lymphocytes, ILCs do not express antigen receptor and do not display lymphocyte expansion or clonal selection. However, ILCs promote the development of immune responses through secretion of a vast array of cytokines following an insult or pathogen signal (Artis and Spits, 2015; Eberl et al., 2015; Fan and Rudensky, 2016). Following pathogen processing, antigen presenting cells (APCs) promote the development of long-term immunity when APCs interact with the adaptive immune lymphocytes (Moticka, 2016). Major players of adaptive immune cells are grouped into two large lineages: T and B lymphocytes that will be activated to induce a robust response to provide memory immunity for subsequent challenges (Anderson et al., 2004). Depending upon the antigen involved, either Th1 or Th2 immune responses will lead to cytotoxic and humoral responses, respectively (Dawson et al., 2005). Hence, Th1 responses are mostly associated with intracellular antigens, whereas Th2 responses are

involved with extracellular pathogens and further collaboration for B lymphocyte development and antibody-based immunity (Liew, 2002; Netea et al., 2005).

Furthermore, Th3 lymphocytes contribute to regulatory mechanisms during immune responses. Another T cell type involved in immune responses are Th17 lymphocytes which are highly responsive and participate in the development of the pro-inflammatory process (Malek and Castro, 2010). In higher vertebrates, T and B leukocytes originate from a common precursor, lymphoblast, in the hematopoietic system.

In contrast to T lymphocyte responses, humoral immunity is led by the production of highly-specific antibody secretion by plasma B cells or plasmocytes, that are the effector arm of B cell populations secreting antibodies that ultimately neutralize pathogens.

Altogether, B lymphocyte populations play a significant role in the transition from acute to adaptive immune responses.

1.4.2.4. Biochemical mediators of inflammation

1.4.2.4.1. Chemokines

Following pathogen invasion, a massive release of cellular by-products from activated host cells participates in pathogen clearance. Most of them are rapidly secreted due to their storage in cellular compartments and contribute to the inflammatory milieu. These molecules play a key role in the arrival of inflammatory cells to further support pathogen clearance. Chemokines are designated based on the amino acid arrangement of cysteine residues involved in disulfide binding. Thus, four groups of chemokines have been described: C, CC, CXC, and CX₃C (Mukaida, 2003). Prostaglandins, lipoxins, leukotrienes and others are grouped into a large group of bioactive complexes known as

lipid mediators. Here, I will briefly cover some chemokines of relevance during acute inflammation.

1.4.2.4.1.1. CXCL-8

A well-known chemokine involved in inflammatory process as mediator for leukocyte migration is CXCL-8, also known as interleukine-8 (IL-8) and it is the prototypical member of CXC family (De Oliveira et al., 2013; Kimmig et al., 1999). CXCL-8 is a potent neutrophil chemoattractant, therefore, it plays a pivotal role in initiation of inflammation. Although, *CXCL-8* is highly expressed following pathogen invasion, it has been shown that other soluble mediators such as tumor necrosis factor alpha (TNF- α) or interleukin 1 beta (IL-1 β) enhance its production, thus *CXCL-8* is inducible rather than constitutively expressed (Kunkel et al., 1991; Mukaida, 2003). Expression of the *cxcl-8* gene encodes for a 99 amino acid protein, that following post-translational modifications yields a 77 or 72 amino acid-sequence product produced by fibroblasts and endothelial cells, and monocyte and macrophages, respectively (Nathan, 2006). *CXCL-8* expression must be highly controlled since its upregulation leads to a chronic inflammation and other developmental disorders such as cancer and inflammatory diseases; or its downregulation might promote the delay of inflammatory responses promoting pathogen survival (Benakanakere et al., 2016). It has been shown that post-transcriptional control is through NF- κ β and AP-1 transcription factors (Mukaida, 2003). Within the wide range of other functions, CXCL-8 promotes L-selectin shedding, regulates β_2 integrin expression and complement receptor 1 (CR1). Also, CXCL-8 stimulates neutrophil degranulation and respiratory burst activity, and activates the

release of leukotriene B₄ and other lipid mediators (Mukaida, 2003; Waugh and Wilson, 2008). CXCL-8 has two main receptors, CXCR1 (IL-8RA) and CXCR2 (IL-8RB), expressed in neutrophils, where the first is considered to be specific for CXCL-8, while CXCR2 is a promiscuous receptor that is also used by other chemokines (Kimmig et al., 1999). Based on their intracellular pathway for activation, it has been proposed that CXCR1 receptor is more important in antimicrobial responses such as ROS production and protease release, whereas CXCR2 is involved in leukocyte recruitment and neutrophil degranulation (De Oliveira et al., 2013; Mukaida, 2003).

In chicken, two CXCL-8 like chemokines have been described. K60 and CAF are the orthologous with the mammalian CXCL-8. Even though both possess the capacity to promote leukocyte migrations, K60 tends to be more efficient in heterophil recruitment, whilst CAF is more prone to induce monocyte migration (Davison et al., 2014). In swine, CXCL-8 has been associated to enteric infections caused by *Salmonella spp.*, where is related to salmonella shedding (Knetter et al., 2014) and following *in vitro* stimulation with porcine respiratory and reproductive syndrome virus (Ladinig et al., 2014).

1.4.2.4.1.2. Leukotriene B₄ (LTB₄)

Leukotrienes are lipid mediators derived from arachidonic acid through 5-lipoxygenase pathway and mainly produced by granulocytes, monocytes and macrophages (Gennaro et al., 2014; Oyoshi et al., 2012). Within the numerous members of this family, leukotriene B₄ (LTB₄) is the most potent phagocytic cell chemoattractant, which promotes T lymphocyte differentiation and dendritic cell migration (Busse, 1998; Iizuka

et al., 2010; Okiji et al., 1991). Other recognized functions of LTB₄ are promoting leukocyte-endothelial interactions, promoting leukocyte aggregation, neutrophil degranulation and production of reactive oxygen species (Claesson et al., 1992; Gennaro et al., 2014; Iizuka et al., 2010; Peters-golden et al., 2005). LTB₄ function is achieved through binding to its surface cell receptor, G protein-coupled receptors (GPCR). The first receptor described for LTB₄ was LTB₄ receptor type-1, also known as BLT1, that belongs to a family of chemoattractant receptors which are expressed in neutrophils, macrophages, dendritic cells, B and T lymphocytes. BLT2 is the second receptor known for LTB₄. It is a low-affinity receptor for LTB₄ compared to BLT1, it has been shown that in addition to its contribution to pro-inflammatory pathways, it is involved in anti-inflammatory mechanisms (Gennaro et al., 2014; Iizuka et al., 2010). In addition to its action in leukocyte function, LTB₄ promotes cytokine expression in other cells types. Thus, it induces MCP-1 and TNF- α in macrophages, and IL-8 in neutrophils (Peters-golden et al., 2005).

1.4.2.4.2. Pro-inflammatory cytokines

1.4.2.4.2.1. Interferon gamma

Interferons are divided into three main groups, type I, type II and type III interferons (Goossens et al., 2013; Kopitar-Jerala, 2017). In addition to their function as antiviral mediators, interferons participate in several cellular processes such as cell proliferation, survival and differentiation (Levy et al., 2011). Type I interferon encompasses more than seven members designated as IFN- α , - β , - δ , - ϵ , - κ , - τ , - ω (Génin et al., 2009; Levy et al., 2011; Pestka et al., 2004; Rauch et al., 2013). On the other hand, the only member

in the interferon type II group is interferon gamma (IFN- γ) and it is the most studied so far. Type III interferons are distantly related to the other groups, they are located in a different chromosomal region and structurally resemble interleukin-10 (*il-10*) genes, and includes three homolog variants *ifn- λ -1*, -2 and -3 (Levy et al., 2011).

IFN- γ is produced by a numerous cell types including Th1-related cells such as natural killer (NK) cells, NK T cells, CD4⁺, CD8⁺ T cells and B cells (John and Darcy, 2016), although it is also expressed by antigen presenting cells, such as neutrophils, dendritic cells, monocyte and macrophages following activation. As a result, IFN- γ , plays a key role in inflammatory processes and autoimmune diseases, intracellular pathogens and tumor development (Murray et al., 2002). The paramount activity of IFN- γ is macrophage activation through its binding to surface receptors. IFN- γ interacts with its cell surface receptors such as IFN- γ R1 and IFN- γ R2, which act as heterodimers.

Following binding, IFN- γ activates Jak-1, Jak-2 and stat-1 pathways, although it has been described that stat-3, stat-5, NF- κ B and others are also triggered by IFN- γ (Pestka et al., 2004). Hence, IFN- γ regulates expression of major histocompatibility complex I (MHC-I) and II (MHC-II), and several inflammatory cytokines such as interleukin-12, -15, tumor necrosis factor alpha (TNF- α), inducible nitric oxide synthase (iNOS), caspase 1 and gp-91^{phox} (Chesler and Shoshkes, 2002). Therefore, along with its pro-inflammatory properties, IFN- γ also possesses anti-inflammatory capabilities inhibiting IL-1 and IL-8, enhances caspase-mediated apoptosis and promotes the expression of suppressors of cytokine signalling (Mühl and Pfeilschifter, 2003).

As a mediator of immune responses, IFN- γ has been found to play a role against viral infections such as Herpes simplex virus-2 (HSV-2), murine hepatitis virus (MHV),

lymphocytic choriomeningitis virus, Theiler's murine encephalomyelitis virus (TMEV), vesicular stomatitis virus (VSV), and influenza viruses (Murray et al., 2002; Parr and Parr, 1999; Schijns et al., 1996; Shtrichman and Samuel, 2001; Whitmire et al., 2005) and others. IFN- γ also plays a role against bacterial and parasitic infections, and has been shown to be involved in intracellular pathogen infections such as *Listeria monocytogenes*, *Toxoplasma gondii*, *Burkholderia pseudomallei*, *Mycobacterium tuberculosis* and *Salmonella enterica* serovar Typhimurium (from now *S. enterica* ser. Typhimurium) (Bao et al., 2000; Buchmeier and Schreiber, 1985; Flynn et al., 1993; Miyagi et al., 1997) where IFN- γ promotes the active secretion of reactive oxygen and nitric species.

As extensively described in mammals, chicken IFN- γ (chIFN- γ) participates in antiviral and antibacterial responses. Despite its similarities in functionality with its mammalian counterpart, chIFN- γ and mammalian IFN- γ share less than 30% homology, but it reaches 50% in functional regions. Hence, both display low biological cross-reactivity. As in mammals, chIFN- γ promotes expression of *mhc-i* and *mhc-ii* genes (Davison et al., 2014; Digby and Lowenthal, 1995; Goossens et al., 2013).

1.4.2.4.2.2. Tumor necrosis factor alpha (TNF- α)

Tumor necrosis factor superfamily (TNFSF) comprises a wide range of molecules that are involved in several biological processes, including cell development, tumoral growth, cell proliferation, immune activation and others (Aggarwal, 2003; Aggarwal et al., 2012; Lang et al., 2016). It includes more than 19 ligands and 29 receptors so far described, thus even when most ligands interact with one receptor, some interact with

more than one, explaining in part, the promiscuous activity of some TNFSF ligands (Aggarwal, 2003; Bodmer et al., 2002). The main feature within the tumor necrosis factor superfamily members is their contribution to pro-inflammatory processes and it is defined that this action is through activation of NF- κ B pathways. Members of TNFSF are transmembrane proteins that potentially could be secreted as soluble forms. To be assigned as part of TNFSF ligands, molecules must contain a conserved C-terminal TNF homology domain (THD) that allow the binding to TNFSF receptors (Bodmer et al., 2002). These receptors are expressed in a wide variety of cells, and apparently no cell type does not express these receptors.

Tumor necrosis factor alpha (*tnf- α*) gene was discovered as a single copy in chromosome 6 in humans (chromosome 17 in mice) (Carswell et al., 1975; Parameswaran and Patial, 2010). Gene structure is defined by four exons and three introns and the mature region of *tnf- α* is encoded in the fourth exon. *TNF- α* is expressed in several cell types such as monocytes and macrophages, epithelial, mesenchymal and other cells (Aggarwal et al., 2012). *TNF- α* regulation is controlled at the transcriptional and translational level. Thus, NF- κ B and nuclear factor activated T cells, are involved in transcriptional regulation, whereas, through UA-rich sequences at the 3' UTR in the translational level (Parameswaran and Patial, 2010). *TNF- α* promotes different intracellular processes such as inducing extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38MAPK), activation of NF- κ B, apoptosis pathways and c-Jun N-terminal kinases (Aggarwal et al., 2012). Primary producers of *TNF- α* are monocytic lineage and following secretion, *TNF- α* interacts with receptors formed by TNFR1 and TNFR2. Interestingly, *TNFR1* is expressed in immune and non-immune cell

types, while *TNFR2* is only expressed in immune cells. Through TNFR1 recruits TRADD to activate caspase-dependent processes. On the other hand, TNFR2 promotes NF- κ B pathway activation through TRAF2 and TRAF3. Following activation in macrophages through TLRs, TNF- α is released and promotes macrophage activation, and along with IFN- γ , promotes cellular activation. As other mediators of immune responses, TNF- α plays a role against intracellular pathogens such as *Mycobacterium tuberculosis*, *Listeria monocytogenes* and *S. enterica* ser. Typhimurium (Rohde et al., 2018).

Even when TNF- α was described in several species such as mammalian, fish and others a long time ago, it was believed absent in the avian genome (Kaiser et al., 2005).

However, presence of *tnf- α* homolog in fish species define that this gene was evolutionarily conserved across species. In fact, recently have been identified the presence of chTNF- α and its corresponding receptors TNFR1 and TNFR2. Interestingly, chTNF- α display almost 50% homology with its mammalian counterpart, and their TNFR1 and TNFR2 receptors share around 30% homology. ChTNF- α has been found in chicken macrophages, macrophage-derived bone marrow, spleen monocytes and CD4⁺ lymphocytes (Rohde et al., 2018).

1.4.2.4.2.3. Interleukin 1 beta (IL-1 β)

The Interleukin 1 family comprises eleven members that share similarities in genomic structure, which suggest that they arose from duplication processes of an ancestral gene. Members of the IL-1 family are involved in innate immune responses, and are largely responsible for acute phase response; but they also participate in adaptive immunity

(Lopez-castejon and Brough, 2011; Lukens et al., 2012; Netea et al., 2010; Santarlaschi et al., 2013). In addition, IL-1 family members also promote expression of integrins in leukocytes and epithelial cells (Rider et al., 2011). IL-1 β is one of the most important cytokines in the IL-1 family, thus, a key factor during antimicrobial response. It is expressed by a various cell types ranging from keratinocytes, fibroblasts, synoviocytes, endothelial cells and immune cells such as neutrophils, monocyte and macrophages (Ren and Torres, 2009; Sims and Smith, 2010). IL-1 β is produced as protein precursor known as pro-IL-1 β after cellular activation through PAMPs. However, for its secretion, it is necessary the cleavage of this precursor by protease caspase-1. This caspase-1 activation is done through a process called inflammasome formation (Brough and Rothwell, 2007; Garlanda et al., 2013; Lopez-castejon and Brough, 2011; Netea et al., 2010; Ren and Torres, 2009). This process is initiated by complex proteins such as NOD like receptors (NLRs). Although, biological basis of inflammasome activation is yet to be clarified, a consensus proposes that reactive oxygen species, induction of hypokalemia and calcium-dependent phospholipase 2 activate the inflammasome. However, it has been described that inflammasome activation is not mandatory for IL-1 β release (Netea et al., 2010). In general, IL-1 β release is through loss of membrane integrity, shedding of microvesicles and multivesicular bodies containing exosomes (Dinarello, 2011). In addition to its contribution to innate responses, IL-1 β participates in the development of adaptive immunity. IL-1 β promotes the activation of CD4⁺ T lymphocytes and it has been demonstrated that mice lacking IL-1 β do not develop anti-sheep red blood cells antibodies. Additionally, IL-1 β has been shown to participates in Th17 polarization in autoimmune diseases.

1.4.2.4.3. Anti-inflammatory cytokines

1.4.2.4.3.1. Transforming growth factor beta (TGF- β)

TGF- β is the most important member of a growth factor superfamily (TGF- β superfamily) that is expressed in every human cell type and essentially each cell possesses a receptor for it. Initially, TGF- β was described due to its capacity to promote fibroblastic differentiation, although, further studies revealed its contribution in other biological processes such as cell proliferation, tissue repair, angiogenesis, embryonic development, regulation of innate and adaptive immune responses (Chin et al., 2004; Khalil et al., 1989; Kulkarni et al., 1993). Even though TGF- β was first characterized as a suppressor of inflammatory process, and it has been described to have effects as pro-inflammatory cytokine (Wahl, 1992; Yoshimura et al., 2010). TGF- β is a 25-kDa homo- or heterodimer peptide that participates in inhibition of T cell proliferation, inhibition of B cell proliferation and function, downregulation of NK cell activity and T cell response, regulation of cytokine expression and macrophage function (Clark and Coker, 1998; Kulkarni et al., 1993; Lacombe et al., 2004). At least five isoforms have been described and three have been characterized in mammals: TGF- β 1 - β 2 and - β 3, however, synthesis and intracellular processing has focused in TGF- β 1. Hence, following gene activation and transcription, pro-TGF- β 1 is cleaved by endopeptidases. After this cleavage, the protein is associated with other proteins to form the latent complex and further stored or secreted (Clark and Coker, 1998; Gentry and Nash, 1990). Monocytes and macrophages, through production of sialidases and proteases, contribute to the activation of TGF- β after secretion. Following activation, TGF- β either binds to cellular receptors (TGF- β RII and TGF- β RI) or several binding proteins. After binding to

its receptor, intracellular signaling promotes nuclear translocation of Smad molecules that induce gene expression (Sanjabi et al., 2009). Even though the latent TGF- β form has a half-life of 90 min, activated TGF- β is metabolized within minutes, revealing an extracellular control of TGF- β (Wahl, 1992)

TGF- β shows high conservation across species, including bovine, porcine and human. Even though *TGF- β* is ubiquitously expressed by all cells, main sources are platelets, macrophages, neutrophils, osteocytes and soft tissues such as placenta, kidneys, endometrium and even cancerogenic cells (Chin et al., 2004). In immune cells, TGF- β decreases macrophage activation, decreasing *iNOS* expression through smad-dependent pathways and protein degradation. TGF- β also downregulates T cell differentiation and development through IL-2 suppression, mainly in naïve cells. In addition, TGF- β inhibits Th1 and Th2 differentiation and Treg development through GATA-3, T-bet and Foxp3 pathways (Bobik, 2006). As pro-inflammatory cells, macrophages are also highly reactive to TGF- β . Hence, it induces monocyte adhesion and promotes chemotaxis through production of IL-1 and IL-6.

As in mammals, chicken TGF- β plays a role in anti- and pro-inflammatory processes and three genes have been described. While *tgf- β 2* and *tgf- β 3* are orthologs for mammalian counterparts, *tgf- β 4* is the ortholog for the prototypical mammalian *tgf- β 1* (Davison et al., 2014; Kaiser et al., 2005).

1.4.2.4.3.2. Interleukin 10 (IL-10)

Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine that regulates immune response and autoimmune disorders (Iyer and Cheng, 2012; Saraiva and Garra, 2010;

Sky Ng et al., 2013). Previously known as cytokine synthesis inhibitory factor (CSIF), IL-10 was initially associated with Th2 responses but is also involved in T regulatory responses. It is widely known that IL-10 is produced by innate and adaptive immune cells including dendritic cells, macrophages, NK cells, neutrophils, B cells, T cells (Couper et al., 2008; Cyktor and Turner, 2011; Moore et al., 2001; Trinchieri, 2007) and others such as non-hematopoietic cells (Zigmond et al., 2014). Expression and secretion of *IL-10* is mediated through the contribution of other cytokine release factors such as IL-12, TGF- β and IL-6 (Ma et al., 2016). Interleukin 10 mediates its action through binding to its cellular receptor IL-10R. This receptor is a heterotetramer molecule composed of two subunits, IL-10R α and two IL-10R β (Moore et al., 2001). Following ligand-receptor interaction, IL-10 induces cellular activation of signal transducer and STAT3, promoting immunosuppression through NF- κ B pathways. Although suppression of *IL-10* expression is beneficial for pathogen clearance in some disorders, it might induce immunopathology in others. In activated macrophages, IL-10 downregulates the expression of MHC-II complex molecules and costimulatory factors (CD80 and CD86) but also the capacity to produce reactive oxygen species and nitric intermediates (Cyktor and Turner, 2011). However, IL-10 promotes development of immune responses via induction of IL-10-secreting T reg cells and enhancing CD8 T cells, NK cells and B cells (Saraiva and Garra, 2010). Anti-inflammatory action of IL-10 leads to the suppression of pro-inflammatory cytokines IFN- γ , TNF- α , IL-1 and IL-2, thus, is a potent mediator of the inflammatory process. Therefore, IL-10 is a key mediator between innate and adaptive immune defenses (Piazzon et al., 2015).

IL-10 regulates immune responses in multiple microbial infections. In fungal infections, IL-10 increases development of naïve Tregs and memory responses to *Candida albicans*. In viral infections, such as HIV, IL-10 suppresses IL-2; in HSV-1, suppress IFN- γ , IL-2 and T cell migration; in LCMV infections, IL-10 downregulates TNF- α , IL-2 and cytotoxic activity. In terms of bacterial infections such as *Mycobacterium spp.* (suppressing macrophages), *Listeria monocytogenes* (inhibits bacteria killing), *Helicobacter spp.* (suppressing IFN- γ), *Bordetella spp.* (downregulates IL-12), *Streptococcus pyogenes* (suppress IL-12 and granzyme B).

In contrast to what is observed in the human genome where the IL-10 family comprises six members, the chicken genome only has four members. *il-10*, *il19*, *il-22* and *il-26* have been defined as members of the chicken IL-10 family. As in mammals, ch-IL-10 also plays a role as an anti-inflammatory mediator (Cheeseman, 2007; Davison et al., 2014).

1.4.2.4.3.3. Interleukin 2 (IL-2)

IL-2 is a 15kDa cytokine that was originally detected and defined as part of T cell mediator responses (Banchereau et al., 2012). Thus, IL-2 is mainly produced by CD4⁺ T cells, although it is also secreted by CD8⁺ T cells, NK cells, NKT cells and dendritic cells in smaller amounts (Krieg et al., 2009; Liao et al., 2013). *IL-2* transcription is mediated through multiple transcription factors such as nuclear factor of activated T cells (NFAT), AP-1, FOS-JUN, NF- κ B and OCT-1. Activation of T cells, through TCR and CD28 via *IL-1*, *IL-2*, *IL-7*, *IL-12*, *IL-15* expression, promotes expression of *IL-2* and IL-2 receptor (*IL-2R*) acting via paracrine or autocrine fashion. Three IL-2 receptor

chains form low, intermediate and high affinity IL-2 receptors (IL-2R α , IL-2R β and IL-2R γ) (Hoyer and Barron, 2008).

IL-2 induces a potent action on lymphoid populations including T, B and NK cells, but it is also demonstrated that IL-2 exerts function in macrophage and neutrophil activity.

In homeostatic conditions, CD4⁺ T cells are the main source of IL-2 in low levels, however, following activation IL-2 release increases. In terms of T cell effect, IL-2 promotes cell differentiation from CD4⁺ T cells to Th1 and Th2, inhibiting Th17 and inducing memory T cells (Hoyer and Barron, 2008; Liao et al., 2013, 2011).

Additionally, IL-2 plays a role in the development of T regulatory cells. On CD8⁺ T cells, IL-2 upregulates CD25 and CD122; and drives the differentiation of naïve CD8⁺ T cells into effector or memory cytolytic T lymphocytes, through antigen activation in inflammatory processes for induction of IFN- γ , perforin and granzymes (Krieg et al., 2009; Malek, 2008; Malek and Castro, 2010; Pipkin et al., 2010).

Chicken IL-2 (*ch-il-2*) has been cloned from spleen samples and revealed that gene structure is identical to the mammalian gene. Hence, Ch-IL-2 has been shown to share low amino acid identity with bovine IL-2. Even though Ch-IL-2 has the same properties as mammalian IL-2, it is only produced by activated T cells (Hilton et al., 2002).

Recombinant Ch-IL-2 has been shown to induce bactericidal activities such as phagocytosis in heterophils (Kogut et al., 2003).

1.4.2.5. Antimicrobial responses

1.4.2.5.1. NF- κ B activation

The NF- κ B transcriptional factor family encompasses a large group of protein complexes that participate in numerous cellular processes including immune responses, apoptosis, cellular growth and development; and as such, they have been involved in several developmental disorders such as cancer, inflammatory diseases and others (Liang et al., 2004; Liu et al., 2017; Tak and Firestein, 2001). Although members of the NF- κ B transcriptional factor superfamily do not exert antimicrobial responses *per se*, indeed they are major players triggering effector mechanisms following pathogen-cell interaction and therefore, tightly linked to cellular immune defenses (Hetru and Hoffmann, 2009; J. Liu et al., 2017; Tripathi and Aggarwal, 2006). NF- κ B transcription factor was first discovered in the 1980s, involved in immunoglobulin light chain gene transcription in pre-B cells following LPS activation (Sen and Baltimore, 1986). NF- κ B transcription factor has been described in metazoans including *Drosophila melanogaster* (Hetru and Hoffmann, 2009), starlet sea anemone (Ryzhakov et al., 2013), chickens (Ikeda et al., 1993) and pigs (Li et al., 2011). NF- κ B/Rel family proteins are sequestered into the cytoplasmic compartment, as hetero or homodimers mainly associated to a I κ B inhibitory family members. The canonical mechanism of NF- κ B activation is achieved through enzymatic degradation of I κ B protein members. Following I κ B protein degradation, NF- κ B complex is translocated to the nucleus where it binds to promotor/enhancer regions of target genes (Caamaño and Hunter, 2002; Finco and Baldwin, 1995; Silverman and Maniatis, 2001). In mammals, NF- κ B complex is composed of five members: NF- κ B₁ (p105/p50), NF- κ B₂ (p100/p52), RelA (p65), RelB

and cRel. In contrast, *Drosophila melanogaster* NF- κ B homologs possess three subunits (Dorsal, Dif and Relish). Despite significant differences in evolutionarily divergent species, higher vertebrates share the common mammalian NF- κ B protein conformation. There are two pathways of NF- κ B activation, the classical NF- κ B activation pathway that is essentially triggered by soluble mediators such as pro-inflammatory cytokines and PAMPs through I κ B phosphorylation and further degradation. A second, most recently described and widely conserved, alternative NF- κ B activation pathway, which is IKK α dependent rather than I κ B degradation mediated. It has been suggested that the classical pathway is involved in the innate immune response, in contrast to the alternative activation pathway where it seems to be associated with development of adaptive responses (Senftleben et al., 2001). Hence, NF- κ B nuclear translocation has been widely used not only to evaluate cellular activation but also the pharmacological effect of drug toxicity in a large variety of biology fields. Thus, there have been developed a vast amount of commercially available antibody against p65 subunit of NF- κ B complex that is used to assess dynamically the nuclear translocation following cellular activation. Furthermore, antibody assay-based confocal microscopy, flow cytometry, and others; provide a sensitive tool for NF- κ B nuclear translocation. As a transcription factor, NF- κ B acts to promote and activate a series of antimicrobial processes after phagocytosis (McDonald and Cassatella, 1997; Zhu et al., 2014), it is associated to nitric oxide production through *iNOS* expression (Arias-salvatierra et al., 2011; Napolitano et al., 2008) and regulation of reactive oxygen species production (Berg et al., 2001; Gloire et al., 2006; Morgan and Liu, 2010; Nakajima and Kitamura, 2013).

1.4.2.5.2. Reactive oxygen species (ROS) production

ROS are free radicals such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO\bullet$) and hypochlorous acid (HOCl); that are normally produced by reduction/oxidation reactions (Ray et al., 2012). Hence, low levels of ROS are an essential part of several intracellular processes including cell development and proliferation, antimicrobial defenses, cell death and others (Dowling and Simmons, 2009; Mittal et al., 2014). However, where the regulatory mechanisms of ROS control are impaired, uncontrolled production of reactive oxygen species might be prejudicial for DNA, RNA and protein synthesis, in a state called oxidative stress. As a result, oxidative stress has been associated with several disorders such as carcinogenesis, neurodegeneration, atherosclerosis and others (Alfadda and Sallam, 2012; Ray et al., 2012). The mechanisms by which ROS affects proteins is through oxidation of aminoacidic residues and altering protein conformation and this is the main feature in antimicrobial response. Sources of ROS for cell signaling are mitochondria, through electron transport chain (ETC), via cytochrome p450. However, ROS production for antimicrobial defenses are derived from NADPH oxidase pathways, in professional phagocytes such as neutrophils and macrophages; and endothelial cells (Mittal et al., 2014).

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a complex that possesses seven homologs, differentially expressed in multiple tissues, and is composed by a membrane catalytic NOX (gp91^{phox}), and intracellular subunits including p22^{phox}, p40^{phox}, p67^{phox}, p47^{phox} and Ras-related C3 botulinum toxin substrate 2 (rac2). In resting cells, these intracellular subunits are isolated, however following activation,

phosphorylation of p47^{phox} leads to subunits assembly (Bae et al., 2009; Ibi et al., 2008; Kohchi et al., 2009). The assembled intracellular complex is further translocated to the membrane component gp91^{phox}, to form the stable complex of NADPH oxidase. There are several kinase inducers that promotes p47^{phox} phosphorylation including pro-inflammatory cytokines such as TNF- α , G-CSF and GM-CSF, LPS, PMA and N-formylmethionyl leucyl phenylalanine (fMLP) (Mittal et al., 2014). Hence, ROS production is essential for pathogen killing in the phagolysosome compartment formed by fusion of phagosome and lysosome.

Mitochondria is another source of ROS production that contributes to total cellular ROS levels, therefore, also contributes to ROS production during inflammation (Hernandez-Garcia et al., 2010). Electron transport carriers with high redox potential are organized as complex I, II, III and IV. Superoxide anion (O⁻) is metabolized by superoxide dismutase to produce H₂O₂ that is further translocated to the cytoplasmic compartment (Alfadda and Sallam, 2012). Hence, these reactive oxygen species will promote activation of transcription factors such as HIF-1 α and NF- κ B for cytokine expression and inflammasome formation. Pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α are activated following LPS stimulation (Mittal et al., 2014). Thus, mitochondria ROS contribution plays a role as antimicrobial defense and intracellular signalling.

As a highly conserved antimicrobial mechanism, ROS production in avian system has been extensively described using several primary and cell line models such as chicken monocyte lineages, HD-11, MQ-NCSU and HTC cell lines (He et al., 2005; Qureshi et al., 1993). Additionally, different triggers have shown to induce ROS production. Hence, zymosan, phorbol myristic acid and different Salmonella serovars are strong

ROS inducers in chicken macrophages (Nerren and Kogut, 2009). For many years, production of ROS by heterophils has been controversial. There has been various research that pointed out chicken heterophils do not produce ROS. However, some research groups described ROS production following *in vivo* and *in vitro* challenges. Even when chicken heterophils do not have the capacity to promote a strong response in ROS production as other mammalian and teleost fish models, chicken heterophils do produce reactive oxygen species following pathogen activation (Davison et al., 2014; Farnell et al., 2003; Genovese et al., 2013, 2007, Kogut et al., 2001a, 2001b, 1995, More-Bayona et al., 2017b, 2017a).

Within the vast antimicrobial mechanisms that cells possess, reactive oxygen species production is a highly conserved tool for pathogen control. Despite their antimicrobial activity, ROS participate as second messengers in cell signaling (Bae et al., 2009) and other intracellular pathways.

1.4.2.5.3. Nitric oxide (NO) production

Nitric oxide (NO) was named the “molecule of the year” in 1992 due to its numerous functions in biology processes. NO is a short-lived free radical and one of the smallest molecular mediator described to date (Sharma and Parvathy, 2007). NO is an important intracellular and intercellular signaling mediator in several physiological processes. In mammals, NO metabolism is an enzymatic process where three isoforms of nitric oxide synthases (NOS) are involved (Bogdan, 2015). NOS are dimeric flavoproteins similar to cytochrome P450 with a high expression in cardiovascular and central nervous tissues. Thus, endothelial-nitric oxide synthase (eNOS), neuronal-nitric oxide synthase (nNOS)

and inducible-nitric oxide synthase (iNOS) play a role in nitric oxide production in different tissues. While eNOS and nNOS are constitutively expressed in normal metabolism in those tissues, iNOS is promoted after cellular activation (Lundberg et al., 1997). In immunity, NO was initially described as a by-product of L-arginine metabolism to L-citrulline, by the enzymatic activity of inducible nitric oxide synthase (iNOS) with antibacterial and antitumor activity (Bogdan, 2015; Coleman, 2001; Connelly et al., 2001; Tripathi et al., 2007a). Although this definition is accepted, other diverse functional activities have been described.

iNOS isoform is mainly activated by pro-inflammatory cytokines such as IL-1 β , IL-2, IFN- γ and TNF- α , endotoxin and LPS activation, through the NF- κ B pathway.

However, other transcriptional factors such as AP-1, Jak-STAT are also involved (Ghosh et al., 1998). Therefore, it promotes the activation of several biochemical mediators, regulating the expression of *iNOS*, constituting a self-regulating pathway. Hence, NO production not only plays a role in pro-inflammatory processes and anti-inflammatory reactions (Connelly et al., 2001; Korhonen et al., 2005).

As pro-inflammatory mediator, NO is produced in monocyte, macrophages, neutrophils and other cells (Lind et al., 2017). After initial NO release in phagocytic cells, specifically in the phagolysosome compartment, a proportion is released to the extracellular compartment where NO acts as a chemical mediator (Moilanen and Vapaatalo, 1995). Over production of NO during this inflammatory process, leads to a downregulation of its NO synthesis. NO also inhibits neutrophil migration and apoptosis (Dal Secco et al., 2004; Nolan et al., 2008). Thus, NO production has a pro-inflammatory and anti-inflammatory effect depending upon its relative concentration

during immune responses (Aktan, 2004; Korhonen et al., 2005; Sharma and Parvathy, 2007).

In chicken macrophages, HD11 cells, LPS induced NO production after 24 hours, and it was the first description of NO production in chicken macrophages (Sung et al., 1991). Interestingly, chickens are unable to metabolize L-arginine due to lack of carbamoyl phosphate synthase I and ornithine transcarbamoylase activity, thus chicken nitric oxide production relies on the nutritional supply of L-arginine (Tamir and Ratner, 1963).

1.4.2.5.4. Phagocytosis

Phagocytosis is a highly evolved process from primitive organisms to higher vertebrates (Gordon, 2016; Metchnikoff, 1921; Richards and Endres, 2014). Although in primordial species, phagocytosis is used as a mechanism for nutrients uptakes, in higher evolved organisms, it is a specialized, tightly controlled process for pathogen elimination (Aderem, 2003; Flannagan et al., 2012; Metchnikoff, 1891; Rosales and Uribe-Querol, 2017; Yutin et al., 2009). Hence, phagocytosis is the paramount process of immune defenses in higher organisms. In short, it is a cellular mediated process where pathogens are engulfed and destroyed in a biochemical coordinated fashion, by specialized leukocytes know as phagocytes including monocytes, macrophages, neutrophils, some lymphocytes and others (Botelho and Grinstein, 2011). These professional phagocytes play a role in innate immunity through elimination of pathogens following invasion and participate in the induction of adaptive responses by antigen presentation to form acquired immunity (Uribe-Querol and Rosales, 2017). In short, phagocytosis can be divided in three general steps: (1) pathogen or microbial recognition; (2) phagosome

formation and (3) phagolysosome formation and pathogen degradation (Aderem, 2003; Flannagan et al., 2012; Uribe-Querol and Rosales, 2017). Particles larger than 0.5 μm are internalized by cellular surface receptors such as MAMPs that interact with PRRs (Freeman and Grinstein, 2014). These receptors are divided into two groups based on the molecule they interact with. Non-opsonic receptors bind directly to MAMPs, while opsonic receptors required the presence of other associated molecules such as antibodies, globulins, complement, fibronectin and others (Uribe-Querol and Rosales, 2017). Following this receptor-pathogen interaction leads to a membrane modification, through actin reorganization, to form a pathogen containing vacuole, called the early phagosome. A dynamic process allows the interaction of endocytic and secretory vesicles that promotes formation of late phagosome. This event precedes the phagolysosome maturation where lysosome integrates to late phagosome, under these conditions, remodeling of membrane, phagolysosome acidification and induction of oxidative milieu promote pathogen degradation (Freeman and Grinstein, 2014; May and Machesky, 2001).

Once the phagolysosome is formed, production of high levels of V-ATPase molecules occurs along with enzymes such as cathepsins, proteases, lysozymes and lipases, and NADPH molecules that participates in the production of reactive oxygen species. Hence, these mechanisms are involved in pathogen elimination and further antigen presentation (Flannagan et al., 2012; Henneke and Golenbock, 2004).

In chicken, several phagocytic cells are described even in embryonic stages. For instance, macrophages with phagocytic capacity are shown in liver and spleen in embryos. Additionally, macrophages are elicited in one-day-old chickens. Furthermore,

several cell lines have phagocytic capacity such as HD11, MQ-NCSU and HTC (Jeurissen et al., 1989; Qureshi, 2003). Phagocytic capacity in chicken macrophages has been demonstrated with a wide range of microbes. Hence, *Salmonella enterica* (Qureshi et al., 1994, 2000; Xie et al., 2000), *Pasteurella multocida* (Harmon, 1998; Harmon et al., 1992) and others fungal pathogens such as *Candida albicans* and lately, zymosan obtained from *Saccharomyces cerevisiae* demonstrated to induce phagocytosis in heterophils and chicken macrophages *in vivo* (More-Bayona et al., 2017b, 2017a).

1.4.3. Inflammation models

1.4.3.1. *S. enterica* ser. Typhimurium

Salmonella spp. are the cause of several systemic and enteric diseases worldwide. In addition, *Salmonella* has a broad range of hosts including cattle, poultry, swine, mice and humans (Dougan et al., 2011; Griffin and Mcsorley, 2011; Santos et al., 2003). One of the most important serovars involved in enteric diseases is *Salmonella enterica* serovar Typhimurium (*S. enterica* ser. Typhimurium) for its capacity to induce enteric illness that affects a broad range of hosts. Due to its importance as a source of food-borne disease and zoonotic problems, *S. enterica* ser. Typhimurium has been one of the most studied pathogenic bacteria to date (Darwin and Miller, 1999). It has been used to get a better understanding in microbial pathogenesis, but also the assessment of immunological responses. In this review, I will focus on the immune responses driven by *S. enterica* ser. Typhimurium rather than other serovars.

S. enterica ser. Typhimurium is a gram-negative, flagellated, facultative intracellular bacteria that causes a self-limiting gastroenteric infections. Following bacteria ingestion,

S. enterica ser. Typhimurium needs to compete with resident microbiota for nutrients (Wray and Wray, 2000). Furthermore, bacteria utilize its virulence factors to promote inflammation and facilitate establishment in the gut. In the intestinal lumen, bacteria establishment goes through two phases. Initially, bacteria must adhere to epithelial cells through adhesins and fimbriae (Santos, 2014; Santos et al., 2003). After adhesion, bacteria can also access the lamina propria via M cells, or access enteric cells through type III secretion system (T3SS), a virulence factor that is encoded in Salmonella pathogenicity island I (SPI-1) (Bruno et al., 2009; Salcedo et al., 2001). This enteric invasion promotes a massive loss of enteric cells. This induces a massive inflammatory process led by neutrophil infiltration. During this inflammatory state, *S. enterica* ser. Typhimurium has the capacity to survive inside infected macrophages, through mechanisms that block intracellular antimicrobial responses such as interfering with phagolysosome fusion (Buchmeiert and Heffront, 1991). Hence, via this process, some animals shed *S. enterica* ser. Typhimurium for prolonged periods after infection (up to 28 weeks in pigs) and is responsible of bacterial dissemination in the environment and other animals.

1.4.3.1.1. Classification

In terms of nomenclature, Salmonella belongs to Enterobacteriaceae family. species have been classified into two groups, *Salmonella enterica* and *Salmonella bongori*. The first group include subspecies differentiated by their biochemical, antigenic and genetic features, and are defined in general as pathogenic subspecies. On the other hand, *S. bongori*, includes a group of opportunistic, non-pathogenic subspecies. Immune

classification uses serological identification based on the presence of O antigen (LPS), H antigen (flagella) and K antigen (capsule). Other subspecies of importance include *S. typhi* and *S. enteritidis* (Brenner et al., 2000).

1.4.3.1.2. Entrance of *S. enterica* ser. Typhimurium into the host

Most Salmonella infections are initiated through oral ingestion. After ingestion, *S. enterica* ser. Typhimurium (and other serovars) face its first physical barrier in the mucin layer. This barrier is composed of several glycoproteins produced by goblet cells (Broz et al., 2012) including glycoproteins, antimicrobial peptides such as cathelicidins, defensins, C-type lectins and others. Altogether, these components are the initial barrier against Salmonella infections. Despite the development of these antimicrobial mechanisms, *S. enterica* ser. Typhimurium has successfully developed means to avoid this response. *S. enterica* ser. Typhimurium possess a *Pho-PhoQ* transduction system that is responsible to sense the presence of antimicrobial peptides (Darwin and Miller, 1999). Through this mechanism, Salmonella is able to modify its lipid A and make them resistant to antimicrobial peptide action (Broz et al., 2012). Furthermore, while T3SS promotes bacteria entry and inflammation in initial stages, *Pho-PhoQ* suppress T3SS in an attempt for extracellular survival (Griffin and Mccorley, 2011; Hughes and Gala, 2002; Patel and McCormick, 2014; Santos et al., 2003). Additionally, *Pho-PhoQ* is also involved into bacterial survival following macrophage uptake (Jones and Falkow, 1996).

1.4.3.1.3. **Salmonella-cell interactions**

The next barrier that *S. enterica* ser. Typhimurium must overcome is the epithelial barrier. Tight junctions allow reduced permeability across epithelial monolayer, however, under inflammatory conditions, driven mainly by IL-1 β , this integrity is affected, and *IL-8* expressed by enterocytes activated by *S. enterica* ser. Typhimurium fimbriae (Broz et al., 2012; Winter et al., 2009). During this process, *S. enterica* ser. Typhimurium impairs the expression of occludins in epithelial cells allowing the translocation of bacteria through epithelial cells (Darwin and Miller, 1999).

Furthermore, altering monolayer integrity promotes active leukocyte migration to the intestinal lumen. This massive leukocyte infiltration is composed of neutrophils and is lead by an active release of chemoattractant mediators along with the effect on the epithelial integrity (Patel and McCormick, 2014; Santos et al., 2003). Concurrently, *S. enterica* ser. Typhimurium translocates several effector proteins, such as SipA, SopA, SopB, SopD and SopE2 that are encoded in SPI-1, to epithelial cells (Bruno et al., 2009). These proteins possess kinase and phosphatase activity that precedes bacteria internalization and modification of gene expression (Santos, 2014; Santos et al., 2003). Additionally, *S. enterica* ser. Typhimurium also utilize M cell in the epithelial layer to access the lamina propia (Ilyas et al., 2017).

After crossing the epithelial barrier, *S. enterica* ser. Typhimurium faces the next barrier of infection. Phagocytic cells from gut associated leukocyte tissue (GALT) such as macrophages and dendritic cells are the first cells involved in restricting bacterial spread (Dougan et al., 2011). These cells internalize *S. enterica* ser. Typhimurium with the goal of destroying it. Once internalized, *S. enterica* ser. Typhimurium express type III

secretion system that allow it bacteria survival into the Salmonella containing vacuole (SCV) (Bruno et al., 2009). This T3SS prevent the movement of nitric oxide, reactive oxygen species and other antimicrobial peptides. Through this mechanism, bacteria can survive and multiply in phagocytic cells. Although, bacteria initially can avoid immune defenses, phagocytic cells express extracellular and intracellular PRRs such as TLRs -2, -4, -5, -6, -9 and others to promote activation of transcription factors, NF- κ B and IRF3, to express pro-inflammatory cytokines (Broz et al., 2012). Through *Pho-PhoQ S. enterica* ser. Typhimurium inhibits the fusion of the SCV with lysosomes and endosomes. During Salmonella infections, TLR-1 along with TLR-2 and TLR-6 participate in the interaction with Salmonella lipoproteins such as lipopeptides, lipoteichoic acids (LTAs); TLR-4 to lipopolysaccharide (LPS), TLR-5 to Salmonella flagellin FliC and TLR-9 to CpG elements in Salmonella DNA (Broz et al., 2012; Roach et al., 2005; Takeuchi et al., 2018, 2001; Zhan et al., 2015). TLR-2 and TLR-4 are particularly important for bacterial survival since *tlr4⁻ tlr2⁻* deficient mice failed to acidify salmonella-containing vacuole (SCV), therefore preventing bacteria killing. In this context, bone marrow-derived macrophages lacking *tlr2* and *tlr4* genes produced significantly lower levels of nitric oxide following Salmonella exposure, highlighting the importance of TLR signalling for antimicrobial responses (Arpaia et al., 2011). Interestingly, TLR-4 signalling during salmonella infections display not only intracellular effects, but compromise dynamics of leukocytes and development of adaptive responses. Thus, TLR-4 ligands stimulate cytokine expression, leukocyte recruitment, activation of phagocytic cells and the transition to long term immunity (Vazquez-torres et al., 2018). In contrast to TLR-4, TLR-2 does not play a significant

role in the resolution of salmonella infections, however, *tlr2* knockout mice revealed higher salmonella burden in infected mice, with no major effect on mortality and antibody responses (Oliveira-Nascimento et al., 2012). Interestingly, cytokine expression following salmonella infections is mainly mediated through TLR-4 and to a minor extent by TLR-2. It has been suggested that this effect is due to a lower efficiency in signal transduction following TLR-2 activation compared to TLR-4. Thus, lack of those receptors diminishes *TNF- α* and suppressed *IL-6* expression. This suggests that LPS plays a significant role in cytokine expression, highlighting the importance of TLR-2 and TLR-4 in cytokine expression against Salmonella (Lembo et al., 2003). During initial stages of immune response, T cell activation is achieved also through IL-17 and IL-22 production in inflamed enteric tissue. In addition, innate lymphoid cells (ILCs) also play a role during initial stages of *S. enterica* ser. Typhimurium infection, including NK cells, $\gamma\delta$ T cells and lymphoid tissue inducers cells (LTi) that are a source of IL-22 (Broz et al., 2012).

1.4.3.1.4. Development of adaptive responses to ST

Following responses of antigen presenting cells (APCs) such as macrophage and dendritic cells, a link with long-term responses (as production of specific antibody response) is mounted. Although intestinal tissues provide limited lymphoid cells to study adaptive immune response development, using mice models has allowed the assessment of CD4⁺ T cells responses (Markey et al., 1996). These cells respond against *S. enterica* ser. Typhimurium challenges within a few hours (3-6 hours) following invasion in Peyer's patch. CD4⁺ T cells seem to play a stronger role than CD8⁺ T cells

(Mittrücker and Kaufmann, 2000). However, it has also been shown that CD8⁺ T cells are relevant for adaptive response. Even though Th1 cells are the major response following *S. enterica* ser. Typhimurium infections, Th2 cells are also part this response. In general, clearance of *S. enterica* ser. Typhimurium after enteric infections is a slow process compared to other enteric pathogens, due to the immune regulatory capacity of this bacteria through nitric oxide suppression (Mastroeni and Me, 2003).

On the other hand, B cells response has been shown to play a part in adaptive responses in mice infected with *S. enterica* ser. Typhimurium. Even though B cells are important in protection for secondary infections, full protection is achieved through a coordinated response with T cell mediated immunity. However, the mechanisms of B cell responses following *S. enterica* ser. Typhimurium infections are not currently fully understood (Cookson et al., 2009; Vazquez-torres et al., 2018).

1.4.3.1.5. *S. enterica* ser. Typhimurium in livestock

Salmonella infect several species including cattle, swine, poultry and humans. Despite the differences in enteric disorders among species, salmonella infections promote notable similarities in immune responses. As observed in other species, in chickens infected with *S. enterica* ser. Typhimurium orally, infection displays enteric process and the clearance of bacteria and immunity is achieved mainly through cellular response within two to three weeks following infection with no systemic compromise (Wigley, 2014, 2013). Similar to what is observed in mammals, polymorphonuclear cells such as heterophils, infiltrate inflamed tissues. Activation of $\gamma\delta$ T cells in chicken is stronger compared to mammals, thus, it is hypothesized that they contribute to the adaptive

responses (Wigley, 2014). Vaccination protocols in chicken have shown diverse results, probably due to the route used and the specific strain and form. For instance, killed *S. enterica* ser. Typhimurium has been shown to induce a strong inflammatory response but seems to promote relatively low protection to secondary challenges. On the other hand, vaccination using attenuated and live *S. enterica* ser. Typhimurium induces protective immunity (Beal et al., 2004).

Infections in swine species is similar to human infections. However, in pig farms, *S. enterica* ser. Typhimurium induce a mild enteric disease with few clinically infected animals, but most importantly, the progress of carrier animals or persistent infected animals is established (Knetter et al., 2015; Riber and Lind, 1999). In pigs, Salmonella infections tends to affect young pigs from 6-12 weeks of age with lower incidence in older pigs (Wray and Wray, 2000). Salmonella infections in pigs not only affect animal health inducing diarrhea in infected animals but also affect weight gain (Kim and Isaacson, 2017).

1.4.3.2. Zymosan

1.4.3.2.1. Origins

Fungal infections activate several immune responses. Zymosan is a yeast cell wall component obtained from *Saccharomyces cerevisiae*. Initially, it was shown to induce hyperplasia and hyperfunction in reticuloendothelial cells, ie. macrophages (Riggi and Di Luzio, 1961). Zymosan, formed by 50-60% of 1-3- β -glucans, is a particulate polysaccharide of D-glucose (Young et al., 2001) and mannan residues (Frasnelli et al., 2005; Sato et al., 2003) which are main components of fungal species (Romani, 2011).

Mannans are formed by a chain of mannose units linked to mannopyranose units. Although this conformation is conserved, the stimulating properties seems to be associated with β -glucans (Riggi and Di Luzio, 1961).

1.4.3.2.2. Stimulating properties

Zymosan has been widely used in studies of inflammation in several species due to its properties in activating a wide range of cell types, including monocytes, macrophages, polymorphonuclear leukocytes and natural killer cells (Sanguedolce et al., 1992; Underhill et al., 1999; Young et al., 2001). Zymosan-cell interaction is achieved through highly conserved cellular receptors such as toll-like receptors (TLRs) and mannose receptors. Surface cell receptors play a role in cellular activation and phagocytosis of zymosan. Thus, TLR-2 in association with CD14 and TLR-6, receptors for zymosan, induce activation of transcriptional factors, such as NF- κ B that further promote gene expression of proinflammatory mediators including *IL-1*, *IL-2*, *IL-6* and *IL-8* (Brown, 2006; Plato et al., 2013). On the other hand, internalization of zymosan by phagocytic cells is achieved through C-type lectin receptors, specifically, dectin-1 that is highly expressed in monocytes, macrophages and neutrophils (Dillon et al., 2006; Frasnelli et al., 2005). Following dectin-1 binding, a group of tyrosine kinases, such as caspase-recruitment-domain containing protein 9 (CARD9) and RAF pathways are activated. Hence, through dectin-1, NLRP3 inflammasome activation is induced and further production of pro-inflammatory mediators (Romani, 2011). Interestingly, dectin-1 expression is induced by IL-4 and IL-13 that leads to Th2 responses (Brown, 2006). Altogether, TLRs and dectin-1 receptors act synergistically to contribute to pro-

inflammatory responses. Interestingly, despite the fact of its pro-inflammatory properties, zymosan induces the expression of anti-inflammatory cytokines such as *IL-10* and TGF-beta, suggesting a role in the anti-inflammatory processes (Dillon et al., 2006).

1.4.3.2.3. Zymosan-induced inflammation

As mention above, zymosan participates in pro-inflammatory and anti-inflammatory processes. Hence, zymosan promotes activation of alternative pathways of complement through production of C3a and C5a; biosynthesis of lipid mediators such as arachidonic acid derivatives, production of reactive oxygen species and lysosomal enzymes (Rao et al., 1994), and activation of pro-inflammatory and anti-inflammatory cytokine. Due to these features, zymosan has been widely used for studies of acute inflammation and resolution phase of inflammatory process. Hence, zymosan represents a reliable tool for assessment of acute inflammation. Through use of zymosan, dynamics of main leukocyte subpopulation such as neutrophils, monocytes, macrophages and lymphocytes during inflammation, are studied and their functionality has been assessed. Zymosan has been used in different species including mice (Rao et al., 1994), teleost fish (Havixbeck et al., 2016, 2014; Rieger et al., 2012), chicken (More-Bayona et al., 2017a, 2017b), rats (Zagorski and Wahl, 1997) and rabbit (Forrest et al., 1986) as a self-resolving inflammation model through peritoneal challenge. Hereafter, I defined some relevant immunological parameters following activation through zymosan in chicken and swine.

1.4.4. Water and health

1.4.4.1. Water in biological processes

Water is the most important molecule for any organism. Thus, its consumption is vital for animal development. Despite its importance in life, we are still far from fully understand its role in biology and animal health; and it has been underestimated in the last decades (Ball, 2017). In the past, water was considered only as a mere vehicle for macromolecules such as proteins or nucleic acids that transit from place to place (Chaplin, 2006). However, now it is well known that water plays an active role in all biological process so far described. Water exhibits diverse and complex structural and dynamic roles in cell biology (Ball et al., 2007). Therefore, it exerts one of the forces that dictates molecular conformations such as hydrophobic attraction and plays a part in chemical transit.

Water inside cells has different characteristics to extracellular water. In general, it is considered that intracellular water has higher viscosity and lower capacity to diffuse through membranes. Although it can be argued that these properties are due to the presence of a diverse array of macromolecules, it seems to be attributed to a multifactorial effect. Hence, it is suggested that cytoplasmic fluid is a more gel-like environment. Therefore, changes in protein conformation inside cells induces modifications will lead to effects on intracellular viscosity. This effect forms high-density and low-density areas exhibiting different metabolic and reactive properties. On this note, it has been suggested that higher intracellular hydration has been linked as primary trigger to malignancy in cancer cells.

1.4.4.2. Drinking water quality

Water plays a role in several biological conditions including gain or loss of body heat, a process called thermoregulation; balance of internal and external electrolytes or osmoregulation; nutrient transport and elimination of waste products. As water is pivotal in organism development, quality of water is essential for normal function. As consequence, poor drinking water quality is the main cause of development disorders and disease such as diarrhea associated with microbial infections (parasites, bacteria, others), which is considered the leading cause of morbidity and mortality in young children under 5 years of age in developing countries. Hence, it is considered that 30% of those cases are related to contaminated water. This groups of disorders are caused by biological entities. On the other hand, industrial activities have increased the risk for environmental pollution since multiple waste contaminants are released without proper processing therefore they become potential threats for environmental health (Xia et al., 2017). This group of disorders is caused by non-biological drivers of disease. Most studies have focused on the disorders caused by microbiological entities that produced clinical disease, however, little is known about non-biological compounds that induces immune effects even in the absence of clinical status. Hence, one of the common targets after pollutants release is the aquatic environment. As this environment receive and store those pollutants, these contaminants are eventually encountered by animals and humans as mixtures. With the development of multiple assays to evaluate the presence of organic and inorganic chemicals, water analysis has gained relevance for assessment of quality control for potable water. Water analysis tests can be divided into three groups based on the pollutant targets. Physical tests, that indicates water properties detectable

by senses such as colour, odor, turbidity, taste, etc; chemical tests, that detect the presence of organic and inorganic chemicals; and microbiological tests that evaluate the presence of microorganisms such as bacteria and parasites including *Salmonella spp.*, *E. coli*, etc. Despite the importance of the drinking water quality related to organic or microbial contamination and its association with health disorders, presence of chemicals or other inorganic compounds in drinkable water has also importance, mainly in subclinical conditions due to effects on nutrient absorption and others. However, the analysis for multiple contaminants represents an expensive approach and ultimately does not assess accurately their cumulative effect in biological entities. Several attempts to provide a reliable approach for assessment of water quality have shown significant progress. *In vitro* assays represent an alternative tool where the evaluation relies on the capacity to detect toxicologic effects on biological processes. However, they lack of consistency and only assess effects on specific known targets (Xia et al., 2017). Alternatively, toxigenomic assessment represents a useful tool that provides a broader approach allowing the evaluation of toxicology effects on multiple biological pathways, therefore a deeper analysis of biological effects using primary cells or highly stable cell lines. In the light of this, *in vivo* assays might provide a unique method for toxicological effects following exposure to contaminated water. Similar to *in vitro* approaches, *in vivo* experiments assess biological effects but using a whole living organism, allowing the analysis of several tissue/organs while analysing the systemic and local effect through the assessment of multiple biological pathways.

1.4.5. Comparative immunobiology of livestock

As mentioned in the sections above, successful animals have evolved dynamically, relying on their immunological capacity to battle potential threats. Despite this concept, livestock are raised in conditions where their goal is to achieve maximum productive performance. Considering several variables such as growth rate, feed conversion, reproductive parameters and others, livestock animals have gone through selection processes by crossbreeding allow them maximum efficiency and benefits for livestock production. Hence, livestock species have added features that provide farmers to take advantage for massive production, such as poultry and swine systems. For example, broiler chickens reach maximum growth at 6 weeks of age (in some highly-industrialized systems, this can be reached at 5 weeks of age) where they must go to slaughterhouse. This represents maximum energy expenditure in growth with potentially less energy resources for other vital biological processes such as immunity. In addition, despite this optimization in productivity, proper designs and tools to assess livestock immune parameters are lacking. Here, I summarize some relevant comparative aspects of immunity in poultry and swine that will provide information of immune performance in livestock.

1.4.5.1. Comparative immunology of swine

Pigs is a relevant model for production system. Their relative short gestational period (114 days), large litter size, high feed conversion efficiency, sensorial and nutritional properties make them a sustainable animal meat for human consumption. In addition to their importance for meat production, pigs have relevance as comparative animal model

for human health due to its immune system closely resembling to human equivalent, even closer than the classical mice model (Fairbairn et al., 2013, 2011). Hence, although there are still limited swine-specific reagents commercially available compared to mice, the increased interest in swine studies has opened up a research field that will provide multiple advantages for comparative biomedical research. For instance, swine thymus participates in T cell development, as it has been observed in other mammalian species such as mice. Additionally, tonsils also display similar contribution to immune defenses as in humans and this provides an advantage over mice models where this organ is absent (Mair et al., 2014). Due to large similarities between human and porcine spleen, pig spleen has been used in several studies of splenectomy. Despite the multiple similarities observed between human and swine immune system, there have been described multiple differences, associated with immune function. For example, swine species possess unique features such as inverted lymph node structures and different organization of Peyer's patches compared to human. As lymphocyte development in the gut plays a mayor role in systemic development of lymphocytes, swine immune system becomes a powerful tool for studies of lymphocyte development.

In terms of innate immunity, mammalian species share large similarities and therefore, main features described for mice and human are also present in porcine innate immunity. Despite this fact, there have been evolutionarily significant differences.

Porcine neutrophils display similar morphology to human neutrophils, however, smaller size and lower granularity and higher activation threshold (Bréa et al., 2012).

Proportions of circulating neutrophils in pigs are similar to those in humans (50-70%), and lower than mice proportions (Meurens et al., 2012). Even though major enzymatic

components are present in porcine neutrophil granules, they have shown variations in concentration levels compared to human neutrophils. Porcine neutrophils contain higher alkaline phosphatase levels compared to human neutrophil levels, while beta-glucuronidase and myeloperoxidase have shown higher proportions in human neutrophil. Also, it seems that lysozyme levels are lower in porcine neutrophils (Styrt, 1989). Similarly, antimicrobial peptides are widely conserved in higher vertebrates, and thus, present in swine and humans. However, swine species have the widest collection of cathelicidins compared to other mammalian species (Sang and Blecha, 2009). In addition, newborn pigs lack maternal antibodies since there is no transplacental maternal transfer during gestation. Hence, swine have to acquire maternal antibodies via gastrointestinal colostral absorption during the first 24 – 48 h of life (Ward et al., 1996). Pig macrophages, as in other mammals, play a pivotal role in the initiation of immune responses. Most mechanisms described in mice and human models are also present in pig macrophages, but differences are observed in the activation level. Hence, susceptibility to pathogen infection in multiple pig breeds are associated with macrophage activation (Fairbairn et al., 2011). While differences between mice and humans are present in the pattern of inducible genes against pathogens, pigs and humans share more of those genes due to a greater proportion of promoter sequences conservation (Fairbairn et al., 2013). Nitric oxide (NO) production plays a key role as antimicrobial response in activated macrophages in several species such as mice and teleost fish. Conversely, swine macrophages do not produce NO following LPS stimulation, even after IFN- γ priming (Meurens et al., 2012). In contrast, pigs and human macrophages produce indoleamine 2,3-dioxygenase (IDO) that participates in the

cellular activation, substituting iNOS production and its contribution for activation of inflammatory cytokines (Fairbairn et al., 2011). Unlike pigs and humans, mice are highly resistant to endotoxic shock during sepsis. Hence, mice are approximately 10^5 more resistant than humans, and lethal dose for mice is 5 to 12 times higher than in pigs (Berczi et al., 1966).

Pig lymphocytes function show multiple similarities with human lymphocytes, however, there are important differences. First, although B lymphocytes in mice have been classified into three subtypes: B-1a, B-1b and B-2, there is not a clear definition in pigs. B lymphocytes in pigs seem to be developed from bone marrow progenitors as in other large mammalian species (Sinkora and Butler, 2016). Studies in swine B lymphocytes have used different differentiation markers through monoclonal antibodies such as CD21, IgM and CD79 α . Along with CD2, stages of B cell differentiation have been proposed due to variations in expression patterns of CD2⁺CD21⁺ within stages (Sinkora et al., 2013). B cell development in the gut has been widely studied in pigs ileal Peyer's patches (IPP) as they play a role for B cell development. Although jejunal Peyer's patches (JPP) develop similarly to other mammalian such as mice, human and rabbits, IPP are fully formed during gestational life, remain in early life and involute after the first weeks of life. This latter contributed to the theory that IPP are the primary B cell organ in pigs (Sinkora et al., 2011). However, multiple studies have shown that gut microbial colonization plays a major role in the development of IPP and JPP in pigs. Peripheral swine $\alpha\beta$ T lymphocytes have been divided into three groups: cytotoxic CD8⁺CD4⁻ T cells, CD8⁻CD4⁺ and CD8⁺CD4⁺ T helper cells. Interestingly, this double positive CD8⁺CD4⁺ T helper cells are present in higher proportions in swine blood

(Bode et al., 2010). Additionally, $\gamma\delta$ T lymphocytes are present in peripheral blood in higher proportions compared to other mammalian species. These leukocyte populations have been associated to effector/memory T functions and involved in adaptive immune responses (Pastoret et al., 1998).

1.4.5.2. Comparative immunology of poultry

In chickens, heterophils represent the main circulating polymorphonuclear cell (Brooks et al., 1996; Montali, 1988). As previously described, heterophils are mostly bilobed, with minor proportion of polymorphic heterophils, which differ from mammalian neutrophils, which are mostly polymorphonuclear. Functional studies have shown that chickens display lymphopenia (decrease of circulating lymphocyte numbers) associated to heterophilia (increase of circulating heterophils numbers) in chickens following corticosteroid administration, revealing that chickens show “stress leukogram” similar to mammals (Harmon, 1998). During acute phase of inflammatory process in chickens, several studies have shown that heterophils are the first migrating leukocytes following challenge (Harmon, 1998; He et al., 2003; Kogut et al., 2005; Swaggerty et al., 2005). In terms of heterophil functionality, heterophil share several similarities with mammals neutrophils, however, it has been shown that chicken heterophils lack myeloperoxidase, a principal enzyme for reactive oxidative species production (ROS) (Penniell and Spitznagel, 1975), therefore, they produce significantly lower levels of ROS compared to other species such as mammals and teleost fish (Havixbeck et al., 2016; More-Bayona et al., 2017b; Rieger and Barreda, 2011). Therefore, chicken heterophils rely more on non-oxidative antimicrobial mechanisms than oxidative responses such as phagocytosis,

antimicrobial peptides and heterophil extracellular traps (HETs) (Chuammitri et al., 2009; Harmon, 1998; Wu and Kaiser, 2011). Interestingly, despite the capacity of chicken heterophils to internalize foreign or microbial pathogens, mechanisms of antigen presentation remain unclear. Additionally, chicken heterophil granules differ from those in mammalian neutrophils. Peroxidase, catalase and alkaline phosphatase are absent in heterophils, while those are abundant in mammalian neutrophils. Despite these differences, protein kinase C (PKC) activation through PMA has been shown in heterophils and neutrophils. These results suggest NADPH oxidase conservation among mammalian and avian species (Genovese et al., 2013).

The monocyte/macrophage lineage in chickens derives from bone marrow progenitors in a similar process to other vertebrates. As in mammals, monocytes are mainly blood circulatory mononuclear cells, while monocyte-derived macrophages are tissue-specific cells. As different subsets of resident macrophages established in multiple tissues, the peritoneal cavity has been used as the primary source of macrophages in several species. Mice, rabbit and teleost fish peritoneal cavity offers a large number of resident leukocytes suitable for *in vivo* and *ex vivo* assays. Contrarily to other species, the chicken abdominal cavity does not possess a large pool of resident leukocytes (Qureshi, 2003), therefore it has been necessary to use stimulants to promote macrophage recruitment (Chu and Dietert, 1988; Qureshi et al., 2000; Sabet et al., 1977). Although this approach allows for recovery of a large number of macrophages, these cells recruited are the result of an active inflammatory process, therefore they do not reflect the basal immune function in healthy chickens. Most of the monocyte/macrophage functions in chickens are similar to their mammalian equivalents, they display

phagocytosis, chemotaxis, cytokine production and antigen presentation (Qureshi et al., 1994; Qureshi, 2003). Although monocyte heterogeneity has been described in avian and mammalian species, lack of specific markers for chickens do not allow clear monocyte/macrophage subset differentiation. Interestingly, like murine models and unlike human, chicken monocyte/macrophages do not express CD4; CD4 expression is restricted to lymphoid cells (Mast, 1998). Nitric oxide production is a major antimicrobial and signalling molecule in macrophage function. As in mammalian systems, chicken monocyte/macrophages produce significant amount of nitric oxide species, however, differential production levels have been observed in various chicken lines (Sung et al., 1991). Studies have shown that this differential production is associated with *iNOS* expression and revealing a genetic control of nitric oxide in chicken lines (Qureshi et al., 2000). Phagocytosis as hallmark of the monocyte/macrophage lineages is also displayed in chicken mononuclear cells, although differential patterns of pathogen internalization have been shown in HD-11, MQ-NCSU and HTC (Chu and Dietert, 1988; Davison et al., 2014). Additionally, inflammatory macrophages display a strong capacity for pathogen phagocytosis, however, it seems that this capacity increases as the inflammatory process progresses, which demonstrates that chicken monocytes/macrophages undergo functional maturation during the inflammatory process (Chu and Dietert, 1988; Davison et al., 2014). Respiratory burst activity is also present in chicken monocyte/macrophages using different cell lines and several stimulants such as phorbol myristic acid (PMA), zymosan A and Salmonella species, where they show differential oxidative species production associated with different serotypes (Chadfield and Olsen, 2001; Desmidt et al., 1996).

The main immune-anatomical differences between mammalian and avian species are the presence of Bursa of Fabricius along with the absence of organized lymph nodes in the avian system (Tizard, 1978). These specie-specific features are directly involved in chicken lymphocyte development. Bruce Glick was the first in defining the Bursa of Fabricius (BF) as the first organ for B cell development (Glick et al., 1955; Ribatti et al., 2006). BF progressively grows after hatching during the first 4 – 10 weeks, followed by a regression process later in life. BF is located dorsal to cloaca and both are directly connected. Therefore, BF is involved in the recognition of environmental antigens and contributes to the induction of associated adaptive responses (Ekino et al., 1985; Toivanen, 1998). Following absorption, these antigens are taken up by mononuclear phagocytic cells and then presented to lymphocytes for long-term and memory responses. Altogether, this shows that environmental antigens might induce changes in mononuclear and peripheral lymphocyte lineages by cloacal-bursal access (Arakawa et al., 2002; Ekino, 1993; Sorvari et al., 1975; Tizard, 1978).

In terms of T lymphocyte subsets, mammalian and avian systems also share multiple similarities. As in mammals, $\alpha\beta$ TCR T lymphocytes are the main effector cells in avians, but $\gamma\delta$ TCR T cells are the most abundant circulating T cells, which differ from their mammalian counterpart (Bridle et al., 2006). $\alpha\beta$ TCR T lymphocytes are recognized using TCR2 and 3 mAb, while $\gamma\delta$ TCR T lymphocytes are detected by TCR1 mAb. In addition to TCR differentiation, chicken T lymphocytes have also been differentiated through other accessory molecules such CD4 and CD8 receptors (Chen et al., 1994), which resembles mammalian CD4 and CD8 T lymphocytes. In chickens, T lymphocytes also differentiate in the thymus and they are defined based on the presence of cellular

receptors using CD4 and CD8, specifically. Chicken lymphocytes as in other vertebrates are mainly associated with adaptive responses. Thus, using the Salmonella infection model, several studies have shown that chickens are able to display antibody and cellular response following Salmonella exposure after one week (Wigley, 2014, 2013).

Chapter II
Materials and Methods

2.1. Animals

For my poultry experiments, three-week-old Ross 708 broiler chickens (*Gallus gallus*) were used. All animals were housed in the Poultry Research Facility of the Agriculture, Food and Nutritional Sciences at the University of Alberta. For my swine experiments, three-week-old crossbred piglets (Duroc x Large White/Landrace) were maintained in pens at the Swine Research and Technology Centre (SRTC) of the Agriculture, Food and Nutritional Science at the University of Alberta. In my objective of characterization of acute inflammation, animals were randomly IP/IA challenged with either zymosan (poultry) or HKST (swine). Following challenge, peritoneal lavages were performed at 0, 4, 8, 12, 18, 24 and 48 h. Additional blood samples were taken in the poultry experiments. These time points were selected because they allow the dynamic assessment of pro-inflammatory (initial hours) followed by anti-inflammatory stages of inflammatory conditions in other higher vertebrates. This approach allows the assessment of the interplay of leukocyte subpopulations and their underlying antimicrobial mechanisms. Despite the strength of this experimental design, this has limitations. Among others, this evaluation only allows the analysis of limited time points under a 48 h period. In addition, it requires experienced personal to perform challenges, sample harvesting, etc.

For my second objective in poultry, animals were grouped into two experimental treatments: 1) raw underground well water commonly used in small poultry farms in Southern Alberta (raw water); and 2) municipal tap water control group (tap water). Chickens were fed *ad libitum* under farm conditions for their correspondent age.

For my second objective in swine, Pigs were grouped into three experimental groups: 1) raw underground water commonly used in small pig farms in Northern Alberta (raw water); 2) same water as 1 with the addition of representative chemicals commonly detected as by-products of oil industry (spiked water); and 3) municipal tap water control (tap water).

Broiler chickens and pigs were maintained according to guidelines specified by the Canadian Council on Animal Care, and protocols approved by the University of Alberta Animal Care and Use Committee. Maximum efforts were made to minimize animal stress.

2.2. Treatments

Animals were randomly distributed into treatment groups. For chickens: underground raw water and tap water control group. For swine: Short-term exposure to different water treatments and controls was administered for 7 days, *ad libitum*. Our experimental design used a short-term exposure (7 days) trial with the objectives: 1) to assess the effects of water contaminants on immunity under acute exposure conditions, and 2) to avoid immune tolerance towards water contaminants mounted under chronic exposure conditions. For chicken experiments, water administration was done using nipple drinkers connected to 80L containers with respective water, as needed. For pig experiments, 120L containers of each experimental water were connected to stainless steel bite drinkers for piglets, allow them access to water *ad libitum*.

For the evaluation of long-term effects of water contaminants in immune response in broiler chickens and pigs, one-week exposure to water exposure previously described

was performed, and samples were taken 2 weeks (poultry) and, 2 and 4 weeks (swine) following HKST challenge. This experimental design allows the characterization of acute inflammation in poultry and swine and evaluates the effects of drinking water quality on acute inflammation, and the effects on long-term immunity development.

2.3. Intraperitoneal / Intrabdominal challenges

2.3.1. Zymosan

Zymosan A (2.5 mg, Sigma-Aldrich) was resuspended in 500 μ l of PBS^{-/-} (no calcium/no magnesium) and administered through injection into the abdominal cavity to induce inflammation. Our primary goal was to select experimental conditions that effectively triggered an acute inflammatory response *in vivo* which, subsequently, were also effectively controlled. *In vivo* stimulation of our birds with 2.5 mg of zymosan intra-abdominally effectively induced this self-resolving process. Both induction and resolution of acute inflammation were clearly observed. Given that the leukocyte responses observed were already significantly faster than those previously identified in mice and fish (e.g. rates of leukocyte recruitment, initiation of cytokine gene expression, activation of antimicrobial responses) we chose to remain at this zymosan dose rather than increase the dose to normalize conditions based on overall body weight.

For *ex vivo* experiments, unlabeled zymosan particles (Molecular probes) were stained in carbonate buffer (0.1 M sodium carbonate, 0.1 M sodium bicarbonate) using 250 ng/ml FITC (Sigma-Aldrich) with continuous shaking overnight at 4 °C, followed by two washes with 1x PBS^{-/-} (no calcium, no magnesium) to remove excess dye.

Zymosan-FITC was resuspended at a concentration of 1×10^6 particles per μl and kept at 4°C until use.

2.3.2. *S. enterica* ser. Typhimurium (ST)

ST strain X4232 was culture on XLD (xylose lysine deoxycolate) agar at 37°C for 24 h. For broth culture, one representative colony was inoculated into Luria-Bertani (LB) broth and incubated at 37°C for 22 h. One ml of culture was used to determine CFU/ml by plating a 10^{-3} to 10^{-9} dilution series on XLD agar. The remaining culture was heat-killed inactivated at 80°C for 1 h. For chicken experiments, heat-killed ST (HKST; 10^9 CFU) was resuspended in $500 \mu\text{l}$ $1 \times \text{PBS}^{-/}$ and kept them at -4°C until injection. For pig experiments, following the procedure described above, heat-killed ST was used resuspended in 1 ml $1 \times \text{PBS}^{-/}$ (to allow a better distribution into the pig peritoneal cavity) and kept them at -4°C until injections were performed.

For *ex vivo* assays, same strain used for *in vivo* challenges was used. Hence, *S. enterica* ser. Typhimurium strain X4232 was grown in LB broth. The plasmid pAT113-GFP (Kindly provided by Dr. Benjamin Willing, AFNS, University of Alberta) was introduced into X4232. Transformed bacteria was confirmed by its growth in kanamycin- containing agar.

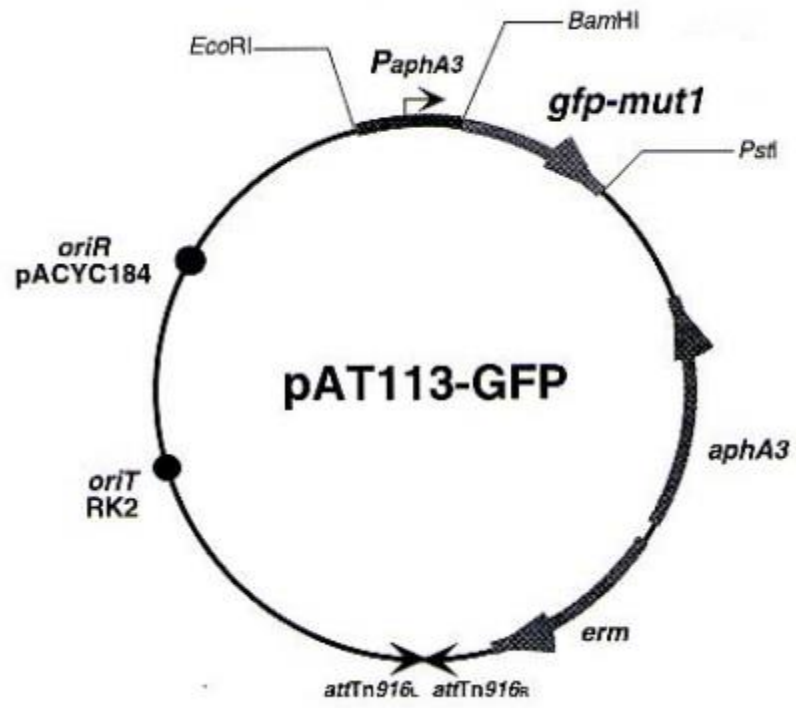


Fig. 2.1. pAT113-GFP inserted into *S. enterica* ser. Typhimurium

2.3.3. Intrabdominal injections in chickens

Chickens were restrained in dorsal recumbency with hind end elevated allowing organs to move cranially away, creating significant space for injection. The site of injection is defined by the area 1 cm cranial to the cloaca and 1 cm lateral to the midline, for personal preferences, left side was chosen for consistency purposes. Once the area was recognized, cleaning with 75% alcohol using paper towel. Skin was pinched, and needle was inserted perpendicularly to the skin, introducing approximately no more than 50% of the needle to avoid internal bleeding and smooth injection.

2.3.4. Intraperitoneal injection in pigs

For peritoneal injection in pigs, an assistant was required for holding the animals while the injections were performed. In short, pigs were held by the hind legs and raised to allow internal organs move cranial away, leaving space for the injection site. The area of injection, lower left abdominal quadrant, was recognized as 5 cm cephalad to the pubis bone and 5 cm lateral to the midsagittal line (middle line). Area of injection was cleaned with 75% alcohol using paper towel. Insert $\frac{3}{4}$ depth of the needle to skin injecting smoothly.

2.4. Abdominal lavage

Chickens were euthanized via cervical dislocation and animals were bled to minimize potential blood contamination into abdominal cavity. After termination, feathers were removed from abdominal area and cleaned using 75% alcohol. 20 ml of cold, incomplete RPMI-1640 media was injected into the lower left quadrant in the abdominal

site and gently massaged to form a homogeneous suspension. A small 1-2 cm incision was made to get access to the abdominal cavity. Using 1 ml sterile, plastic droppers, abdominal fluid was harvested. A 50 ml falcon tube was used to recover the fluid and kept at -4 °C until further experimental process. Non-injected chickens were used as negative controls.

Pig terminations were performed using a captive bolt gun by a certified technician from the University of Alberta Swine Research and Technology Centre. Immediately after, the abdominal area was cleaned with 75% alcohol and paper towels. An 8 cm incision was made in the midsagittal line to get access to the peritoneal cavity and 500 ml of 1x PBS⁻ was added, followed by a gentle massage to ensure homogeneous suspension. At least 400 ml of peritoneal fluid was recovered and kept at -4°C until further analysis.

2.5. Blood samples

Chicken blood samples were obtained using the wing vein. In short, chickens were held horizontally on their back. The wing was pulled towards the person performing the blood collection, inserting the needle carefully. Approximately 3 ml of blood were obtained from each animal, mixed with EDTA to avoid clotting and kept at -4°C.

For pig blood sample, before termination, samples were taken from jugular vein which allows the fastest and easiest access to blood. Approximately 7 ml of blood were collected from each animal, maintained in EDTA to avoid coagulation and maintained at -4°C.

2.6. Samples for gene expression analysis

RNA quality is a major aspect in molecular studies. RIN (RNA integrity number) allows the assessment of RNA quality for molecular purposes. Thus, samples with RIN > 7 are required to accurately measure RNA expression. Hence, abdominal/peritoneal samples were harvested immediately after collection. 14 ml of samples were placed into 15 ml tubes and centrifuged at 311 x g for 5 min at 4°C. Immediately after, supernatant was discarded, and cellular pellet was resuspended in 1 ml of Trizol, transferred to 2 ml tubes and maintained in liquid nitrogen until RNA extraction procedure.

2.7. Definition of immune cell populations

Leukocyte subpopulations were determined using flow cytometry cell sorting analysis (FACS), imaging flow cytometry (ImageStream) along with modified Wright-Giemsa staining (Hema3) and antibody-specific markers, explanation follows.

2.7.1. Modified Wright-Giemsa staining (Hema3)

For Hema3 staining (Fisher Scientific), 100 µl of samples (approximately 10⁵ cells) were transferred to properly mounted Shalton cytofunnels with a glass slide, placed into a cytocentrifuge (Cytospin, Thermo Scientific) and centrifuged at 55 g for 6 min.

Leukocyte cytopins were stained according to manufacturer's specifications and analysed through light microscopy. In short, cells were fixed to slides by dipping it into fixative solution, followed by staining with Hema 3 solution I and II for 5 seconds each and rinsed with distilled water (DI) to remove excess to drain and slides were left to dry. Two hundred and fifty cells were counted under light microscopy and proportions were

determined based on cytomorphological features (staining properties, cellular size, nuclear morphology and size, nucleus/cytoplasm ratio).

2.7.2. Flow cytometry cells sorting (FACS)

At least 10^7 cells/ml of samples were used for sorting cells to allow cellular differentiation. In short, after collection cells were resuspended in incomplete RPMI media to maintain cellular integrity and viability. Cells were sorted using a FACSAria flow cytometer (BD Biosciences). Three main leukocyte subpopulations were sorted out based on their size (forward scatter, FSC) and granularity or internal complexity (side scatter, SSC) dot plots. Sorted cells were recovered in 1x PBS^{-/-} for staining as described above.

2.7.3. Imaging Flow cytometry

Samples were also run in imaging flow cytometry analysis (ImageStream^x MkII instrument, Amnis). Thus, a dot plot of 10000 events using area (size, x-axis) vs intensity channel 6 (internal complexity, y-axis) was created to define different subpopulations using leukocyte morphology and nuclear staining (Draq5). Altogether, sorted cells, conventional hematological staining and imaging flow cytometry methods allowed faster and reliable leukocyte differentiation to any of those previously mentioned alone.

2.7.4. Antibody staining

In addition, we used an array of monoclonal antibodies to define specific leukocyte subpopulations. Monocyte/macrophage labelling was performed using the KUL01 antibody marker (Abcam). This antibody recognizes a homolog of the mammalian mannose receptor C-type, MRC1 (also called CD206) (Mast, 1998; Staines et al., 2014). The KUL01 antibody was added to a final concentration of 1:1000, followed by 30 min incubation at 41°C. Cells were washed in PBS^{-/-} and fixed in 1% of formaldehyde. PE anti-chicken CD4⁺ and Cy5 anti-chicken CD8⁺ (Abcam) were used for CD4⁺ and CD8⁺ T lymphocytes staining, respectively. Anti CD4⁺ antibody was added at 1:1000 dilution and anti CD8⁺ T lymphocytes antibody at 1:2000 dilution. Leukocytes were incubated at -4°C for 30 min and followed by 20 min at room temperature. Cells were washed with PBS^{-/-} and fixed in 1% formaldehyde. Twenty minutes prior analysis, 5 µl of Hoechst 33342 was added as nuclear staining.

2.8. Effect of apoptotic leukocytes on chicken immunity

2.8.1. Induction of apoptotic leukocytes

Activated leukocytes were recovered from chickens 12 h post zymosan injection. Cells were resuspended in complete RPMI-1640 (10% FCS, 5% chicken serum, 1% penicillin/streptomycin) and incubated for 24 h in the presence of 10 mg/ml of cyclohexymide (Sigma - Aldrich) at 41 °C, 5% CO₂ to induce apoptosis. After 24 h, cell viability was examined to evaluate the percentage of apoptotic cells, as described below. Cells were washed twice with 1 x PBS^{-/-} to remove media and incubated or injected individually as described.

2.8.2. *Ex vivo* effect of apoptotic leukocytes

In vivo activated intra-abdominal leukocytes were isolated 12 and 48 h post-challenge and incubated *ex vivo* with pro-inflammatory particles and/or apoptotic leukocytes at different ratios (5:1, 1:1, 1:5, apoptotic leukocyte: zymosan ratios). The goal was to evaluate the relative sensitivity of chicken leukocyte functional responses to those particles. Briefly, activated leukocyte were incubated in cRPMI-1640 and co-incubated with apoptotic leukocyte and zymosan particles for 2 h. After incubation, CellROX (5 mM, Molecular Probes) was added and incubated for 30 min. Cells were washed twice with 1x PBS^{-/-} and fixed. Ten thousand events were analysed by flow cytometry.

2.8.3. *In vivo* effect of apoptotic leukocytes

Apoptotic cells were administered at different time points (0, -2 and -4 h, prior to zymosan challenge). The goal was to mimic a classical physiological scenario at an inflammatory site where apoptosis of spent leukocytes (the majority of which are infiltrating neutrophils/heterophils) contributes to a shift from pro-inflammatory to pro-resolution/anti-inflammatory phenotypes. After 12 h of zymosan administration, leukocytes were recovered by intra-abdominal lavage. ROS production and leukocyte infiltration were measured as previously described.

2.9. Analysis of leukocyte function

In addition to leukocyte determination, an array of cellular markers was used to assess leukocyte function. Reactive oxygen species production (through DHR oxidation and

CellROX), nitric oxide levels and NF- κ B nuclear translocation were used to evaluate leukocyte performance *in vivo* and *ex vivo*.

2.9.1. Reactive oxygen species

ROS production was measured by Dihydrorhodamine 123 (DHR; Molecular Probes), CellROX (Molecular Probes) and where stated, nitroblue tetrazolium assay (NBT). DHR is an uncharged and non-fluorescent reactive oxygen species (ROS) indicator that can passively diffuse across membranes where it is oxidized to cationic rhodamine 123 which localizes in the mitochondria and exhibits green fluorescence. CellROX is a fluorogenic probe that measures ROS production through its oxidation and offers strong fluorescence signal, which is stable even after fixation. For DHR oxidation, 1×10^6 cells were incubated with this reagent for 5 min (10 μ M final concentration), followed by an additional 30 min incubation with phorbol myristate acetate (PMA; Sigma-Aldrich, 100 ng/ml). Samples were appropriately staggered with respect to timing to avoid transient DHR oxidation. For CellROX analysis, cells were incubated with 5 μ M of CellROX for 30 min, washed twice with 1x PBS^{-/-} and fixed with 1% formaldehyde. All incubations were performed at 41 °C and 5% CO₂. The NBT assay was used as previously described (Neumann and Belosevic, 1996). Briefly, 2.5×10^5 intra-abdominal leukocytes were placed in 96-well microplates. NBT (100 mg/ml, Sigma Aldrich) was diluted at a final concentration of 2 mg/ml in 1x PBS^{-/-}. PMA was added to the NBT at 100 ng/ml final concentration. Fifty μ l of NBT/PMA solution was added to the cell suspension and incubated for 25 min at 41 °C. The microplate was spun down and fixed with 200 μ l of 70% methanol for 1 min and washed twice with the same solution to remove unreduced

NBT. Potassium hydroxide (2 M) was used to dissolve reduced NBT by pipetting vigorously. Seventy μ l of DMSO was added and optical densities were read at 600nm.

2.9.2. Nitric oxide (NO) production

NO production was quantified using DAF-FM diacetate (Molecular Probes) oxidation staining. DAF-FM diacetate is not fluorescent until they react with NO to form a fluorescent benzotriazole. DAF-FM diacetate can passively diffuse through cell membranes and deacetylated inside cells. DAF-FM diacetate reagent was diluted 1:25 in 1x PBS^{-/-} prior the experiment. 1×10^6 cells were incubated with 4 μ l of DAF-FM added for incubation for 30 min at 41°C. Cells were washed with 1x PBS^{-/-} and fixed in 1% formaldehyde. Ten thousand events were measured by imaging flow cytometry.

2.9.3. Cell viability

After abdominal/peritoneal leukocyte collection, annexinV/propidium iodide (PI) staining was performed in order to determine cell viability, as previously described (Havixbeck et al., 2015; Rieger et al., 2010). In short, one hundred microliters of abdominal/peritoneal lavages at different points were washed twice with Annexin V binding buffer (BD pharmigen) and resuspended in 100 μ l of AnnexinV binding buffer. AnnexinV (2.5 μ l, eBioscience) was added according to the manufacturer. Propidium iodide (PI, Sigma) was added at a final concentration of 4 mg/ml, followed by 30 min incubation at 41°C. Following incubation, cells were washed twice in AnnexinV binding buffer to remove excess dyes. Cells were fixed in 1% formaldehyde. Draq5 or Hoechst

33342 were used as nuclear stain. Ten thousand events were analysed by imaging flow cytometry.

2.9.4. NF- κ B nuclear translocation

Abdominal/peritoneal cells (2×10^6 /ml) were fixed in 1% formaldehyde overnight. Cells were incubated in permeabilization buffer (1x PBS^{-/-} with 2% calf serum and 0.1% saponin) for 20 min and washed twice in permeabilization at 311 g for 8 min. Cells were resuspended in 100 μ l of permeabilization buffer, following by incubation in unlabelled rabbit IgG anti-mouse NF- κ B p65 (Santa Cruz Biotechnology) for 30 min at 4°C followed by 20 min at RT. Following the primary staining, cells were washed with permeabilization buffer and stained with a rabbit anti-mouse FITC secondary Ab (Jackson ImmunoResearch) for 20 min at RT. After incubation, cells were washed once with permeabilization buffer, followed by a wash step with 1x PBS^{-/-}. Prior to acquisition, DRAQ5 nuclear stain (Molecular Probes) was added as per manufacturer's recommendations. Ten thousand events were analysed on an ImageStream MK II system and analyzed using IDEAS software (Amnis).

2.10. Gene expression

Abdominal leukocytes were kept in Trizol (Thermo Fisher Scientific) and stored in liquid nitrogen. Total RNA was extracted following manufacturer's specifications. RNA concentration and quality were evaluated using Nanodrop ND-1000 (Thermo Fisher Scientific) and Bioanalyser-2100 with the RNA 6000 Nano Kit (Agilent Technologies), respectively. Samples had a RIN higher than 7.5. cDNA was synthesized with 650 ng of

total RNA in a final volume of 20 μ l using iScript Kit (BioRad). Transcripts of r28s and ACTB were used as reference genes for quantification purposes. qPCR was carried out using SYBR Green (prepared by Molecular Biology Services Unit staff at the University of Alberta), 500 nM of primers and 2.5 μ l of cDNA previously diluted (1:10 for target mRNA and 1:100 for the reference gene) in 10 μ l of final volume; and evaluated using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). Primers for qPCR are described in table 1. Relative quantification was performed according to Livak method. This method allows for quantification (fold-change) of gene expression in a sample compared to an untreated sample. A normalization step using a set of reference genes are needed to avoid differences of RNA quantity among samples. (Livak and Schmittgen, 2001). Samples were run in triplicates and results were statistically analyzed using Two-way ANOVA followed by Sidak's multiple comparison test to estimate differences between treatments.

2.11. Statistical analysis

Conventional flow cytometry data was analysed using FCS Express software v4 (DeNovo Software) and FACSDiva v6.1.3 (BD Biosciences). Imaging flow cytometry data was analysed using IDEAS software (Amnis Corporation, EMD Millipore). GraphPad Prism program was used to determine statistical difference and significance between groups using: one-way ANOVA and Tukey's multiple comparison tests were used for characterization of acute inflammation (variable: time). Two-way ANOVA and Sidak's multiple comparison tests were used to effect of drinking water quality on acute

inflammation (variables: time, water treatment). We used an alpha level (threshold of significance) of 0.05 for all statistical tests.

Table 1. Primers used for qPCR analysis in chickens

Gene	Primer name	Sense	Sequence	Accession number
Interleukin 2	IL-2	Fw	ACCGGAAGTGAATGCAAGAT	AF000631
Interleukin 2	IL-2	Rv	AGTGGTCCCAGAATGGACAG	AF000631
Interleukin 8	IL-8	Fw	GGCTTGCTAGGGGAAATGA	AJ009800
Interleukin 8	IL-8	Rv	AGCTGACTCTGACTAGGAAACTGT	AJ009800
Tumoral necrosis factor alpha	TNF-α	Fw	GTTGACTTGGCTGTCGTGTG	AY765397.1
Tumoral necrosis factor alpha	TNF-α	Rv	TCAGAGCATCAACGCAAAAAG	AY765397.1
Interleukin 1 beta	IL-1β	Fw	AGGTCAACATCGCCACCTAC	NM_204524.1
Interleukin 1 beta	IL-1β	Rv	ACGAGATGGAAACCAGCAAC	NM_204524.1
Transforming growth factor beta	TGF-β	Fw	CGACCTCGACACCGACTACT	NM_001318456.1
Transforming growth factor beta	TGF-β	Rv	CCACTTCCACTGCAGATCCT	NM_001318456.1
Inducible nitric oxide synthase	i-NOS	Fw	CTCTCACAGGCCTTGACATATT	D85422.1
Inducible nitric oxide synthase	i-NOS	Rv	CAGTCTCTGTTTGTCTCCTTCC	D85422.1
Ribosomal 28 subunit	r28S	Fw	GGCGAAGCCAGAGGAAACT	FM165415
Ribosomal 28 subunit	r28S	Rv	GACGACCGATTTGCACGTC	FM165415
Actin beta	ACTB	Fw	CCAGACATCAGGGTGTGATGG	AJ719605
Actin beta	ACTB	Rv	CTCCATATCATCCCAGTTGGTGA	AJ719605

Chapter III

Characterization of Acute Inflammation in poultry¹

¹ Portions of this chapter have been published in

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More-Bayona, J.A., Kumar, A., Barreda, D.R., 2017b. Contribution of leukocytes to the induction and resolution of the acute inflammatory response in chickens. *Dev. Comp. Immunol.* 74, 167–177. doi:10.1016/j.dci.2017.04.018

3.1. Introduction

Avian species are located in an evolutionary position between fish and mammals, representing a relevant group of animals for comparative studies (Kumar and Hedges, 1998). Thus, birds possess strategies of immune defences widely shared with other vertebrates, while exhibiting unique innate immune mechanisms. Among others, avian MHC complex (B locus) and TLRs are less polymorphic than human homologs, conferring them differential function for antigen presentation in early immune responses (Wigley, 2017). Moreover, chicken heterophils seem to have limited, if any, oxidative antimicrobial responses, while displaying strong phagocytic and non-oxidative defenses (Genovese et al., 2013). Furthermore, proportions of circulating $\gamma\delta$ T lymphocytes in chickens are higher than mice and human values and appear to play a role in innate defenses (Bridle et al., 2006). Altogether, these features suggest alternative capabilities for chickens to gain immunity compared to mammals. However, it is still unclear the dynamic immune processes triggered following *in vivo* challenges, their antimicrobial responses and how those mechanisms are regulated during acute inflammation. In this chapter, we characterized the molecular and cellular events underlying pro-inflammatory and anti-inflammatory responses during acute inflammation. In addition, we pointed out relevant comparative features of chicken immunity to classical mammals and teleost fish. Hence, these results provide new insights into how avian species have gain immunity. Also, they allowed a better understanding of how avian species transit from acute inflammation into long-term immunity. Lastly, this section offers a platform of immunoassays that can be used for assessment of immune performance in chickens. Thus, the objectives of this chapter were 1) to characterize a self-resolving abdominal

challenge model using zymosan as pathogen mimic, 2) to evaluate the dynamics of leukocyte populations and their antimicrobial contributions during acute inflammation and 3) to study the regulation of chicken acute inflammation by homeostatic particles (apoptotic cells). Our results revealed a large leukocyte infiltration dominated by heterophils following zymosan challenge. We detected an increase of heterophil infiltration as ROS levels increased. However, these ROS levels were lower than those observed in other animal models. Additionally, our results revealed that downregulation of ROS levels along with the increase of apoptotic cells defined the transition to resolving phases of acute inflammation. Moreover, we discovered that chicken heterophils can internalize apoptotic cells, a feature present in mammals but absent in teleost fish. Our results also demonstrated that heterophil capacity to uptake pathogen-mediated particles decreased at the resolving phases of acute inflammation in a process different from teleost fish. These findings seem to be associated to higher sensitivity of inflammatory leukocytes towards apoptotic cells than pathogen-mediated particles as the inflammation progresses. These findings were confirmed when apoptotic cells induced downregulation of leukocyte migration and antimicrobial responses *in vivo*. Taken together, my findings demonstrated: 1) a self-resolving acute inflammation model elicited by zymosan, and 2) unique features among higher vertebrates of avian acute inflammation to achieve immunity.

3.2. Results

3.2.1. Intra-abdominal injection of zymosan induces leukocyte infiltration in chickens.

In a local acute inflammatory process, rapid cell migration and infiltration are characteristic of the immune response (Gordon, 2016; Havixbeck et al., 2015). As this initial response must be closely regulated, the immune response needs to be driven towards a pro-resolving state following pathogen control. In this context, a self-resolving inflammatory process can be used as a tool for study of multiple underlying immune mechanisms. Using zymosan as a well-characterized pathogen mimic in evolutionary distinct models such as mice and teleost fish, it might be useful for studies of inflammation in avian species. Thus, the zymosan challenge induced an acute inflammatory process *in vivo* and evaluated the infiltrating phagocytes into the peritoneal challenge site as well as changes in functional antimicrobial responses in the early and late phases of this acute inflammation. In conjunction with cell sorting and morphological characteristics such as nuclear size and shape, internal complexity, and cell size, three dominant leukocyte subpopulations infiltrating the peritoneal cavity were identified: heterophils, monocyte/macrophages and lymphocytes (Fig. 3.1). Basal levels of resident leukocytes within the peritoneal cavity prior to *in vivo* challenge were lower compared to those found in mice in the same compartment (Rieger et al., 2012). However, leukocyte numbers climbed rapidly after intra-peritoneal challenge (Fig. 3.2A). Leukocyte infiltration increased as early as 4 h with the peak of leukocyte infiltration 12 h post-zymosan injection (4-fold at 4 h $p < 0.05$; 10-fold at 8, 12 and 18 h $p < 0.01$). This was driven primarily by an increase in heterophil numbers within the

peritoneal cavity. As early as 4 h, the percentage of heterophils shifted dramatically to become the dominant subpopulation (10-fold, $p < 0.001$) compared to 0 h (Fig. 3.2B). At its peak, total leukocyte numbers within the peritoneal cavity increased by more than 80-fold. This was dramatically faster than those previously described for other vertebrate species. For example, previous experiments indicated that under equivalent challenge conditions, murine leukocytes reached a peak of infiltration at 48 h, whereas bony fish (*Carassius auratus*) displayed a peak of infiltration at 18 h post zymosan challenge (Havixbeck et al., 2016). The latter also showed lower rates of leukocyte recruitment compared to the current avian results, with numbers increasing by approximately 12-fold at the peak of cell infiltration (Havixbeck et al., 2016).

3.2.2. Maximum capacity to mount a respiratory burst closely mirrored the peak of leukocyte infiltration into the challenge site.

ROS production through the respiratory burst represents a widely conserved antimicrobial mechanism among vertebrates (Gilroy and De Maeyer, 2015; Havixbeck et al., 2016; Hernandez-Garcia et al., 2010; Jones et al., 1998; Rieger et al., 2012). However, some studies have concluded that chicken are less reliant on ROS production to kill microorganisms compared to mammals (Genovese et al., 2013; Wigley, 2013). Our results indicated that ROS producing capacity closely mirrored the kinetics of leukocyte infiltration. Maximal levels of ROS production were also reached at 12 h following zymosan intra-abdominal challenge (2-fold at 4h, $p < 0.05$; 4-fold at 8 h and 5-fold at 12 h $p < 0.01$; Fig. 3.2C). As expected, heterophils and monocyte/macrophages were the primary contributors to ROS production (Fig. 3.2C). Also expected is that they showed

differential capacity for ROS production during induction and resolution phases of acute inflammation (12 h and 48 h, respectively). Our results were corroborated using two independent methods for examination of ROS production, following co-incubation of isolated leukocytes with DHR or CellROX (Fig. 3.2C). The DHR oxidation test represented a widely utilized flow cytometry-based technique for the study of cell activation (Emmendorffer et al., 1990; Hernandez-Garcia et al., 2010; Qin et al., 2008). CellROX, in contrast, was a more recently available reagent which offered a simple approach to evaluate ROS production. It can be used directly in cell culture media and remains vibrant after fixation steps, which minimizes the need to stagger samples in order to prevent differential levels of activation among examined leukocytes (Apolloni et al., 2013; Choi et al., 2015; Dupre-Crochet et al., 2013). Taken together, these results demonstrated that heterophils, as well as monocyte macrophages, contribute to ROS production during acute inflammation.

3.2.3. Higher levels of apoptotic leukocytes are detected in later phases of avian acute inflammation

Accumulation of apoptotic leukocytes at the inflammatory site is associated with the down-regulation of immune responses and resolution phases of inflammation in mammalian and teleost fish systems (Esmann et al., 2010; Havixbeck et al., 2016). As avian species have distinct systemic physiological features (e.g. higher body temperature) and locally (as abdominal cavity contains fewer number of resident leukocytes), apoptotic leukocytes emerging during acute inflammation might have different contribution to homeostasis return. In our model, cell viability of inflammatory

leukocytes collected at different time points following peritoneal zymosan injections was measured using a modified propidium iodide/annexinV staining method. Overall, inflammatory apoptotic leukocytes were around 20% during the first 18 h post zymosan injection (Fig. 3.3B). As expected, the proportion of apoptotic cells increased after 24 and 48 h (30% at 48 h, $p < 0.05$). There is a correlation between the downregulation of antimicrobial ROS production and an increase in the number of apoptotic leukocytes. This is consistent with previous reports in other models showing a role of leukocyte apoptosis in the resolution of local inflammation. Our results suggest that this mechanism is conserved in chickens. Taken together, these results suggest that decrease of infiltrating leukocytes impact down-regulation of ROS production and accumulation of apoptotic cells to switch immune responses from a pro-inflammatory to a pro-resolving during acute inflammation in chickens.

3.2.4. Multi-parametric analysis via imaging flow cytometry highlights divergent responses to pathogen-derived and homeostatic particles in chicken phagocytes.

Phagocytosis provides a primary tool for cell turnover and removal of foreign invaders across all animal groups (Buchmann, 2014; Davison et al., 2014; Gordon, 2016; Moticka, 2016; Soehnlein and Lindbom, 2010). It therefore provides a good stage to examine the evolution of leukocytes through their contributions to homeostasis and host defenses. *Bona fide* neutrophils and macrophages (or neutrophil and macrophage-like cells) represent professional phagocytic leukocytes in all vertebrates examined to date and their role during acute inflammation has been extensively described (Rieger et al.,

2012; Rieger and Barreda, 2011; Soehnlein and Lindbom, 2010). In chickens, heterophils and macrophages are the primary leukocytes responsible for phagocytosis (He et al., 2005; Kogut et al., 2012, 2005; Swaggerty et al., 2005) but their contributions from early to late phases of the acute inflammatory response remains unclear. Further, much remains to be defined with regards to their changing phenotypes and functional contributions during this process.

The phagocytic capacity for leukocytes infiltrating the avian peritoneal cavity was examined *ex vivo*. Using IDEAS software, a mask was generated to define the cellular membrane. Subsequent analyses allowed discrimination of internalized and membrane-bound events in X- Y- and Z- axis based on degree of internalization measurements. As shown in Figure 3.4, total chicken leukocytes displayed equivalent levels of phagocytosis for pathogen-derived and homeostatic particles at early and late phases of the acute inflammatory response. There was no significant difference for internalization of either zymosan or apoptotic cells following comparison of total peritoneal leukocytes at 12 h and 48 h after zymosan intra-peritoneal challenge (Fig. 3.4A and B). There was also no difference in the level of surface binding for these particles at any of the time points examined. Co-incubation of isolated total peritoneal leukocytes with both zymosan and apoptotic cells *ex vivo* looked to examine the potential for differential preference of these avian phagocytes for pathogen-derived or homeostatic particles at early and late phases of acute inflammation. Again, no difference was identified with regards to internalization of these particles following co-incubation at either 12 h or 48 h post-challenge. Interestingly, a minority (< 5%) of these phagocytes exhibited internalization of both zymosan and apoptotic cells (Fig. 3.4C), which has important

implications for regulation of pro-inflammatory and homeostatic pathways at the single cell level of the avian phagocyte. These implications are related to efficiency to battle secondary infections at the cellular level, the capacity to timely downregulate inflammation and developing of adaptive defenses (Fig. 3.4C). Representative images are shown in the Fig. 3.4D.

Multi-parametric analysis of cell morphology and phagocytosis via imaging flow cytometry supported the consistency in the level of total phagocytic responses described above. However, the added level of depth provided by this analysis identified a selective decrease in the phagocytic capacity among heterophils for zymosan between 12 h and 48 h (20% at 12 h, 10% at 48 h $p < 0.05$; Fig. 3.5), which is consistent with a transition between pro-inflammatory and resolution phases of acute inflammation. It will be important to determine if this decreased uptake is conserved upon encounter with other pathogen-derived particles. Interestingly, our results showed that chicken heterophils are able to uptake apoptotic cells, a feature shared with mammalian neutrophils but not with the lower vertebrate neutrophils of bony fish (Rieger et al., 2012). These results indicated that chicken heterophils are likely contributors to both antimicrobial and resolution mechanisms during acute inflammation. Also, notable and consistent with recent descriptions in several other animal species was the phagocytosis exhibited by infiltrating lymphocytes (Fig. 3.5A and B).

Multi-parametric examination of phagocytosis and ROS production in mixed inflammatory leukocyte populations using imaging flow cytometry added further depth to our analyses through characterization of divergent functional responses following internalization of pro-inflammatory and homeostatic particles (Fig. 3.6). Cellular

respiratory burst served as a marker for the induction of pro-inflammatory antimicrobial responses among leukocytes infiltrating the avian abdominal cavity. As expected, internalization of zymosan induced a strong respiratory burst among infiltrating leukocytes (60%, $p < 0.05$; Fig. 3.6A). Importantly, this was limited to those leukocytes that had internalized zymosan, suggesting the importance of intrinsic cell regulatory mechanisms downstream of zymosan internalization for modulation of the phagocyte pro-inflammatory phenotype. Conversely, phagocytosis of apoptotic cells resulted in a significant decrease in the production of ROS both within phagocytic and total leukocyte subsets. Results were consistent across early (12 h) and late (48 h) phases of the acute inflammatory response (20% at 12 and 48 h, $p < 0.05$; Fig. 3.6A). Finally, we were interested in assessing these responses under conditions which more closely mimicked those at the inflammatory site. Thus, recruited leukocytes were isolated and subsequently exposed to both pro-inflammatory stimuli and dying cells (Fig. 3.6B, C and D). Among others, this allowed us to discern four unique populations based on imaging flow cytometry (cells containing no particles; phagocytes containing only zymosan; phagocytes containing only apoptotic cells; and phagocytes containing both zymosan and apoptotic cells). It also permitted assessment of ROS production under conditions that allowed for cellular cross-talk between these four cellular groups. Similar to leukocytes that were incubated independently with zymosan (Fig. 3.6A), those internalizing only zymosan under co-incubation conditions showed a marked increase in ROS production (40% at 12 and 48 h, $p < 0.01$; Fig. 3.6B). Internalization of both zymosan and apoptotic cells also showed enhanced ROS production, suggesting a dominance for phagocyte pro-inflammatory responses among avian phagocytes when

both pro-inflammatory and homeostatic particles are available. In contrast, leukocytes displaying apoptotic cell internalization showed equivalent levels of ROS production as those which did not internalize any particles. Finally, consideration of the total number of leukocytes within the peritoneal site at 12 h and 48 h post-challenge (Fig. 3.6C) provided a necessary perspective of the scale of leukocyte ROS production in this model. The total number of ROS producing leukocytes shows the expected dominance of this antimicrobial mechanism early in the avian acute inflammatory response. Representative flow imaging system panels are shown in the Fig. 3.6D.

3.2.5. Impact of exogenous apoptotic leukocytes on chicken leukocyte antimicrobial mechanisms at the inflammatory site

As primary infection is being dealt with by the immune system, inflammatory processes that result must be highly regulated to regain homeostasis during the resolution of infection (Maderna and Godson, 2003; Poon et al., 2014; Soehnlein and Lindbom, 2010). Thus, leukocyte activation and ROS production should effectively be down-regulated. As such, the presence of apoptotic cells in later phases of inflammation activates homeostatic signals and contributes to its downregulation. Here, we evaluated the effect of chicken apoptotic leukocytes on the activation level of inflammatory leukocytes, when apoptotic cells were administered along with or prior to zymosan injection by measuring total ROS production in infiltrating leukocytes. Over 65% of infiltrating leukocytes produced ROS in the positive control group. In this context, I observed a decrease in ROS producing cells (to 35% from total leukocytes collected, $p < 0.05$) 12 h post zymosan injections in 0, -2 and -4 h groups with apoptotic leukocyte

administration prior the zymosan challenge. Interestingly, the reduction of ROS production levels in inflammatory leukocytes in the 0, -2 and -4 h groups correlate with the reduction of heterophil populations at the inflammatory site 3-fold at 0 h ($p < 0.01$) and -2 h, ($p < 0.05$; Fig. 3.7A). However, these apoptotic cells did not affect leukocyte viability (Fig. 3.7C). This reduction in inflammatory leukocyte activation by apoptotic leukocytes in chickens is less dramatic compared to that observed in fish model; but similar to the effect previously described in the murine model.

3.2.6. Chicken apoptotic leukocytes do not affect phagocytic capacity in inflammatory leukocytes

In the late phase of inflammation, apoptotic cells are cleared up from the inflammation site by resident and infiltrating phagocytes (Esmann et al., 2010; Soehnlein and Lindbom, 2010). To evaluate the effect of apoptotic cells on the phagocytic capacity of inflammatory leukocytes, we harvested inflammatory leukocytes from chickens injected intraperitoneally with zymosan alone or with apoptotic leukocyte injection at 0, 2 and 4 h prior and collected the inflammatory cells at 12 h post zymosan injection. The phagocytic capacity of total leukocytes was assessed through their ability to uptake zymosan particles *in vitro*. We observed that total phagocytic leukocyte proportion was 18% in the positive control group which corresponded to animals injected with zymosan only (Fig. 3.8B). Surprisingly, the 0 h group, injected with a mixture containing both apoptotic leukocytes and zymosan, did not show altered phagocytic activity in inflammatory leukocytes compared to the positive control group. Thus, it highlights that exposure to apoptotic particles did not alter that phagocytic activity of avian

inflammatory leukocytes following challenge.

3.3. Discussion

Avian species are in a key evolutionary position between fish and mammals. They share some physiological and anatomical features, while have gained their own characteristics that make avian class a unique group of successful animals. For example, body core temperature in birds is significantly higher (41°C) than mammals and fish. Therefore, it is reasonable to consider that avian species might display different mechanisms to achieve immunity, as temperature plays a crucial role in inflammation (Hori et al., 1991; Tesch et al., 2018). Hence, a series of questions arise on how those defence mechanisms are mounted in avian species following pathogen invasion and what the cellular contribution is under inflammatory processes while it transits to homeostasis return. First, I defined basal immune characteristics under normal conditions to characterize a homeostatic stage, as this helped to draw an immunological threshold for *in vivo* challenges. Low numbers of resident leukocytes in the avian abdominal cavity suggest that normal leukocyte distribution/development into tissues in avian species is displayed at different rates or deficient than in other vertebrates where, a large pool of leukocytes is present (Eid et al., 2010; Rieger et al., 2012). This might also suggest that during this period (3-4 weeks of age), the avian immune system must prioritize other tissue sites of primordial relevance to chickens rather than the abdominal cavity. Yet, these resident leukocytes found, are the sentinels for the abdominal compartment, composed by a proportion of monocytes/macrophages and lymphocytes, defined by morpho-cellular characteristics. Although my experimental setup did not allow the assessment of cell-to-

cell interaction between these two groups, major events following *in vivo* challenge suggest that these cells might contribute to inflammatory processes similarly as in mammals and teleost fish. Hence, following zymosan challenge, a massive leukocyte infiltration was dominated by heterophils. Despite this prominent heterophil migration and their active antimicrobial responses such as phagocytosis and others, they did not exhibit robust oxidative defenses. These results provide evidence that chicken heterophils use ROS production as intracellular signaling molecule rather than an antimicrobial defense *per se*, under zymosan challenge. This shows that oxidative-antimicrobial capacity in heterophils was lost in the avian immune evolution. In contrast to previous studies (Penniall and Spitznagel, 1975; Stabler et al., 1994), I demonstrated that avian heterophils indeed exhibit ROS production following pathogen-heterophil interaction but it appears to use them as a mechanism of cellular activation.

Dynamics of infiltrating leukocytes in avian inflammation showed that leukocyte influx is faster compared to other vertebrates such as teleost and murine models (Havixbeck et al., 2016, 2014; Rieger et al., 2012). In those species the peak of leukocyte infiltration following zymosan challenge was 18 and 24 h hours, respectively. Although there is not clear explanation for this differential dynamic, it seems that an increase in body temperature in avian species (41°C) might play a role. Increase of body temperature (fever) is favoured by a systemic release of multiple soluble mediators as pro-inflammatory cytokines that subsequently accelerate immune defences (Hori et al., 1991). Hence, further studies are needed to analyse whether chicken core body temperature also promote an additional release of other pro-inflammatory mediators in a synergistic process. Furthermore, it needs to be defined to what extent this has

implications in the normal avian physiology.

PMN infiltration to inflammatory site is followed by a supply of monocytes and macrophages in murine (Cash et al., 2009). In the current avian model, the lack of significant arrival of circulating monocytes suggests that these cells do not play a major role during initial phases of inflammation against zymosan. However, I observed an increased level of monocyte/macrophage infiltration later in the inflammatory process. These results revealed that monocyte infiltration contributes to the resolution phase of chicken acute inflammation. Additionally, this evidenced that circulatory monocyte migration might occur between 12 and 48 h of zymosan challenge. Alternatively, a local self-proliferation might also play a role in the increase of monocyte/macrophage subsets, a process observed in murine model (Davies et al., 2011; dos Anjos Cassado et al., 2015) but not described in avian species.

Following pathogen clearance, increase of lymphocytes marks the transition from innate responses into adaptive responses in mammals. T and B2 lymphocytes have shown to appear days after peritoneal challenge in mammals (Braunstein et al., 1976). Thus, I wanted to address whether avian lymphocytes display differential dynamics since monocyte/macrophage and lymphocyte interactions are linked to the resolution phases of inflammation. My results reveal that levels of lymphocytes returned to homeostatic proportions with no major increment. These results highlight that even when major players of inflammation are returned to homeostatic state, lymphocytes do not develop to a larger proportion within that period. Rajakariar et al. pointed out that replenishment of lymphocytes occurs between 12 and 24 h post zymosan peritoneal challenge (Rajakariar et al., 2008). However, this was not observed in the current avian model.

Altogether, this suggests that mammals and avian species display differential lymphocyte contributions to resolve inflammation.

The progress of inflammation to homeostasis is driven, among others, by the leukocyte sensitivity to respond to homeostatic elements such as apoptotic cells (Gilroy and De Maeyer, 2015). Indeed, as the inflammation progresses to pathogen control, a gradual accumulation of apoptotic cells participates in the release of anti-inflammatory mediators (Maderna and Godson, 2003). The results demonstrate the gradual appearance of apoptotic cells as the inflammation progresses, suggesting similar mechanistic contributions to the resolving stage of chicken inflammation as other vertebrates. Thus, it was reasonable to believe that these chicken apoptotic cells contributed to the development of adaptive responses in chicken. Equal capacity of responding against pathogen-mediated particles and apoptotic cells at pro- and anti-inflammatory phases demonstrates chicken leukocytes can recognize, bind and engulf both inflammatory and homeostatic particles at similar proportions. A deeper analysis shows that heterophils have phagocytic capacity to internalize apoptotic cells, a feature shared with mammals (Esmann et al., 2010) but absent in teleost fish (Rieger et al., 2012). These findings suggest that PMN capacity to internalize apoptotic cells developed later in the divergency of these animal classes. Furthermore, it demonstrates the downregulatory function of heterophils in chickens is through phagocytic-dependent mechanisms, alternatively to those in fish, where an independent mechanism is observed. Lastly, this evidences the overall contribution of chicken apoptotic cells in the dynamics of avian acute inflammation. Additionally, diminished heterophil capacity to internalize pro-inflammatory particles at later phases of inflammation contradicted what has been

observed in teleosts, where neutrophil capacity to internalize pathogen-mediated particles is not affected. My findings suggest that internalization of apoptotic particles by heterophils during inflammation plays a role in the downregulatory phagocytic capacity to pathogen-derived particles, in the downregulation of oxidative-mediators and promoting anti-inflammatory responses. Furthermore, this reveals higher sensitivity of heterophils to homeostatic than pro-inflammatory particles as the inflammation resolves. These features in chicken heterophils are similar in mammalian models (Esmann et al., 2010), but they display differential performance compared to early vertebrates such as teleost fish. Altogether, this highlights the differential functional dynamics of heterophils from PMNs in other vertebrates, adding novel functional features to those already assigned to chicken heterophils.

As expected, abdominal leukocytes internalizing zymosan displayed increased levels of ROS, while leukocytes internalizing apoptotic cells had a decreased ROS production capacity. Interestingly, my findings of low ROS production in cells internalizing both zymosan and apoptotic cells confirmed my previous findings that chicken inflammatory leukocytes display higher sensitivity to apoptotic cells than pathogenic particles. This added to reveal the chicken apoptotic cell contribution in downregulation of inflammation. Furthermore, I wanted to address whether this had implications during an *in vivo* challenge as additional soluble factors might play a role. My results of simultaneous intra-abdominal administration of apoptotic leukocytes and pathogen-derived particles (zymosan) demonstrated a downregulation of leukocyte (heterophil) infiltration and ROS production. Interestingly, similar observations were obtained when apoptotic cells were administered prior to inflammatory particles (2 and 4 h prior)

although to a lesser extent. Altogether, these findings highlight the importance of apoptosis in the resolution of chicken inflammation and the contribution of the apoptotic milieu in the development of pro-inflammatory responses even prior to the invasion of pathogens. Further studies are needed to address the contribution of homeostatic particles in early stages of chicken acute inflammation, but those were beyond the scope of the current study.

In summary, this chapter provides a characterization of the relevant processes driven following pathogen invasion in chickens. It shows that major parameters during initial stages of chicken acute inflammation are conserved among higher vertebrates.

Interestingly, chicken acute inflammation reveals quicker dynamics (e.g. leukocyte migration, phagocytosis, ROS production, and others) than mammals and early vertebrates. Furthermore, it highlights differences of ways to resolve chicken inflammation (heterophil phagocytosis and apoptosis). Additionally, I characterized some unique features such as ROS production in heterophils and contribution of monocyte/macrophages to chicken acute inflammation. Taken together, these results offer a reliable platform of immunoassays with potential practicality for assessment of immune capacity in chickens

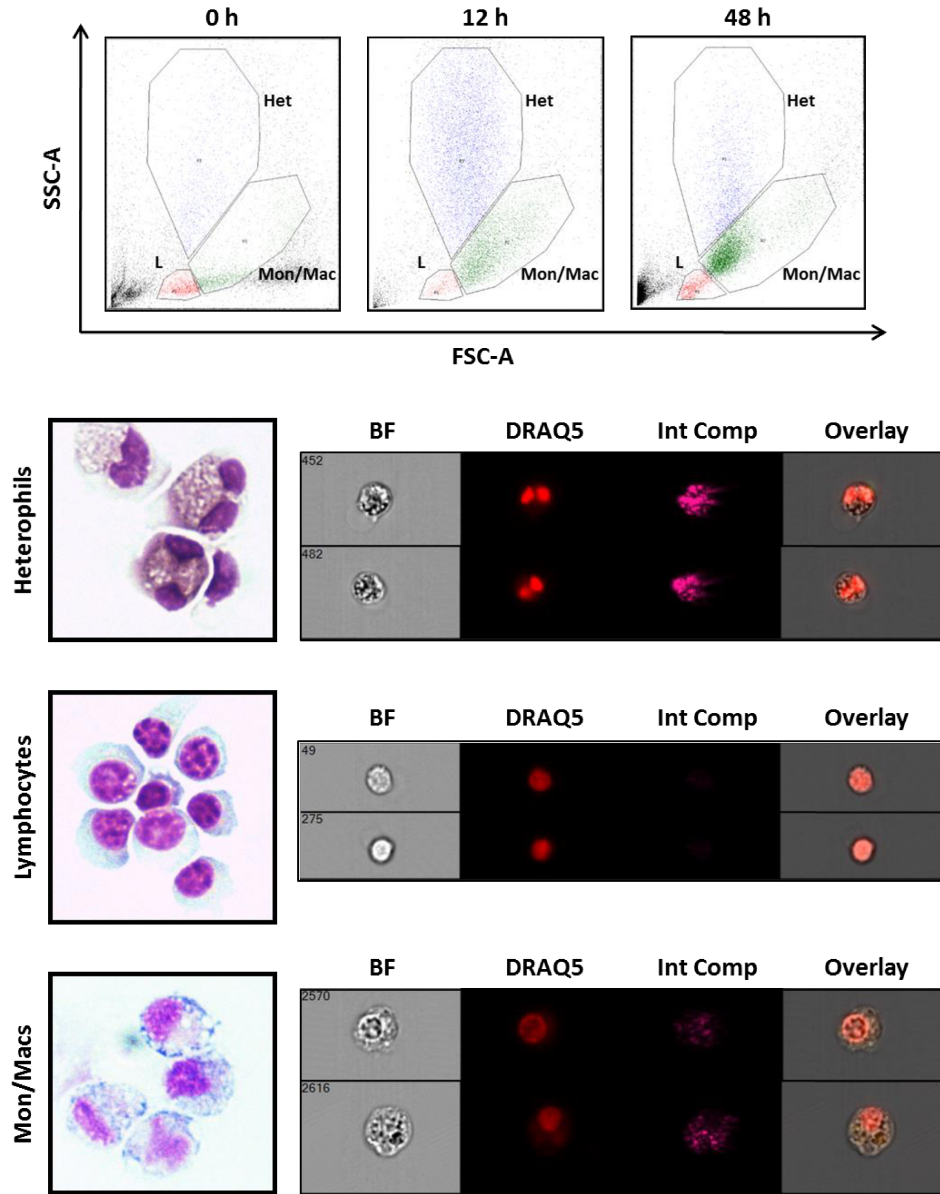


Fig. 3.1. Zymosan intra-peritoneal challenge induces recruitment of heterophils, lymphocytes and monocyte/macrophages in chickens. Animals were injected intraperitoneally with 2.5 mg of zymosan and leukocytes were recovered at 0, 12 and 48 h post-challenge; these correspond to unstimulated, pro-inflammatory, and pro-resolving phases of acute inflammation, respectively. Dot plots showing the control and stimulated peritoneal cells based on FSC-A (size) and SSC-A (internal complexity) flow cytometric characteristics are shown. Infiltrating leukocytes corresponding to the 12 h pro-inflammatory time point, were sorted using a FACS Aria flow cytometer. Three main subpopulations were distinguished using Imaging flow cytometry based on their size (brightfield), internal complexity (darkfield) and nuclear morphology (DRAQ5): heterophils (Het), lymphocytes (L) and monocyte/macrophages (Mon/Mac).

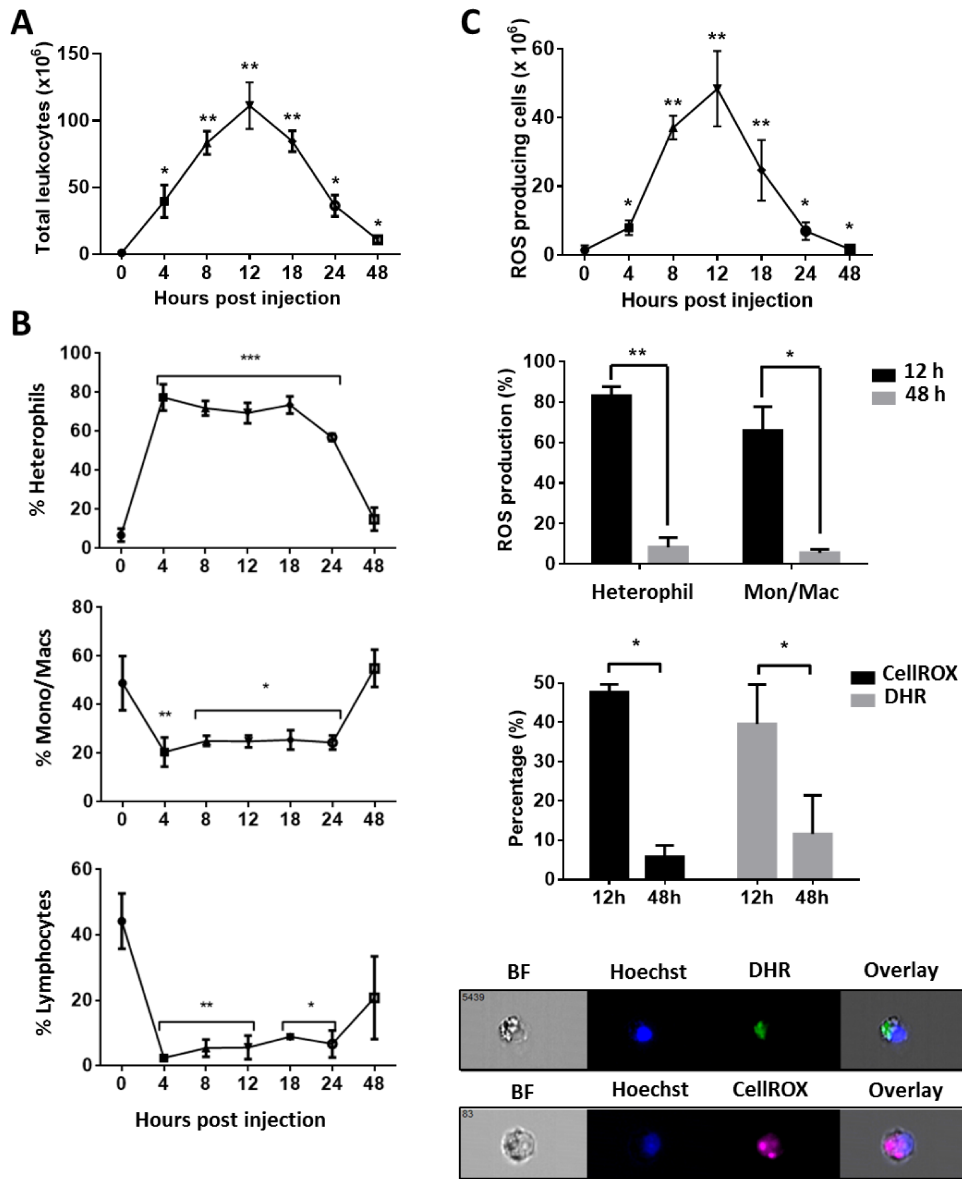


Fig. 3.2. Zymosan intraperitoneal challenge induces ROS production that correlates with the peak of leukocyte infiltration. (A) Zymosan was injected intraperitoneally and leukocyte recruitment was analyzed at indicated time points. $n = 9$. (B) The kinetics of infiltration for heterophils, monocyte/macrophage and lymphocytes based on cytochemical HEMA 3 staining are shown as a percentage of the total leukocyte population. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (C) Absolute number of leukocytes producing ROS was examined using DHR. $n = 4$. Values among the two primary ROS producing leukocyte subsets are shown for 12 h (peak for induction phase of acute inflammatory response) and 48 h (resolution phase of acute inflammation). Two distinct staining methodologies confirmed maximal ROS production at 12 h and 48 h time points post-challenge.

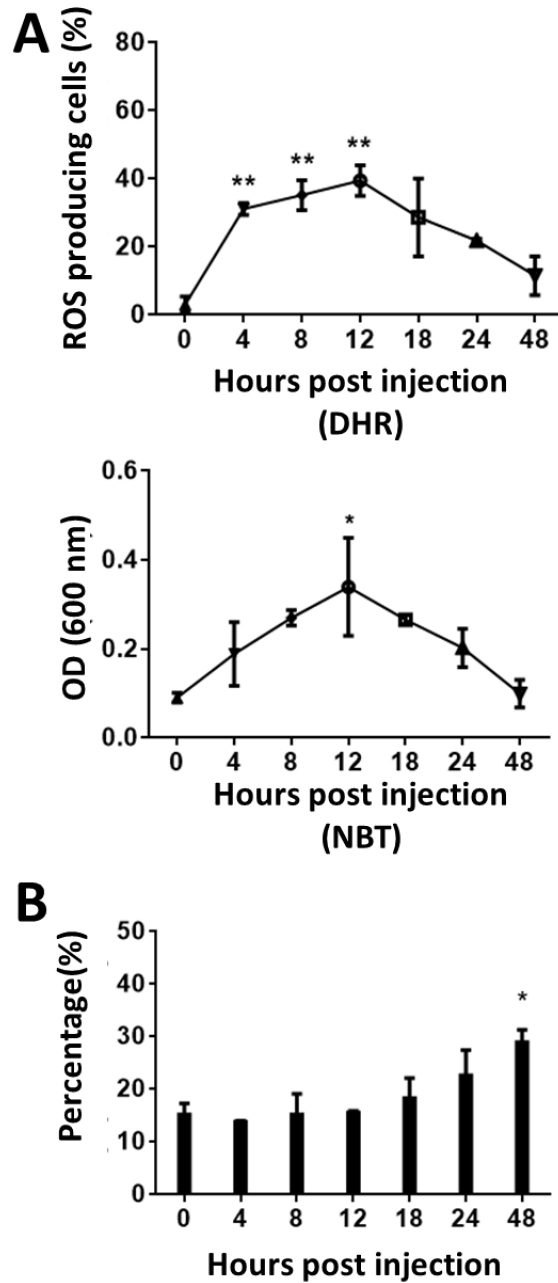


Fig. 3.3. Kinetics of ROS production during the chicken acute inflammatory response. A) The percentage of ROS producing leukocytes was measured following *in vivo* administration of zymosan into the abdominal cavity of chickens. DHR oxidation (top) and the NBT assay (bottom) were used; n=5. Both assays were able to detect early activation of ROS production and subsequent down-regulation in later phases of inflammation. B) Percentage of apoptotic leukocytes was examined through induction (0-12 h) and resolution phases (12-48 h) of the chicken acute inflammatory response; n=3. *p < 0.05, **p < 0.01. OD = optical density

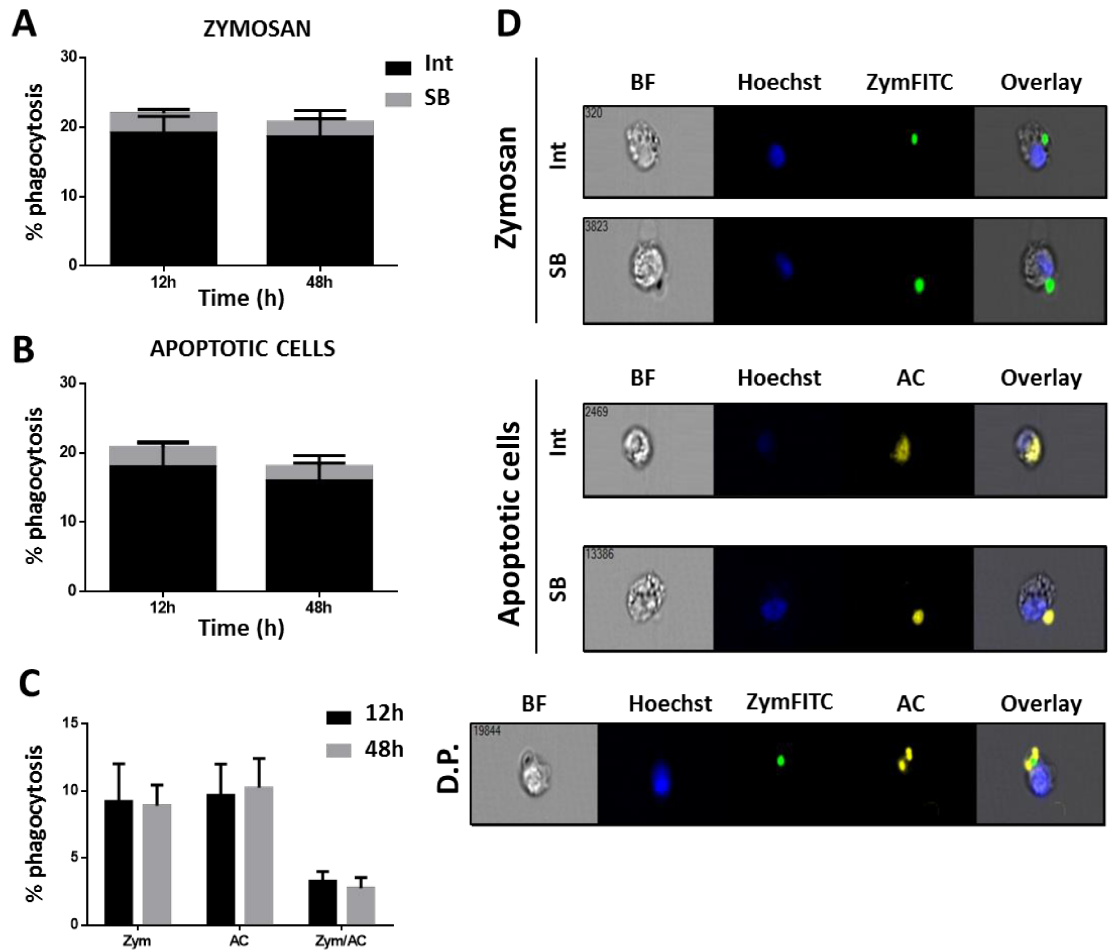


Fig. 3.4. Chicken leukocytes display equivalent capacity for uptake of pro-inflammatory and homeostatic particles at early and late phases of the acute inflammatory response. Twelve and forty-eight hours after intraperitoneal zymosan challenge, leukocytes were harvested and incubated separately for 2 h with zymosan-FITC (A) or apoptotic cells- WGA (B) at a 5:1 particle to cell ratio. The percentage of phagocytic leukocytes was determined as described. Data was acquired using an ImageStream MkII multispectral flow cytometer. Black bars correspond to internalization events (Int), whereas grey bars denote particle surface binding (SB). n = 5. (C) Isolated leukocytes were cultured in the presence of both zymosan-FITC and apoptotic cells-WGA for 2 h (5:1 ratio for both) and analyzed at 12 and 48 h post-challenge based on their capacity for particle internalization: cells containing only zymosan (Zym), cells containing only apoptotic cells (AC) or cells containing both zymosan and apoptotic cells (Zym/AC). n = 5. Representative images are shown in panel D. BF – brightfield; Int – Internalized; SB – surface bound; D.P. – double positive.

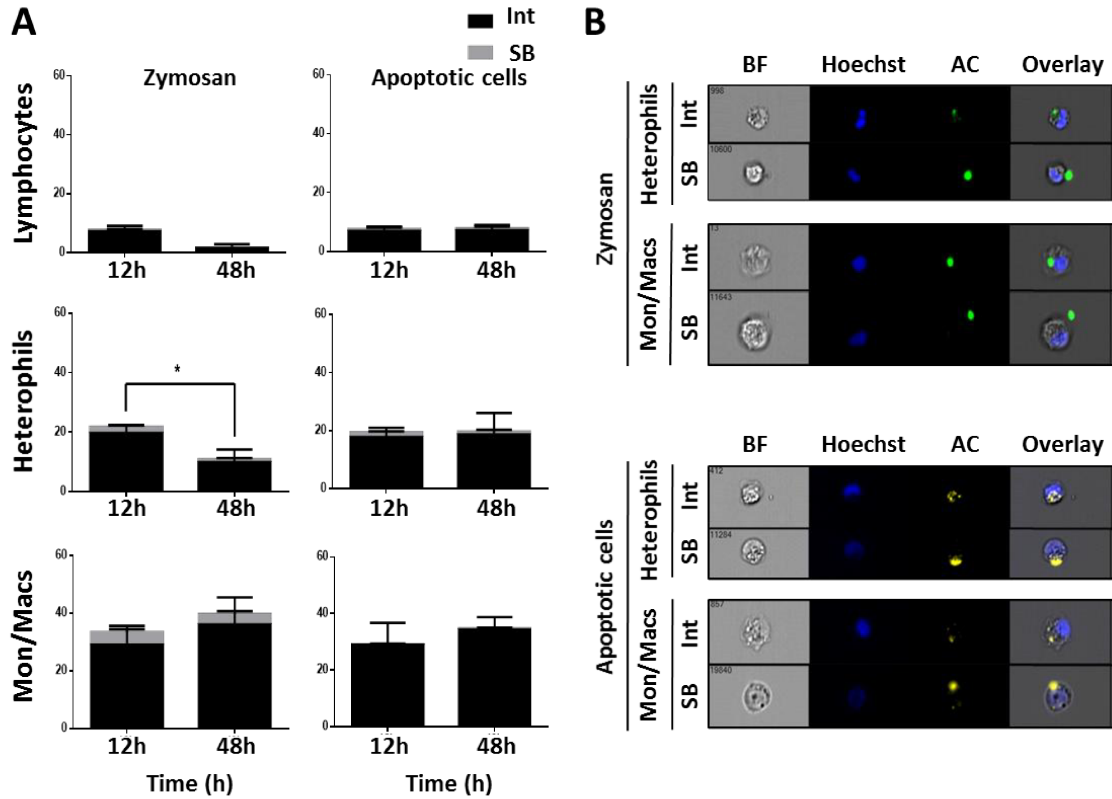


Fig. 3.5. Imaging flow cytometry shows selective decrease in zymosan phagocytosis among heterophils infiltrating the acute inflammatory site at 48 h post challenge.

Animals were injected intraperitoneally with zymosan, and leukocytes were subsequently harvested 12 or 48 h post-challenge and incubated separately for 2 h with zymosan-FITC or apoptotic cells-WGA at a 5:1 particle to cell ratio. Data was acquired using an ImageStream MkII multispectral flow cytometer. Lymphocyte, heterophil, and monocyte/macrophage (Mon/Macs) leukocyte subsets were defined based on size and internal complexity parameters. The percentage of phagocytic leukocytes among each subset was determined as described. Black bars correspond to internalization events (Int), whereas grey bars denote particle surface binding (SB). n = 5. Representative images are shown in panel B

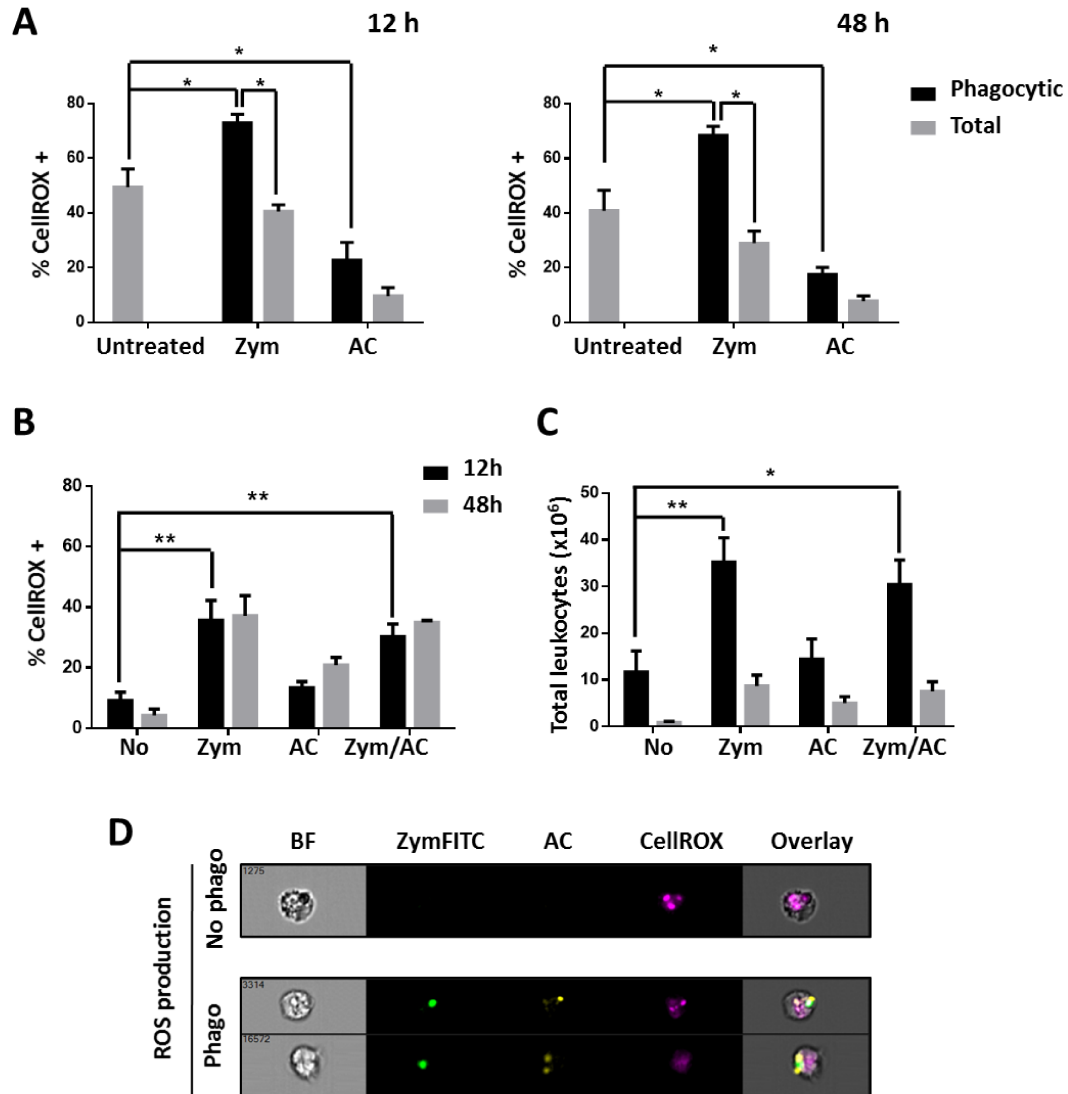


Fig. 3.6. Chicken phagocytes display divergent functional responses to pathogen-derived and homeostatic particles. (A) Animals were injected intraperitoneally with zymosan, and leukocytes were subsequently harvested 12 or 48 h post-challenge and incubated separately for 2 h with zymosan-FITC or apoptotic cells-WGA at a 5:1 particle to cell ratio. Data was acquired using an ImageStream MkII multispectral flow cytometer and evaluated for ROS production based on positive CellIROX staining. Grey bars correspond to ROS production for total leukocyte population; black bars correspond to ROS production among phagocytic leukocytes. $n = 5$. (B) Isolated leukocytes were also cultured in the presence of both zymosan-FITC and apoptotic cells-WGA for 2 h (5:1 ratio for both) and analyzed at 12 and 48 h post-challenge based on their capacity for particle internalization: cells containing only zymosan (Zym), cells containing only apoptotic cells (AC) or cells containing both zymosan and apoptotic cells (Zym/AC). The corresponding number of ROS producing total leukocytes was also determined (C). $n = 5$. Representative images are shown in panel D. * $p < 0.05$, ** $p < 0.01$. BF – brightfield; Zym – Zymosan; AC – apoptotic cell

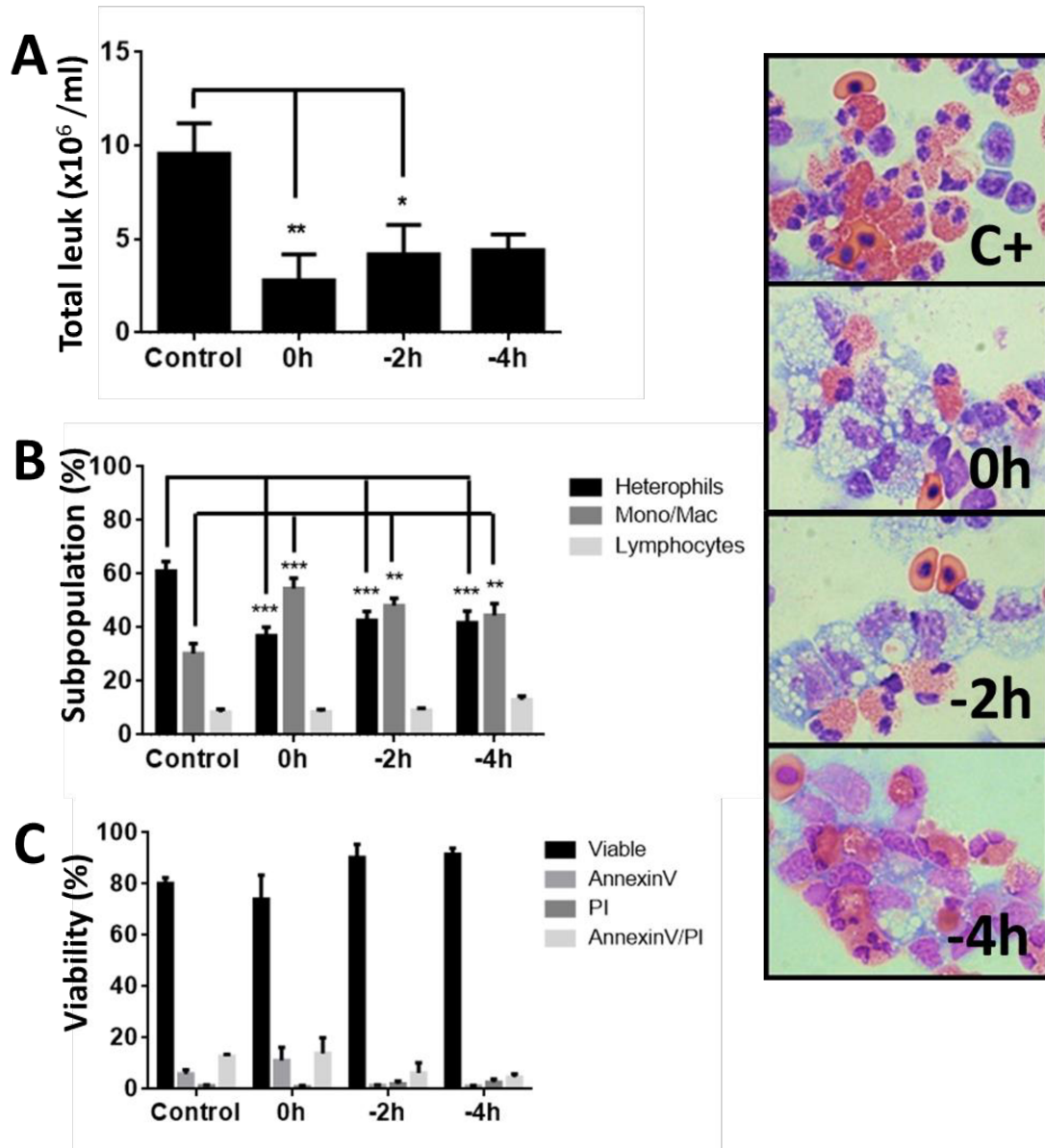


Fig. 3.7. Introduction of apoptotic leukocytes differentially inhibit leukocyte recruitment into the avian challenge site. Chicken apoptotic cells were injected intra-abdominally at different time points (0, -2 and -4 h) of zymosan *in vivo* challenge. Leukocytes were harvested after 12 h and leukocyte counts were determined (A). Leukocyte sub-populations were calculated using conventional Hema3 staining (B). Cell viability was determined at different time points to ensure that down-regulation of ROS production was not associated with a loss of viability (C). Representative images are shown in D. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. $n=4$.

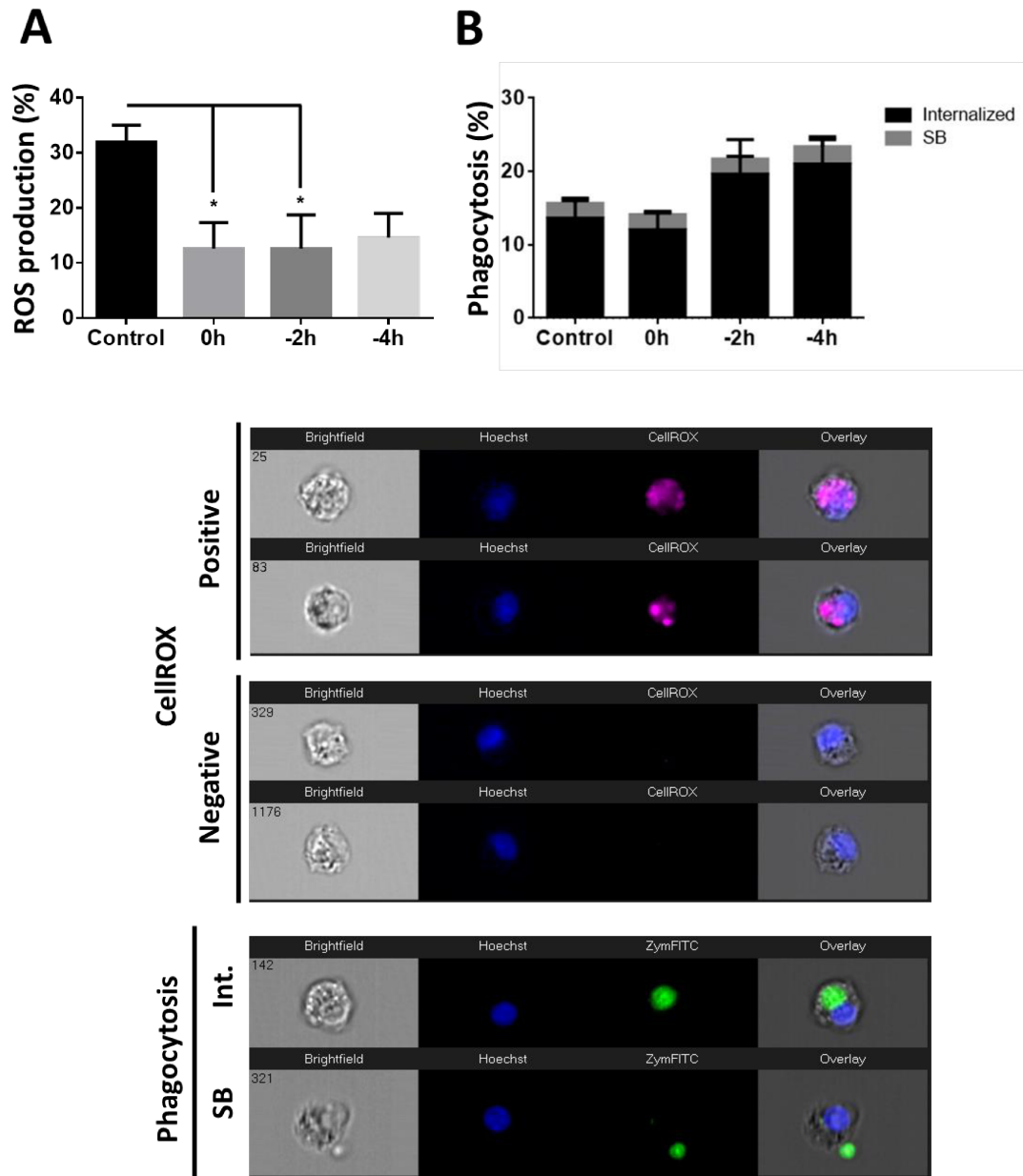


Fig. 3.8. Chicken apoptotic leukocytes downregulate ROS production but do not affect the phagocytic capacity of inflammatory leukocytes. Chicken apoptotic leukocytes were injected at different time points (0, -2 and -4 h) of zymosan challenge. Leukocytes were harvested after 12 h and leukocyte counts were determined. Inflammatory cells were harvested and ROS production (A), using CellROX, and phagocytosis of zymosan (B) were measured. The apoptotic cells decreased ROS production by inflammatory cells, however, the phagocytic capacity was not decreased at any time point. * $p < 0.05$. $n=4$.

Chapter IV

Characterization of Acute Inflammation in Swine

4.1. Introduction

Pig (*Sus scrofa domestica*) is of importance for immunological studies due to its closely-related immune system with humans, even closer than the well-studied murine model (Bailey et al., 2013; Bode et al., 2010; Chevaleyre et al., 2016; Meurens et al., 2012). Thus, studies of swine inflammation represent a useful model providing a closer comparative view to human immunity than other models. Hence, swine becomes a powerful tool for assessment of multiple disorders or diseases shared between swine and humans. Despite this close evolutionary relationship between swine and humans, there are some clear differences and gaps that need to be filled and are crucial under infection conditions. Salmonellosis is a common enteric disease affecting livestock and human, that has been studied mainly through intestinal approaches (Drumo et al., 2016; Kim and Isaacson, 2017; Wray and Wray, 2000). Since the peritoneal compartment provides a sterile environment to study inflammation, it is a powerful tool for the study of cell-pathogen interaction under Salmonella infections and the underlying process throughout inflammation. (Balaji et al., 2000; Donné et al., 2005; Knetter et al., 2015). The current approach took advantage of these conditions to add a depth into the understanding of acute inflammation processes using a widely-known peritoneal challenge model. Thus, this chapter sheds light upon the dynamics of infiltrating leukocytes and their antimicrobial responses following an HKST intraperitoneal challenge. Additionally, I evaluated how those leukocytes contribute to the transit from a pro-inflammatory stage to the resolving phases of acute immune responses.

The main objectives of this chapter were to 1) design a self-resolving model using peritoneal challenge approach for the study of acute inflammation; 2) the assessment of

dynamics of antimicrobial responses *in vivo*; and 3) evaluation of acute immune responses against salmonella using an *in vivo* approach. My findings determined a massive leukocyte infiltration dominated by neutrophils at early stages of inflammation, reaching maximum levels at 12 h and a gradual return to homeostatic levels between 18-48 h. These dynamics were parallel with robust ROS production that downregulated at later phases of inflammation. Taken together, these results demonstrated conserved mechanisms of acute inflammation among mammals, while establishing a self-resolving peritoneal model in swine for assessment of immune capacity to battle infections under *in vivo* conditions.

4.2. Results

4.2.1. Intraperitoneal injection of HKST induce a self-resolving inflammatory process lead by neutrophils

The peritoneal cavity of mammals is a large and sterile compartment that provides an environment for the study of multiple biological processes. It has been widely used in the study of development of acute inflammation because it provides a large number of resident leukocytes while they go through all facets of acute inflammation (Bain and Jenkins, 2018; Davies et al., 2013; Leyboldt et al., 2007). Hence, pro- and anti-inflammatory features can be assessed *in vivo*. Even though various studies demonstrate its reliability in classical models such as mice and others, little is known about these responses in swine. Here, we took advantage of intraperitoneal challenges using HKST for assessment of inflammation. Our hypothesis is that HKST will represent a reliable trigger of a self-resolving peritoneal inflammatory process adding a depth into the

understanding of Salmonella infections in porcine with benefits for swine and human health. Basal levels of resident leukocytes revealed a large and heterogenous cellular component. Lymphocytes and monocyte/macrophage subpopulations were detected prior to intraperitoneal challenges, with reduced levels of neutrophils. Following challenge, massive leukocyte infiltration, dominated by neutrophils, was observed as soon as 4 h. Active leukocyte migration was observed until 12 h post challenge, followed by leukocyte depletion up to 48 h. Through cell sorting, imaging flow cytometry and conventional staining I was able to differentiate lymphocytes, monocyte/macrophages and neutrophils (Fig. 4.1).

4.2.2. HKST inflammatory process resolves within 48 h post challenge

Following pathogen invasion, a strong and effective immune response must be displayed for pathogen control. This mechanism must be efficiently and tightly regulated to timely return to homeostasis. This return to homeostasis is led by a shift of cellular pro-inflammatory function to a pro-resolving stage, through release of anti-inflammatory mediators and pathogen control in an integrative fashion. Thus, this inflammatory complex must be properly assessed to understand the real contribution of those cellular players. In this context, a multiparametric analysis enables the timely assessment of cellular dynamics and function, their antimicrobial contribution, and processes of pathogen control towards resolution of inflammation. Hence, massive neutrophil infiltration was observed as soon as 4 h post challenge (10-fold increase, $p < 0.0001$). Neutrophil proportions progressively decreased after 18 h post challenge (5-fold compared to 0 h, $p < 0.001$), however, these proportions remained significantly higher up

to 48 h compared to 0h (3-fold, $p=0.034$). Monocyte/macrophage proportions decreased up to 48 h where they remained close to 20% ($p<0.001$). In contrast, development of lymphocytes showed lower values within the first 18 h post challenge and gradual increase between 24 and 48 h post challenge when it reached significant higher values (35% at 48 h, $p<0.001$) (Fig. 4.2). These results showed an integrative *in vivo* approach of leukocyte dynamics following bacterial challenge, where an active pro-inflammatory response is led by neutrophils during the first 18 h post stimulus and a gradual shift to anti-inflammatory phenotype within 24 and 48h. This shift is mainly achieved through significant changes in neutrophil and lymphocyte pools and a lesser monocyte/macrophage contribution.

4.2.3. *In vivo* HKST challenge induces active ROS production during pro-inflammatory state and down-regulation during pro-resolving phase.

Dynamics of leukocyte populations during acute inflammation differentiate two phases of immune response. The initial phase, a fast, active and robust response is dominated by neutrophils infiltrating the inflammatory site within the first 18 h post HKST challenge. The second phase is characterized by a progressive drop in leukocyte numbers correlated with reduction of neutrophil and increase of lymphocyte subpopulations. As leukocytes migrate to the inflammatory site, these cells also displayed a wide array of antimicrobial responses. Here, I evaluated the reactive oxygen species (ROS) production since it is a fast-triggered response following cell-pathogen interaction, that function as antimicrobial mediator and intracellular signalling molecule (Ray et al., 2012). As ROS is rapidly activated following pathogen entrance, it is

reasonable to use them as marker for inflammation as representative of antimicrobial mechanism to battle infections. Basic levels of ROS showed that out of total resident leukocytes, 20% were ROS producing cells prior to Salmonella exposure. However, these proportions reached 80% of ROS producing cells after *ex vivo* incubation with *S. enterica* ser. Typhimurium ($p < 0.0001$). These results revealed that even in normal conditions a proportion of resident cells display ROS production. Also, resident leukocytes respond strongly following antigen exposure. *In vivo* dynamics of ROS showed maximum proportions at 4 h post challenge (80% producing cells, $p < 0.0001$) with significant higher levels up to 18 h ($p = 0.035$), and gradual reduction up to 48 h ($p = 0.03$). ROS production levels returned to homeostatic levels at 48 h post challenge (Fig. 4.3A). Interestingly, higher values of ROS correlate with the progressive increment of leukocyte infiltration, suggesting that higher levels of ROS play a role in the initiation of inflammation, and lower ROS levels is involved in resolving phase of inflammation (Fig. 4.3B).

Our multiparametric analysis of phagocytosis using Imaging flow cytometry adds a higher resolution for internalization assessment. Hence, using this approach, dynamics of phagocytosis can be evaluated in a cell-by-cell analysis. *Ex vivo* incubation with GFP-ST showed 40% of phagocytosis in unelicited resident leukocytes. This analysis revealed that close to 90% of those uptaking cells had internalized GFP-ST after 30 min incubation and 10% remaining had surface bound GFP-ST and both equally contribute to total ROS production. Interestingly, not only cells interacting with GFP-ST (internalizing and surface bound cells) but cells with no GFP-ST interaction displayed ROS production levels (Fig. 4.4). Even though pathogen-cell interactions are relevant

for activation of antimicrobial responses through ROS production; these findings show that pro-inflammatory phenotype also promotes cellular activation through chemical mediators that potentiate immune responses in swine.

Additionally, leukocyte differentiation through imaging flow cytometry allows the evaluation of phagocytic capacity during pro- and anti-inflammatory state of immune response. Leukocyte obtained at 12 h and 48 h post challenge were incubated *ex vivo* to assess their phagocytic capacity at representative time points of induction and resolution phases of acute inflammation following Salmonella challenge. Results showed that pro- and anti-inflammatory neutrophils displayed relatively similar capacity of phagocytosis. Significantly higher phagocytic capacity of macrophages was observed later the inflammatory process compared to pro-inflammatory state (40%, $p=0.0021$; Fig.4.5). Interestingly, small proportion of lymphocytes-like cells showed phagocytic events with no ROS production response (Fig. 4.5). To the best of my knowledge, this is the first description of phagocytic lymphocytes in swine model.

4.3. Discussion

Swine are considered, genetically and functionally, a closer comparative model of the human immune system than classical murine models. As a result, immunopathological studies of several diseases such as Salmonella infections in swine are increasing. Most of these studies using gastrointestinal approaches integrate local mucosal responses with their implications in the development of long-term immunity (Broz et al., 2012).

Alternatively, the peritoneal cavity offers a niche for evaluation of *in vivo* Salmonella-cell interactions, the assessment of inflammation in a timely manner, and provides a

better immunological perspective for comparative purposes with human defences. The current approach allowed an integrative view of inflammation dynamics and antimicrobial functions, as it transits from pro-inflammatory stages to restore homeostasis. The large homeostatic proportion of resident leukocytes determined in the swine peritoneal cavity suggests a major systemic distribution of leukocytes in the peritoneal compartment distinct from avian. It also appears that self-proliferation within the peritoneal compartment is a process that plays a role in the renewal of resident leukocytes, in a similar process to that in other mammals. Higher proportions of basal ROS levels in swine, compared to avian and teleost fish, suggested that these cells have an active role in homeostasis cellular maintenance in the peritoneal environment. Furthermore, this might be related to local cell proliferation and involvement into leukocyte contribution to systemic immune development described in other studies (Davies et al., 2011). Altogether, these results indicated that swine immune system has equipped the peritoneal compartment with a large group of leukocytes, dynamically active cells that regulate homeostasis in non-challenge conditions.

Ex vivo phagocytosis assays demonstrated that although internalizing bacteria is the main intracellular signal to promote oxidative-defenses, soluble mediators stimulate ROS production in non-phagocytic leukocytes. Further studies are required to better understand the interplay among these cellular communities and evaluate whether phagocytosis promotes differential release of soluble mediators following pathogenic invasion. Nevertheless, these results highlight the integrative role of resident leukocytes in antimicrobial defenses and reflect the initial first response against pathogens.

My next step was to examine the underlying mechanistic processes of inflammation

during an *in vivo* challenge. Dynamics of infiltrating leukocytes and their antimicrobial responses evidenced that inactivated pathogens are potent stimulators of immune defences as the inflammation produced it is closely regulated in a timely manner. Anti-inflammatory mediators such as decreasing number of neutrophils and downregulated levels of ROS evidenced the transition to resolving phase of acute inflammation. However, it appears that neutrophil numbers only decrease after 48 h post challenge. Nevertheless, the increment of lymphocyte-like cells at 48 h marks the entrance to the resolving phase of acute inflammation. These findings suggest that lymphocytes play a regulating role in the resolution of inflammation. Lymphocytes are known to play a role in intracellular communication to promote homeostasis in a murine model (Kolaczowska et al., 2008). The results suggest that this mechanism is also employed in inflammatory regulation in swine and has implications in the development of long-term immune local and systemic responses. However, is still unclear the interplay between pro-resolving neutrophils and emerging lymphocytes during late phases of swine inflammation. Further studies are needed to get a better understanding of the specific lymphocyte type involved in this process, their functional and molecular profile and interaction with other leukocytes.

The findings of equal neutrophil activity to internalize pathogen-mediated particles at pro- and anti-inflammatory stages demonstrated that this ability is maintained among mammals and differ from early vertebrates. This reveals the importance of neutrophils to recognize and battle potential triggers even when inflammation resolves. In addition, this evidences an advantageous feature in higher vertebrate immunity gained throughout evolution. On the other hand, increasing proportions of macrophages internalizing

pathogens at anti-inflammatory phases demonstrated that the macrophage polarization process observed in murine is also present in swine. This macrophage polarization is a process that drive macrophage lineage towards one of the macrophage phenotypes (pro-inflammatory, M1 or anti-inflammatory, M2). In murine models, this polarization process shows that M2 has higher phagocytic activity, low levels of pro-inflammatory cytokines while high levels of anti-inflammatory mediators (Kim et al., 2017). These results suggest that monocyte/macrophage lineage undergoes a polarization process towards pathogen-mediated particles that increases the sensitivity to pro-inflammatory particles in the absence of apoptotic cells. Although this polarization process has been described in other models driven by the abundance of either pathogen-mediated or homeostatic particles (Devitt and Marshall, 2011), this is the first description in swine inflammation. Further studies are needed to determine whether this polarization feature is maintained in the presence of swine apoptotic cells and how it influences the resolution of inflammation in swine. Finally, lymphocyte-like cells internalizing *Salmonella* displayed morphological and functional lymphocyte features at pro- and anti-inflammatory stages in similar low rates. Phagocytic B cells are described in multiple higher vertebrates such as teleost, amphibians and mice. These cells are considered, in opposite to the classical B cells, innate-like B cells (B-1 B cells) (Li et al., 2006; Parra et al., 2013). However, there is no description of this type of cells in swine, neither their function under *in vivo* inflammatory conditions. Like in avian species, these results suggest that avian and swine phagocytic lymphocytes share common characteristics that have been acquired and fixed in the immune system even before evolutionary divergency. Furthermore, deeper studies are needed to determine

phagocytic lymphocyte contribution during acute inflammation and their progression to adaptive responses.

Altogether, this chapter establishes a self-resolving method to study Salmonella-cell interactions and their underlying antimicrobial processes during acute inflammation.

Although major defence mechanisms are described in murine models (such as leukocyte dynamics and ROS production), some differences were determined in swine that might be of benefit for comparative studies with human (phagocytic sensitivity). Finally, this chapter provides a set of highly-sensitive array of immunoassays for analysis of immune effects under multiple conditions (e.g. drinking water contaminants, antibiotics effects on immunity, microbiota composition, among others).

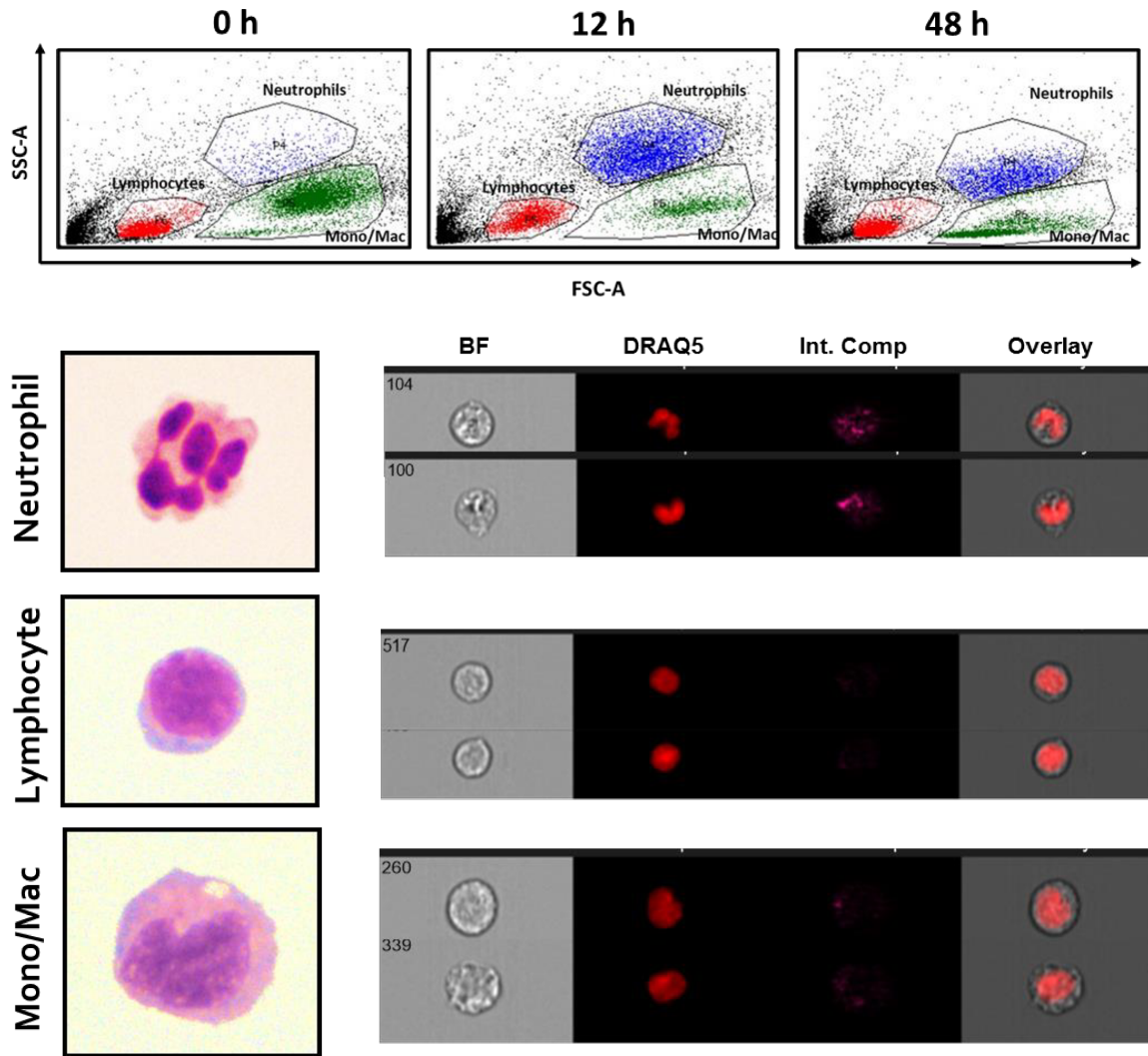


Fig. 4.1. HKST intra-peritoneal challenge induces recruitment of neutrophils, lymphocytes and monocyte/macrophages in swine. Animals were injected intraperitoneally with 10^9 CFU of HKST and leukocytes were recovered at 0, 12 and 48 h post-challenge; these correspond to unstimulated, pro-inflammatory, and pro-resolving phases of acute inflammation, respectively. Dot plots showing the control and stimulated peritoneal cells based on FSC-A (size) and SSC-A (internal complexity) flow cytometric characteristics are shown. Infiltrating leukocytes corresponding to the 12 h pro-inflammatory time point were sorted out using a FACSaria flow cytometer. Three main subpopulations were distinguished using Imaging flow cytometry based on their size (brightfield), internal complexity (darkfield) and nuclear morphology (DRAQ5): neutrophils, lymphocytes and monocyte/macrophages.

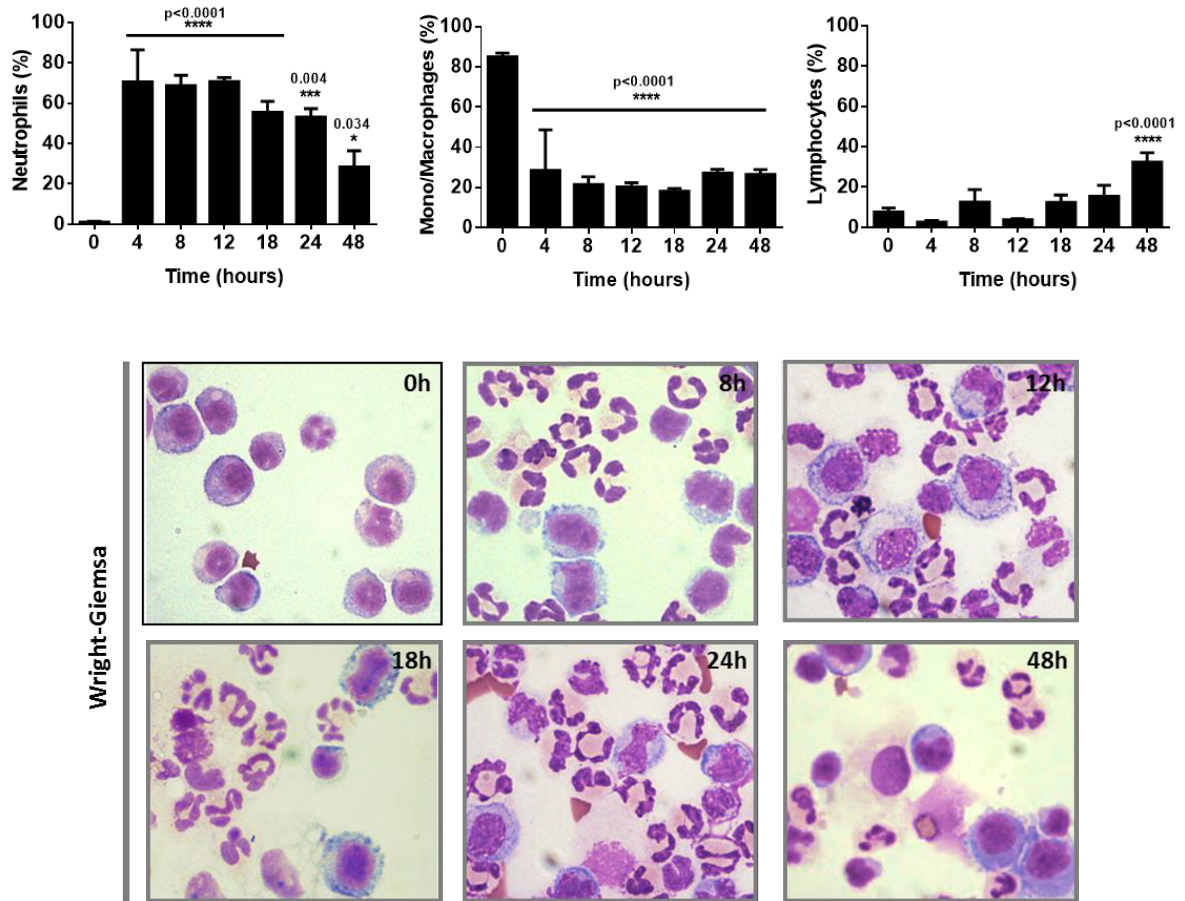


Fig. 4.2. HKST intraperitoneal challenge induces self-resolving inflammatory process. HKST was injected intraperitoneally and leukocyte recruitment was analyzed at indicated time points. $n = 9$. The kinetics of infiltration for neutrophils, monocyte/macrophage and lymphocytes based on cytochemical HEMA 3 staining and Imaging Flow cytometry approach are shown as a percentage of the total leukocyte population.

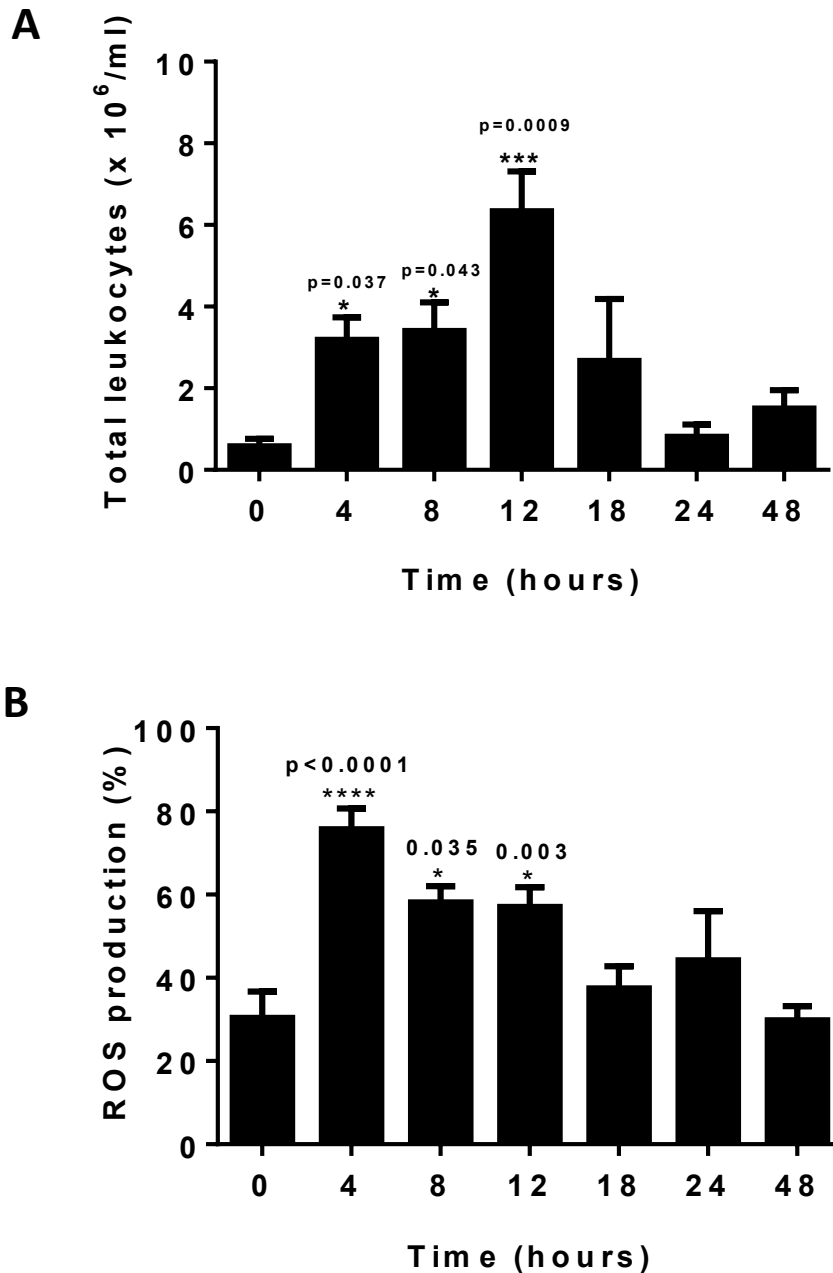


Fig. 4.3. HKST intraperitoneal challenge induces ROS production that correlates with the peak of leukocyte infiltration. HKST (10^9 CFU/ml) was injected intraperitoneally and leukocyte recruitment was analyzed at indicated time points. CellROX staining was used to measure the levels of ROS producing cells at different time points following challenge. Values demonstrated that there is a massive leukocyte infiltration (A) that correlates with higher proportions of ROS producing cells up to 12 h. Later, ROS producing cells decrease as number of infiltrating leukocytes dropped (B). n=9.

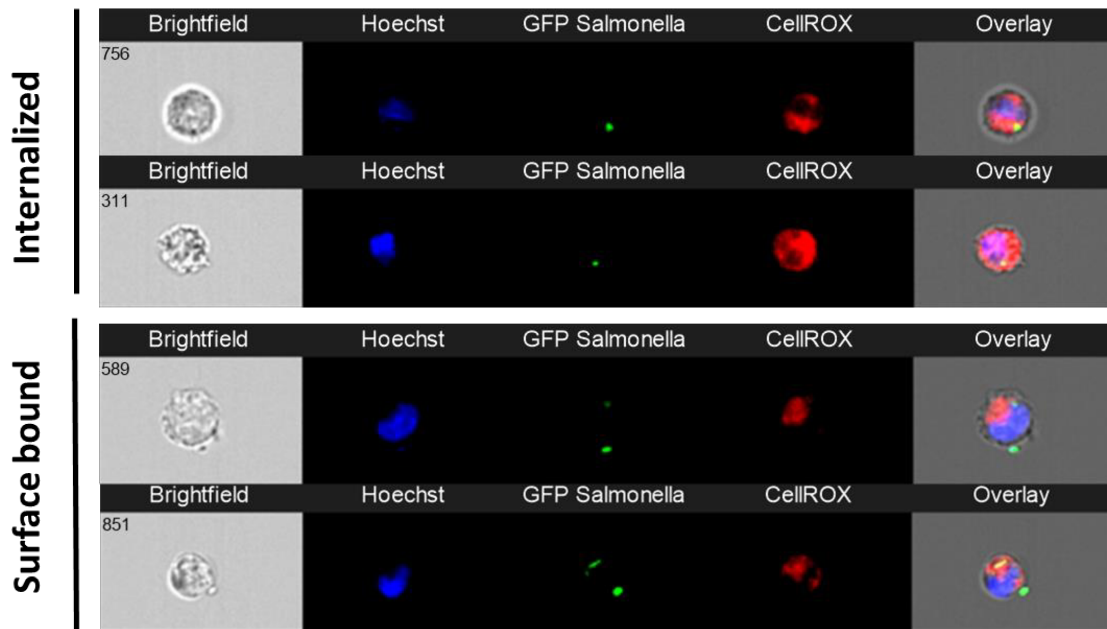
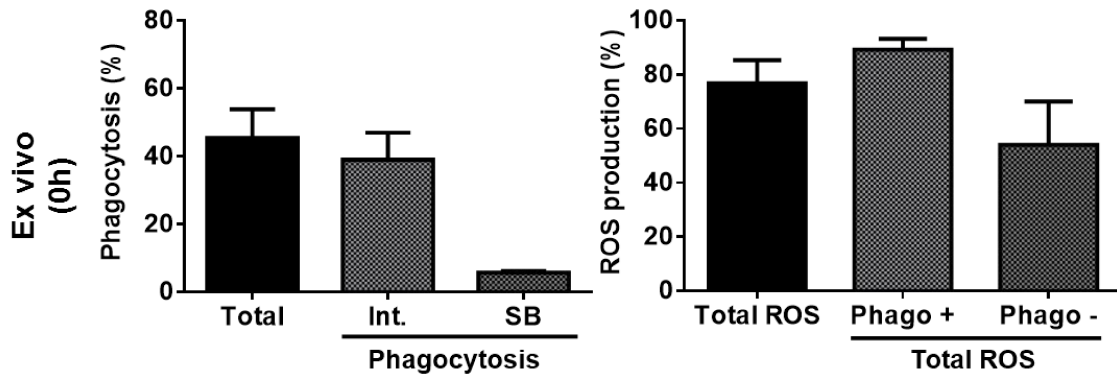


Fig. 4.4. Porcine peritoneal leukocytes are a reliable tool for assessment of antimicrobial responses against *S. enterica* ser. Typhimurium. Peritoneal leukocytes were harvested and incubated *ex vivo* with GFP-ST. After 1h incubation, phagocytosis and ROS production were multiplexed through Imaging Flow cytometry analysis. Multiparametric approach allows robust assessment of GFP-ST phagocytosis (internalizing and surface bound cells) and their respective ROS producing cells capacity. Interacting GFP-ST cells as well not interacting contribute to total ROS production. Int = Internalization, SB = Surface bound.

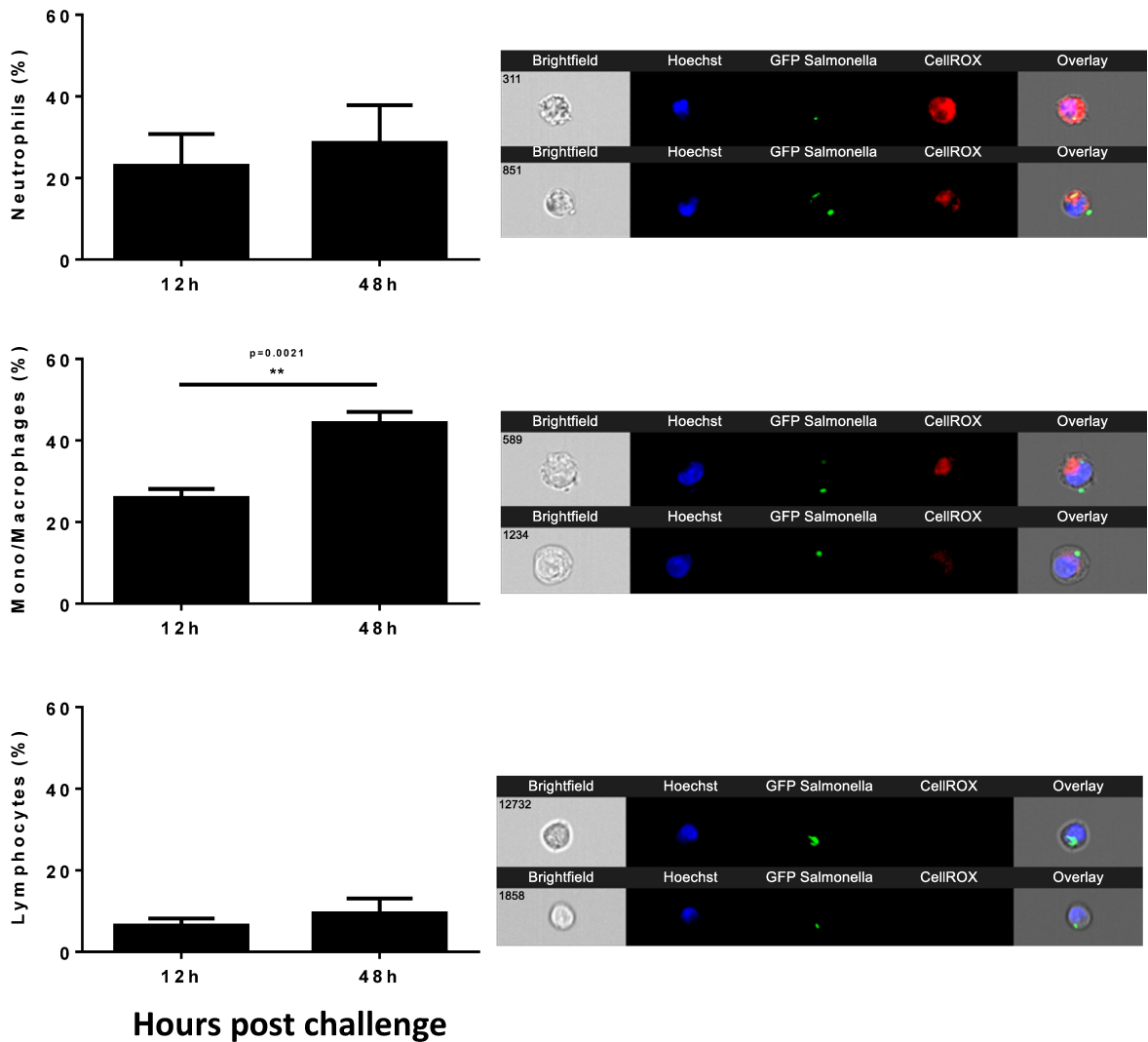


Fig. 4.5. Assessment of antimicrobial responses against *S. enterica* ser. Typhimurium in different leukocytes during inflammation. Porcine peritoneal leukocytes were harvested at pro (12 h)- and anti (48 h) inflammatory stages of acute inflammation and exposed to GFP-ST *ex vivo*. Results revealed differential phagocytic capacity at those stages in different subpopulations. Interestingly, phagocytic capacity increased at 48 h post challenge in the monocyte/macrophage subpopulation.

Chapter V

Contributions of drinking water quality to livestock immune parameters²

² A portion of this Chapter is in preparation for publication

More-Bayona JA., Torrealba D., Thomson C., Wakaruk J., Barreda DR. Differential effects of drinking water quality on the immune responses of broiler chickens against fungal and bacterial challenges.

5.1. Introduction

Water contamination has become a rising concern worldwide in recent years due to an exploding development of industrial activities such as oil and gas extraction systems. Several implications have been described in the disruption of ecosystems by unregulated release of industrial pollutants and their effects on biological systems. Despite increasing number of studies focused on these alterations at population levels, it is still unclear how those contaminants act at the tissue or cellular level. Although several contaminants have been shown to induce detrimental effects in immune response following long-term exposure in several species such as mice, rats, fish and others; little is known in the effect on other divergent species such as poultry and swine. As these species have developed unique immune systems, they represent unique models for assessment of water contaminants on the immune responses and therefore become a reliable tool utilized in comparative approaches for effects in human health. In addition, these species can be used as sentinels for assessment of water contamination for pollution monitoring. Altogether, livestock immunity offers multiple advantages for assessment of human and animal health effects of environmental contamination. Firstly, they are the first animals exposed to contaminants in drinking water compared to humans. Secondly, animal physiology has several conserved mechanisms for homeostasis maintenance among higher vertebrates. Third, easy access to multiple individual (replicates) for testing and *in vivo* experiments. Hence, this chapter provides an integrative assessment of basal and post *in vivo* challenge of cellular immunological responses after a short-term exposure to distinct water sources used in poultry and swine

farms. Furthermore, this chapter offers a platform for evaluation of immune parameters affected by drinking water quality in two distinct livestock animals.

The main objectives of this chapter were 1) to assess the effect of different water sources on basal immune parameters in poultry and swine, 2) to compare the effects of water contaminants in the development of acute inflammation and 3) to define the effects of water contaminants in the development of immunity following acute inflammation process.

5.2. Results

5.2.1. The poultry model

5.2.1.1. Raw water source selection and analysis

Raw water was obtained from a small poultry producer in South East Alberta, Canada (SE-24-22-02) located in an area of high chemical (Agricultural and Agri-Food Canada, 2001a) and fertilizer use (Agricultural and Agri-Food Canada, 2001b), and close to a source of natural gas contamination. Importantly, a local producer uses this source of water as the main primary source for his chicken coop. The same producer also reports that a nearby coop that uses municipal water shows better productive parameters such as growth rates, compared to the one that receives raw water. Thus, this raw water source offers a reliable candidate for evaluation of immune performance.

Water analysis was CALA (Canadian Association for Laboratory Accreditation Inc.) certified and ISO17025. Parameters selected for analysis are known contributors to the impairment of human and animal health (MacDonald et al., 1999). Higher levels of ammonia, phosphates or potassium for the area selected indicated the presence of

fertilizer and contaminants stemming from agricultural activities. Levels of xenobiotics and other industrial chemical parameters were determined using the Ontario Minister of Environment (MOE) regulation for human drinking water (Canadian Environmental Law Association, 2011). A summary of the results found in raw water source are indicated in Table 2, other parameters were significantly lower than maximum acceptable levels, therefore not included in the table.

5.2.1.2. Raw water increases abdominal macrophage numbers in broiler chickens

Individual xenobiotics have been shown to induce detrimental effects on individuals exposed. For example, acting as pro-inflammatory activators in mice models (Lamb et al., 2016; Pande et al., 2005; Vogel et al., 2007). However, assessment of synergistic combinatorial effects of multiple contaminants in water, even in levels below the maximum acceptable, remains unclear. Thus, we evaluated whether acute exposure to raw water promotes changes in basal levels of chicken immune performance. We took advantage of the chicken abdominal compartment to address whether our underground raw water alters levels of resident leukocytes as representative systemic immunological performance. We used a high-sensitive and robust approach based on cellular morphometric characteristics along with an antibody marker (KUL01) for monocyte/macrophage recognition. Through this platform, we determined a higher proportion of abdominal resident monocyte/macrophages in animals exposed to contaminated water (40% resident cells) compared to control animals exposed to normal tap water (20%) ($p < 0.05$, Fig. 5.1). Short-term exposure to multiple xenobiotics present

in drinking water, even with values below the maximum acceptable, induced a pro-inflammatory state that promoted a higher systemic leukocyte migration as observed in chicken abdominal cavity.

5.2.1.3. Drinking water treatment induced changes in leukocyte proportion dynamics during acute inflammation

As previously described, initial phases of acute inflammation are dominated by heterophil migration to the inflammatory site. As inflammation resolves, it must return to homeostasis, which is driven by a sustained decrease of heterophils and progressive increase of lymphocytes and monocyte/macrophages. As water is shown to affect multiple biological process, it is reasonable to believe that water contaminants have effects on the normal immune defenses, since this is a principal process for homeostasis maintenance. Herein, the next objective was to assess whether short-term exposure of broiler chickens to contaminated raw water impacts dynamics of leukocyte infiltration during *in vivo* acute inflammation. Hence, following 7 days exposure to raw water, an *in vivo* approach with zymosan and HKST through intra-abdominal challenge was used. After 4, 12 and 48 h; abdominal leukocytes were harvested. In the bacterial challenge model, we observed a rapid heterophil migration in early stages of acute inflammation (4 and 12 h post challenge) like the control group. However, levels of heterophils remained significantly higher (40% of heterophils at 48 h, $p < 0.05$) in chickens exposed to raw water compared to lower values in the control group. Concurrently, proportions of monocyte/macrophages remained lower at the same time point (40% of monocyte/macrophages at 48 h, $p < 0.05$). On the other hand, we did not observe

significant differences in leukocyte dynamics in the fungal model between both contaminated water and control groups. Interesting to note, total leukocyte migration values were not significantly different ($p > 0.999$) due to water treatment, with slight differential patterns in leukocyte migration in both fungal and bacterial challenge models. This result suggested that contaminated water treatment might induce a differential effect on leukocyte dynamics during inflammatory responses under bacterial and fungi conditions (Fig. 5.2).

5.2.1.4. Acute raw drinking water exposure alters innate antimicrobial responses in chickens

As previously described, zymosan and *S. enterica* ser. Typhimurium are two distinct, representative microbial activators with strong capacity to promote antimicrobial responses in multiple species (Chadzinska et al., 2008; Ipinza et al., 2014; Kaiser et al., 2000; Kolaczowska et al., 2008; Leypoldt et al., 2007; Rao et al., 1994; Rieger et al., 2012). We used these pathogen mimics to assess whether contaminated water treatment not only increased numbers of resident leukocytes but also induced a detrimental effect in their antimicrobial responses following *ex vivo* assays. We measured ROS production as a highly conserved antimicrobial mechanism of immune defence, as it is fast-triggered following pathogen-cell interaction, strongly activated and down-regulated after pathogen control. As a result, it is a reliable marker of antimicrobial responses and cellular function. Indeed, following incubation with these two pathogen mimics, I detected higher proportions of ROS producing cells in leukocytes obtained from chicken exposed to contaminated water (60% ROS producing cells) compared to 30% of ROS

producing cells in control group when stimulated with fungal mimic ($p=0.0009$).

Conversely, this up-regulatory effect was not observed when leukocytes were incubated with the bacterial trigger (40% of ROS producing cells, $p=0.294$) (Fig. 5.3A).

The next experiment was to evaluate whether this higher number of ROS producing cells were due to the higher numbers of KUL01 macrophages observed or if this antimicrobial effect was related to another cell type. Hence, I took advantage of our high-resolution cell by cell analysis through Imaging Flow cytometry to assess whether ROS producing cells were those macrophages increased in the abdominal cavity.

Interestingly, I observed that ROS producing levels in those KUL01 macrophages were similar in both raw water and control groups. Remarkably, higher proportions of ROS producing cells were KUL01- cells (30% of KUL01- cells, $p<0.05$). Again, this effect was only observed with the fungal trigger and no differential effect when stimulated with bacterial mimic (Fig. 5.3B). Altogether, these results reveal the combinatorial effect of multiple contaminants in drinking water on the homeostatic immunological state of chickens, detected by an increased leukocyte numbers in tissues (abdominal cavity) and their antimicrobial functions.

5.2.1.5. Raw drinking water induced changes in NO production during chicken acute inflammation

NO production is a cellular antimicrobial response triggered against extracellular and intracellular pathogens in immune cells (Coleman, 2001; Tripathi et al., 2007b). NO production is dependent on *iNOS* expression and it is produced as a by-product in the metabolism of L-arginine to L-citrulline (Tripathi et al., 2007b). Here, the goal was to

evaluate whether the presence of multiple contaminants in drinking water modulates the dynamics of NO production during acute inflammation in chickens. In the fungal model, levels of NO production in heterophils were downregulated at 12 h post challenge in animals exposed to raw water (50% in tap water, 25% in raw water; $p=0.032$), while it remained higher in our control group. In contrast, raw water did not induce this downregulatory effect in the bacterial challenge model. As we expected, variations in the patterns of NO production during acute inflammation against are related to different fungal and bacterial challenge models (Fig. 5.3C).

5.2.1.6. Raw water induces changes in the expression of inflammatory mediators during acute inflammation

Short-term exposure to raw water induced changes in leukocyte dynamics and antimicrobial responses during acute inflammation. I wanted to address whether this altered functional performance was complemented with cytokine gene expression profile during the inflammatory process using a qPCR platform. Indeed, raw water induced an upregulation of interleukin-8 (*IL-8*) expression when challenged with both fungal and bacterial triggers, at 4 h post challenge compared to control group (300-fold in the fungal models, $p<0.0001$; 350-fold in the bacterial model, $p=0.0227$).

Additionally, raw water also altered the dynamics of *iNOS* expression during acute inflammation in the fungal model. Thus, maximum *iNOS* expression levels were found to be at 4 h post fungal challenge in exposed animals (100-fold, $p<0.0001$), however, *iNOS* expression reached its maximum levels at 12 h in the control group (100-fold, $p<0.001$). Furthermore, raw water also induced upregulation of *IL-1 β* (110-fold,

$p=0.0013$), *TGF- β* (3.5 fold, $p<0.0001$) and *TNF- α* (90-fold, $p<0.0001$) at 4 h post fungal challenge compared to tap water control group. No effect on *IL-2* expression indicates water contaminants do not alter the lymphocyte development, following fungal stimulation. However, other molecular markers are needed to be analysed.

On the other hand, raw water treatment downregulated *IL-2* expression at 4 h post bacterial challenge (10-fold, $p<0.0394$), however, raw water did not induce an effect on *IL-1 β* , *iNOS*, *TGF- β* and *TNF- α* expression in this model. Altogether, these results reveal that short-term exposure induced molecular changes in cytokine expression, differentially regulated against bacterial and fungal models (Fig. 5.4).

5.2.1.7. Short-term exposure to raw water induces changes in the chicken early adaptive immune responses

So far, these results provide that raw water exerts cellular and molecular alterations of antimicrobial responses during acute inflammation. Since innate and adaptive immune responses are tightly linked, it is reasonable to consider that those effects might induce changes in the adaptive immunity. I observed changes in the populations of abdominal resident macrophages but also in their functional activity. Additionally, I found significant changes in the leukocyte dynamics, mainly in the heterophils and monocyte/macrophages later in the inflammatory process under bacterial challenge. Hence, I wanted to address whether these altered patterns in the acute inflammatory responses influenced the development of acquired immunity. For this purpose, I harvested peripheral leukocytes 2 weeks following challenge from animals previously exposed to water treatment. I evaluated the phagocytic capacity of peripheral blood

mononuclear cells (PBMC) to internalize GFP-ST and their capacity to produce ROS as representative antimicrobial mechanisms in chicken monocytes. Additionally, I evaluated the proportions of CD4⁺ and CD8⁺ T lymphocytes in peripheral blood. Overall, although total PBMC proportions, total KUL01⁺ cells, total ROS production and total phagocytic capacity were not affected by water treatment (Fig. 5.5A and B); raw water treatment altered peripheral macrophage (KUL01⁺) antimicrobial responses where they had increased phagocytic capacity (30% raw water, 5% tap water; p=0.0054) but lower ROS production (60% raw water, 90% tap water; p=0.0315) (Fig. 5.5C). On the other hand, CD4⁺/CD8⁺ T lymphocytes ratio were found to be significantly lower in animals exposed to raw water compared to our control group (2:1 raw water, 4:1 tap water; p=0.047; Fig. 5.6). These results provide new insights in the long-term effect of acute exposure to xenobiotics in water. Hence, I observed that this effect on short-term immunity impairs the antimicrobial capacity of monocyte/macrophages (KUL01⁺ cells) later when the inflammatory process returned to homeostasis. Moreover, this evidence that water contaminants impairs the development of adaptive immunity, mainly in the balance of CD4⁺/CD8⁺ T lymphocytes. Further analysis needs to be done to establish whether this effect on CD4⁺/CD8⁺ T lymphocytes modifies patterns of B lymphocytes and humoral responses such as *S. enterica* ser. Typhimurium-specific antibodies levels.

5.2.2. The swine model

5.2.2.1. Raw and spiked water

Since raw water selected for swine experiments had different physicochemical characteristics than those used in chicken experiments, I expected a differential effect in

swine acute inflammation. Additionally, I wanted to address the effects of water contaminants of major relevance for human consumption, commonly found nearby extractive industries, using a close comparative model of human immunity. Thus, I included a third spiked water group, that used raw water in combination with some chemicals of importance in environmental pollution. Raw water was obtained from an area of intense industry activity in the Northern Alberta (NW-60-01-01) known for its high presence of heavy metals. The source of water was selected based on the perceived concern of Alberta producers of the presence of water contaminants and their influence on animal health on farm. Once selected, physicochemical assessment was completed by Maxxam Analytics Inc. (CALA certified and ISO 17025). After analysis, I found high levels of sulphates (1500mg/L), total dissolved solids (TDS, 3000mg/L), chloride (260mg/L), iron (2.6mg/L), manganese (0.18mg/L) and sodium (680mg/L). For spiked water, I added some relevant chemicals widely detected in areas of oil and gas production, such as gasoline and sulfolane to raw water. Hence, I found ethylbenzene (7.3µg/L), styrene (0.56µg/L), toluene (33µg/L), 1,2,4-trimethylbenzene (13µg/L), 1,3,5-trimethylbenzene (3.5µg/L) and xylenes (43µg/L). This approach looked for the combinatorial effect of these xenobiotics even when individually, they fall under permissible values.

5.2.2.2. Water contaminants modulated leukocyte activation in basal resident swine leukocytes

Following a 7-day period of exposure to raw, spiked and tap (control) water, pigs were euthanized and cellular components from peritoneal cavity were harvested through

peritoneal lavage. The objective was to evaluate whether these water treatments altered basal peritoneal leukocyte numbers, and whether any of leukocyte subpopulations were depleted/increased, as representative measurement of immune impairment. In addition, alterations in their activation state and antimicrobial responses were measured. Based on my physicochemical assessment, I hypothesized that spiked water will display a stronger effect on immune parameters compared to raw water and our control group, due to higher presence of chemicals associated to immune compromise following exposure. Unexpectedly, no water treatment induced significant changes in total numbers of resident leukocytes, however, I observed alterations in their activation state. Through ROS measurement, levels of ROS producing cells in tap water group were around 35% of total resident leukocytes. Likewise, ROS producing cells were slightly increased in raw water group with around 40% of total resident cells. However, proportions of ROS producing cells were depleted to 15% of total peritoneal leukocytes in the spiked water group ($p=0.0111$). Furthermore, our analysis allowed a deeper determination that this downregulation effect was due to a reduction of ROS production in the macrophage pool (15% of ROS producing cells in spiked water, $p=0.0002$). Hence, these results revealed that water contaminants present in drinking water did not induce changes in resident leukocyte numbers following acute exposure, however, it modulated the capacity of ROS production in resident peritoneal macrophages. These results also suggest that this modulatory effect is associated with the levels and type of elements present in the water tested, as I did not observe major changes in the raw water group. As we observed a downregulatory effect in basal levels of ROS production *in vivo*, I wanted to address whether this depleted ROS production capacity could be restored

following an *ex vivo* stimulation with *S. enterica* ser. Typhimurium. Hence, peritoneal leukocytes were incubated for 30 min with GFP-ST to evaluate their capacity for bacterial internalization and ROS production. Results revealed that ROS producing cells reached 75 and 65% of total resident cells in tap and raw water control groups, respectively. Interestingly, proportions of ROS producing cells increased from 15% to almost 80% following *ex vivo* incubation in the spiked water group ($p=0.049$) (Fig. 5.7). These results revealed that even though water treatment modulated leukocyte capacity to produce ROS production, leukocytes did not lose this capacity and can be further stimulated following pathogen activation.

5.2.2.3. Water contaminants upregulated basal NF- κ B nuclear translocation in swine resident leukocytes

Nuclear factor kappa-B (NF- κ B) is a hallmark protein complex for cellular activation in a vast array of cellular types including the immune system. In unstimulated cells, NF- κ B is arrested in the cytoplasm compartment due to I κ B complex activity. Following cellular activation and enzymatic activity that degrades the I κ B complex, NF- κ B subunits are aggregated and further transported to the nucleus where they bind DNA promoter regions promoting gene expression. Hence, several tests are used to evaluate the rate of NF- κ B mobilization to the nucleus, or NF- κ B nuclear translocation, following cellular activation. Here, it was evaluated whether water treatments modulate the basal levels of NF- κ B nuclear translocation in resident peritoneal leukocytes. As mentioned above, resident leukocytes were harvested after water exposure and processed using an anti-NF- κ B antibody and the rate of NF- κ B nuclear translocation

was evaluated via an imaging flow cytometry platform. Interestingly, basal NF- κ B nuclear translocation was around 10% in both tap and raw water experimental groups. However, values of NF- κ B nuclear translocation reached almost 40% in animals exposed to spiked water ($p < 0.0001$). As observed in ROS production levels, this total NF- κ B nuclear translocation effect was associated with an upregulation in macrophage NF- κ B nuclear translocation (40% in spiked water, $p < 0.0001$) (Fig. 5.8). These results suggest that water treatment induces a higher cellular gene activation through NF- κ B signalling pathways and therefore will induce changes in response to pathogen stimulation.

Altogether, ROS production and NF- κ B nuclear translocation revealed that water treatments modulate basal levels of antimicrobial responses and cellular activation, and these effects are associated with the presence, concentration and type of elements present in the water treatment, suggesting a combinatorial effect on basal immune parameters.

5.2.2.4. Water contaminants altered leukocyte dynamics following *in vivo* *S. enterica* ser. Typhimurium challenge

Detection of changes in basal antimicrobial responses and cellular activation following exposure to distinct water sources, led to address whether these changes had modified patterns of immune defences (leukocyte dynamics and antimicrobial function) during a self-resolving inflammatory process induced by an *in vivo* *S. enterica* ser. Typhimurium stimulation. First, the leukocyte dynamics during this induced inflammatory process was analysed. Although initial stages of acute inflammation were not affected by water

treatments, I did observe significant alterations later on in the inflammatory process. Hence, neutrophil proportions increased rapidly at 4 h, remained higher at 12 h and decreased 48 h post *S. enterica* ser. Typhimurium challenge in both tap and raw water groups. However, despite slight decrease in neutrophil proportions at 48 h post challenge, these values were significantly higher in the spiked water group compared to tap group for the same time period (55% in spiked water, 30% tap water; $p=0.0001$). Meanwhile, proportions of monocyte/macrophages (15% in spiked water, 25% tap water; $p=0.0041$) and lymphocytes (25% in spiked water, 40% tap water, $p<0.01$) remained lower compared to tap water control group. Interestingly, spiked water also induced a decrease in macrophage numbers at 12 h post *S. enterica* ser. Typhimurium challenge compared to tap water group (10% in spiked water, 20% tap water; $p=0.0142$). Thus, these results revealed that spiked water induced a down-regulation of macrophage development following *S. enterica* ser. Typhimurium challenge and seemed to be associated with the higher proportions of neutrophil present later on in the inflammatory process (Fig. 5.9). Altogether, these results showed that water contaminants slowed down the return to homeostatic stages and further pathogen control during the resolution phase of acute inflammation.

5.2.2.5. Water treatment did not alter ROS production during *S. enterica* ser.

Typhimurium *in vivo* challenge

I observed alteration in basal levels of ROS producing cells (homeostatic levels) following exposure to spiked water compared to raw and tap water groups. I evaluated whether these changes induced significant changes in the ROS production levels

following an *in vivo* IP challenge with *S. enterica* ser. Typhimurium. In addition to leukocyte dynamics, I also measured the proportions of total ROS production, as well as the levels of ROS production for neutrophils, monocyte/macrophages and lymphocytes at 4, 12 and 48 h post *S. enterica* ser. Typhimurium challenge. As expected, I observed rapid neutrophil ROS production at 4 h, followed by a gradual decrease of ROS production 12 and 48 h post challenge. Conversely, low proportions of ROS producing macrophages were obtained at 4, 12 and 48 h, mainly associated with low macrophage counts at those time points. Likewise, the lymphocyte pool showed minimum ROS production levels at those time points. Nevertheless, raw and spiked water treatments did not induce changes in levels nor in dynamics of these proportions of ROS compared to tap control group (Fig. 5.10). These results revealed that even though water treatment, specifically spiked water induces changes in basal levels of ROS production; these levels are not affected following *in vivo* stimulation. Additionally, these results suggest that ROS downregulation in basal levels might have implications in other alternative pathways of cellular activation rather than oxidative antimicrobial responses.

5.2.2.6. Spiked water altered leukocyte activation through NF- κ B nuclear translocation following *in vivo* *S. enterica* ser. Typhimurium challenge

Changes in basal levels of cellular activation through NF- κ B nuclear translocation were observed followed exposure to spiked water but no effect was observed on tap and raw water groups. Hence, we wanted to address whether these alterations in cellular activation regulate NF- κ B nuclear translocation further an *in vivo* *S. enterica* ser. Typhimurium challenge. This leukocyte activation was measured at the same time

points that ROS production and leukocyte dynamics (0, 4, 12 and 48 h post challenge). Interestingly, despite higher proportions of NF- κ B nuclear translocation in basal levels induced by spiked water, following *S. enterica* ser. Typhimurium challenge, I observed lower levels at 4, 12 and 48 h. However, only levels at 12 h post challenge was significantly lower compared to control group (15% spiked water, 35% tap water; $p=0.0015$). Furthermore, these downregulatory levels correlated with lower values in neutrophils (10% spiked water, 30% tap water; $p=0.0036$), monocyte/macrophages (20% spiked water, 50% tap water; $p<0.0001$) and lymphocyte (5% spiked water, 20% tap water; $p=0.0096$) pools at the same time point. In addition, NF- κ B nuclear translocation levels in the neutrophil pool was decreased 4 h post challenge in the spiked water group compared to tap water (10% spiked water, 30% tap water; $p=0.0178$). Additionally, lymphocyte NF- κ B nuclear translocation tended to increase at 48 h post *S. enterica* ser. Typhimurium challenge, however, these proportions were significantly lower in the spiked group (10% spiked water, 30% tap water; $p=0.0056$). Interestingly, macrophage NF- κ B nuclear translocation levels were also decreased in the raw water group at 12 h mimicking the effect of spiked water at the same time point (30% spiked water, 50% tap water; $p=0.0013$). Altogether, these results revealed that spiked water downregulated the dynamics of total NF- κ B nuclear translocation following *S. enterica* ser. Typhimurium challenge, suggesting a shorter dynamic of cellular activation at 12 h compared to control where this decrease was observed at 48 h (Fig. 5.11). These results seemed to be linked mainly to neutrophil downregulation during acute inflammation against *S. enterica* ser. Typhimurium challenge; and monocyte/macrophages and lymphocytes later on in the inflammatory response. This downregulatory effect might

induce alterations in the antigen presentation for the development of adaptive responses associated to lymphocyte types. Similarly, raw water also induced this downregulatory effect only in the macrophage pool, which altered the normal dynamics of NF- κ B nuclear translocation in macrophages that play a role in homeostatic responses and might have implications for further development of immune responses.

5.2.2.7. Raw water induced changes in early development of long-term immunity.

As I observed multiple effects of raw and spiked water on the acute inflammation against *S. enterica* ser. Typhimurium challenge, I was interested in looking at alterations in the development of long-term immunity. In this experimental design I focused on raw water effects due to: 1) effects in macrophage activation through NF- κ B nuclear translocation, since these leukocytes are involved in pro-resolving states of acute inflammation; and 2) its relevance for swine production industry, as small farms commonly use this water source. To this purpose, following 7-days water exposure, pigs were IP *S. enterica* ser. Typhimurium challenged and blood samples were taken 2 and 4 weeks after. After blood collection, peripheral blood mononuclear cells (PBMC) were isolated and monocyte functionality and T lymphocyte determination was evaluated. Interestingly, proportions of total PBMC number progressively increased 2 and 4 weeks after challenge, with significant higher values at 4 weeks in tap control group ($15 \times 10^6/\text{ml}$ at 4 weeks, $p=0.0302$; $6 \times 10^6/\text{ml}$ in control: $p=0.0265$). Conversely, total PBMC numbers despite their sustained increase, values were not significantly higher at 2 nor 4 weeks. Additionally, no effect on IgM leukocyte dynamics was observed, as

well as no effect observed in the CD4⁺ and CD8⁺ T lymphocytes at the same time points. Conversely, levels of CD4⁺ CD8⁺ lymphocytes, a unique swine lymphocyte population, significantly increased at 4 weeks (15% at 4 weeks, p=0.0013; 5% control: p=0.0017); this effect was not observed in tap control group (Fig. 5.12). Altogether, these results revealed that acute exposure to water contaminants induces detectable changes in the long-term immune responses, associated to T regulatory/memory responses.

5.3. Discussion

In recent years, there has been an expansion of oil and gas industrial activities worldwide. These intensive activities have increased the uncontrolled release of multiple by-products and contaminants to the environment. As such, these contaminants accumulate into aquatic environments where they exert detrimental effects on resident organisms. Species affected are not restricted to aquatic animals but it has also implications for terrestrial species such as livestock and humans through drinking water. Within the multiple effects described are: effects on growth performance, developmental disorders and gastrointestinal problems, among others, including immunological effects. Most immunological effects are related to long-term pollutant exposure, therefore, these become detectable later in life. Furthermore, these studies also assess effects under single-contaminant approaches. Even more, some those toxicological effects are observed in trials designed using xenobiotic concentrations higher than maximum acceptable levels. Although these studies have provided valuable information of common pollutants effects on animal and human health, they do not reflect what happens in nature. Thus, this leaves important gaps that need to be addressed. First, lack

of information on the implications on the early immune responses when these animals encounter those contaminants. Second, due to multiple factors, these contaminants might be encountered in levels below maximum acceptable but in mixture of multiple contaminants that might have additive or synergistic effects. Hence, my objective here was to address to what extent short-term exposure to acceptable levels of multiple xenobiotics/contaminants in drinking water promoted changes in the early immune responses against pathogens. I took advantage of the peritoneal/abdominal challenge model (Chapters III and IV), as it allows the assessment of multiple facets of acute inflammation and the early development of long-term immune responses. Thus, this offers an integrative view of immune responses under inflammatory conditions while providing a general view of animal capacity to battle infections following water contaminant exposure. Hence, homeostatic levels of immunity were evaluated following water contaminants exposure.

Regarding the chicken experiments, higher proportions of monocyte/macrophages in the abdominal cavity following exposure to raw water, suggest activation of the systemic immune response stimulating leukocyte migration to multiple tissues. Alternatively, this might suggest that water contaminants increased the self-proliferation rate of monocyte/macrophage lineage in the chicken abdominal cavity. Hence, these findings reveal the implications of water contaminants in the overall chicken immunological status. Additionally, this also altered their cellular functional activity against different bacterial and fungal pathogens in terms of ROS production. ROS production can be used as a marker of these antimicrobial responses since 1) it is fast-triggered following cellular activation, 2) it is robustly produced into intracellular compartments, and 3) it is

downregulated following pathogen control. Effects of raw water on ROS production against fungal but absent towards bacterial agents suggest that these water contaminant effects also rely on other factors such as pathogenic control. As these factors can be difficult to analyse through *ex vivo* assays, I took advantage of the characterized acute inflammation to perform *in vivo* abdominal challenges following water contaminant exposure. Within the dynamic of inflammation, I selected key time points that I considered of relevance during the acute inflammation process. Hence, 4 h post-challenge was considered the earliest time point when I was able to detect significant numbers of migrating leukocytes while they are displaying strong antimicrobial responses. Twelve hours post challenge, which defines the time point where the maximum cellular infiltration was observed along with maximum antimicrobial responses. Finally, 48 h post challenge that define the phase of inflammatory resolution. Lack of effects on the total leukocyte trafficking following challenge but alterations in the leukocyte proportions at late stages of inflammation (48 h) revealed that water contaminants prolonged inflammation, affecting the events of resolution. Altogether, these results suggest that water contaminants do not affect the overall dynamics of leukocyte migration following challenge but display effects on the mechanisms of resolving inflammation. Interestingly, these findings are linked to an upregulated *IL-8* expression. *IL-8* is the most important cytokine for leukocyte trafficking into inflamed tissues (Kunkel et al., 1991; Mukaida, 2003). Altogether, these results suggest that water contaminants modulate the pro-inflammatory cytokine profile, and this is responsible for alterations in leukocyte infiltration during inflammation. Furthermore, my interest went beyond the cellular dynamics and evaluated their functional activity. Thus, nitric oxide

(NO) production was used as marker to evaluate the effects of water contaminants on immune parameters. Since NO production is linked to molecular/enzymatic control and gradually increase under inflammation conditions, it represents a powerful biomarker of inflammatory progress at the cellular and molecular level. Downregulation of *iNOS* prior to NO production decrease shows that water contaminants affect dynamically and functionally, even at the molecular level of the inflammatory process under fungal stimulation. These results showed the effects of water contaminants promote differential alterations in immune responses. While, under fungal conditions, these effects are displayed shortly after challenge (nitric oxide); under bacterial conditions, these effects are observed at the anti-inflammatory stages (heterophil levels). Further studies are needed to define which factors are involved into these differential effects under bacterial or fungal stimulation.

The prolonged inflammatory process observed under water contaminants exposure appears to be associated with alterations in the long-term immunity. These alterations appear to be linked to antimicrobial responses in peripheral leukocytes revealing that these effects are not restricted locally (abdominal) but systemically. Moreover, water contaminants effects are not limited to monocytic lineage but also occur in lymphocyte. These findings suggest that these alterations affected the development of memory responses and/or regulatory function by lymphocytes. Altogether, these results revealed that water contaminants even at low concentrations induce long-lasting effects in chicken immune defenses. Altogether, this demonstrates the systemic effect in the innate immunity might impair the chickens capacity to battle infections during initial stages and promoting adaptive immune dysfunction in T lymphocytes populations. Moreover,

low levels of water contaminants induce long-term effects that are precisely detectable by cellular assays even in the absence of clinical symptomatology.

In the swine model, raw water had different physicochemical characteristics from the raw water used in the chicken experiments. Hence, I decided to include a third experimental group (spiked water). My rationale was: 1) to determine the effect of these chemicals on animal immunity since they are of relevance for environmental pollution and have implications in animal and public health, and 2) to evaluate their combinatorial immune effects under a short-term exposure trial in swine, a closely-related system to humans for comparative purposes. Homeostatic immunological levels following water exposure demonstrated that water contaminants in spiked water group lowered the basal levels of ROS production. As these levels of ROS production represent the intracellular signalling processes (due to lack of pathogenic trigger), this suggests that water contaminants play a detrimental role in the normal leukocyte function during homeostatic stages. Interestingly, increased levels of NF- κ B nuclear translocation support my prior findings that water contaminants alters the intracellular mechanistic process since NF- κ B participates as a transcriptional factor in multiple cellular pathways. To evaluate whether this has implications in antimicrobial defenses, I exposed those cells to Salmonella in an *ex vivo* assay. Upregulation of ROS production following Salmonella stimulation demonstrates that even when water contaminants diminished the homeostatic levels of ROS production, these cells still maintain their antimicrobial capacity. Hence, these findings evidenced that those resident cells had an unaffected antimicrobial capacity but diminished intracellular signalling that might undergo alterations in the interactions with other leukocyte types during inflammatory

conditions. Hence, I used the peritoneal challenge model, previously characterized (Chapter IV). As in my chicken experiments, 4, 12 and 48 h post Salmonella challenge were used as they represented similar key points during acute inflammation in swine. Similar to my observations in the avian model, water contaminants in (spiked water) promoted a prolonged inflammatory status compared to control. This demonstrates that water contaminants do not exert major changes in the leukocyte dynamics at the initial stages but affects the resolution phase of acute inflammation following Salmonella challenge.

As in the avian model, water contaminants appear to prolong the inflammatory process, due to maintenance of higher levels of PMN cells and lower levels of monocyte/macrophages and lymphocytes, as representatives of pro-resolving stages. These have multiple implications in both species, since this 1) represents an unnecessary continued physiological stress for these animals, 2) undergoes a misuse of energy intake, 3) affects the normal physical performance and development, and 4) implications to immunological responses to secondary infections. These have relevance in any living organism but gain major importance in livestock animals since those parameters affects farm productivity. These results also provide evidence that dynamics of leukocyte subpopulations can be useful in both species as biomarkers of immune alteration under multiple pathogens/toxicants. Nevertheless, characterization of normal acute inflammation under specific pathogens must be defined prior to use as a biomarker. Lack of alterations in ROS production following Salmonella challenge supports my findings that although water contaminants affect homeostatic levels of oxidative derivatives in leukocytes, they retain their ROS antimicrobial capacity against

pathogens. However, ROS production levels following water contaminants exposure reveals that has implications at the intracellular signalling pathways. Indeed, NF- κ B nuclear translocation was downregulated throughout the acute inflammation process. Downregulation of NF- κ B activation suggests that neutrophils might have compromised their cellular function to regulate the inflammation. NF- κ B activation in neutrophils is linked to apoptotic pathways and further release of soluble mediators play a role in this downregulation effect (Castro-Alcaraz et al., 2002; Freire and Van Dyke, 2013). Further research is needed to clarify whether this diminished neutrophil NF- κ B nuclear translocation is involved in regulation of neutrophil apoptosis in swine. Additionally, to determine which specific contaminant-derived molecules are involved in this NF- κ B effect. At the monocyte/macrophage and lymphocyte levels, this downregulated NF- κ B nuclear translocation effect seem to correlate with the slow appearance of these lineages later in the inflammatory process. Interestingly, NF- κ B activity is associated with cellular development and might be involved in the mononuclear (monocyte/macrophage and lymphocyte) development and function during acute inflammation and the transition to adaptive immune responses. Similarly, this downregulatory effect in NF- κ B was observed in the raw water group.

Similar to my chicken findings, water contaminants promoted long-lasting effects detectable by our approach. However, these findings encompass effects only in the lymphocyte populations. Interestingly, water contaminants seem to delay the gradual increase of peripheral mononuclear cells in the raw water group. Although I did not analyze the effects of spiked water, I believe that this would display similar or perhaps stronger effects on leukocyte patterns in circulation. Additionally, specific double

positive T lymphocytes are linked to the development of regulatory and memory responses in swine (Zuckermann and Gaskins, 1996; Zuckermann and Husmann, 1996). An increase of this population found in raw water animals, suggest alterations in the long-term immunity as they interact with other lymphocyte subsets to confer proper long-term immunity. This factor might have implications in the total peripheral leukocyte increment, but this was beyond my focus of interest. Hence, additional studies might elucidate their interactions with other cell types and their functional contributions to the development of these adaptive responses.

Altogether, this chapter provides novel insights in the effect of mixed water contaminants on the development of poultry and swine acute inflammation. Despite common effects induced even with different contaminant mixtures in poultry and swine immunity, differential responses were also noted. Moreover, these effects are observed even when single contaminants are present in low concentrations. Although, I observed multiple immunological effects at the cellular and molecular levels, further studies are required to assess whether these effects have implications in disease development.

Altogether, these findings offer a platform of immunoassays for assessment of immune defenses in livestock and potential use in human health.

Table 2. Relevant xenobiotics found in raw water for chicken experiments.

Parameter name	Units	Results	Max. acceptable concentration
Total dissolved solids	ppm	970	1000
Chloride	ppm	23	200
pH	pH	8.4	6.8-7.5
Alkalinity (CaCO ₃ , Bicarb)	ppm	570	300
Nitrates	ppm	<0.01	25
Sulphates	ppm	220	200
Iron	ppm	0.06	0.3
Calcium	ppm	6.6	60
Copper	ppm	0.0034	0.6
Magnesium	ppm	0.93	125
Manganese	ppm	0.015	0.05
Sodium	ppm	390	150
Zinc	ppm	0.0061	1.5
Total Penta CDD*	pg/L	1.54	2**- 2.3***pg/Kg/day
Octa CDD*	pg/L	5.7	2**- 2.3***pg/Kg/day
Faecal coliforms	CFU	0	0

* chloro-dibenzo-p-dioxin

** World health organization (WHO, 2002)

*** European Commission's Scientific Committee on Food (EC, 2001).

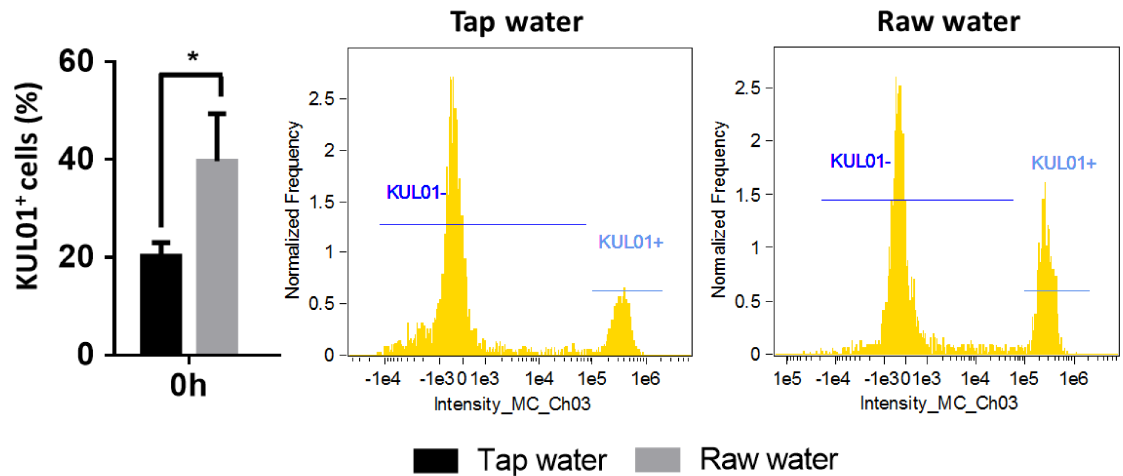


Fig. 5.1. Short-term exposure to raw water increases numbers of abdominal macrophages and increases their antimicrobial responses in chickens. Following raw water exposure, abdominal leukocytes were incubated with anti-PE-KUL01 antibody, to determine the proportions of monocyte/macrophage resident cells. Basal proportions of macrophages (KUL01+ cells) were significantly higher than the control group following exposure to raw water. Data represents mean \pm SEM (n=6). Differences in proportions of KUL01+ cells were subjected to one-way analysis of variance and Sidak's multiple comparison test. *p<0.05.

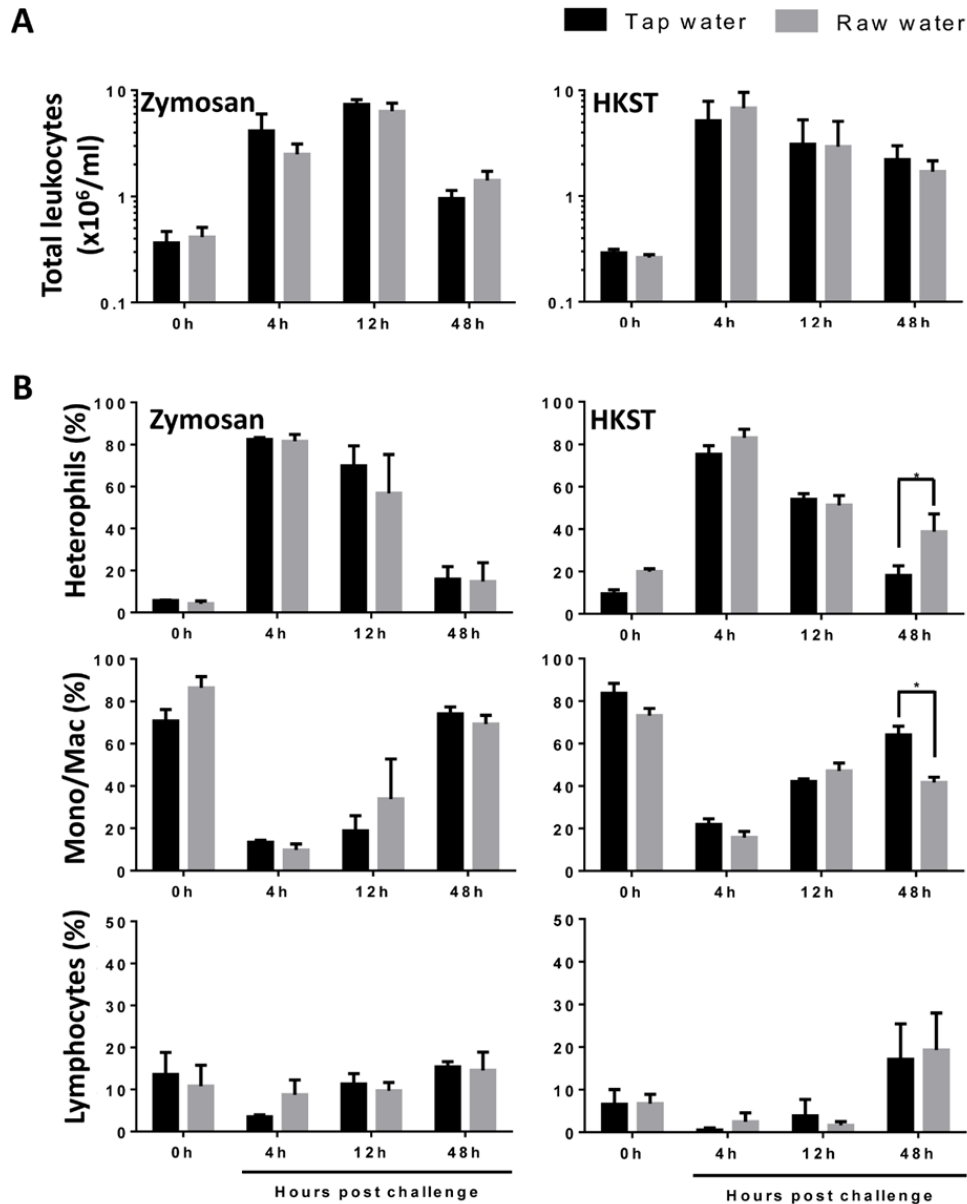


Fig. 5.2. Raw water differentially induces changes in leukocyte migration during acute inflammation. Following raw water treatment, intra-abdominal challenges were performed using fungal and bacterial mimics. Abdominal lavages were performed at 0, 4, 12 and 48 h post intra-abdominal challenge. Overall, I observed different leukocyte migration with those two different pathogens. However, there was no effect of raw water treatment on total values of leukocyte dynamics at any time point (A). Additionally, proportions of heterophils, monocyte/macrophages and lymphocytes were determined using Imaging flow cytometry along with conventional Wright Giemsa staining. No effect on leukocyte subpopulations were observed after fungal challenge, however, higher heterophil and lower monocyte/macrophage proportions were observed after 48 h of bacterial challenge (B). Data represents mean \pm SEM (n=7). Differences in leukocyte proportions were subjected to a two-way ANOVA and Sidak's multiple comparison test. *p<0.05.

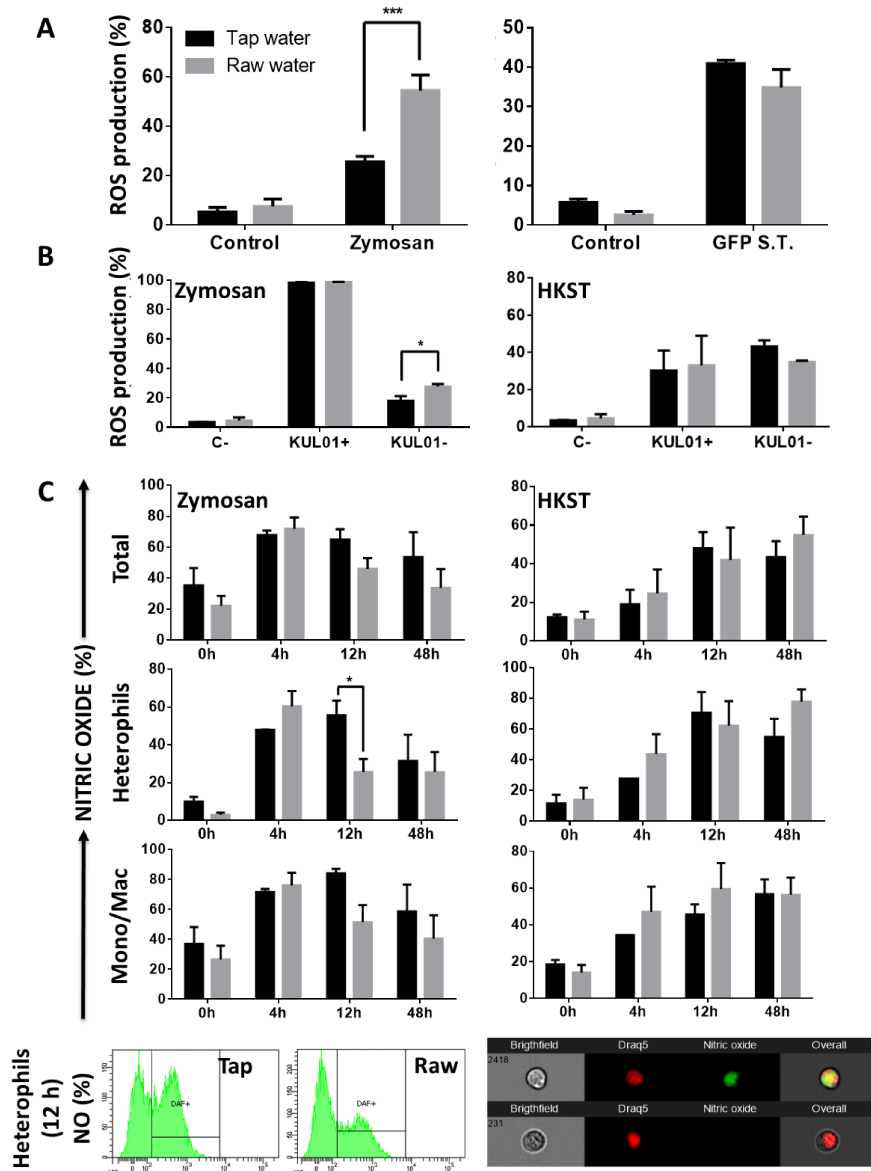


Fig. 5.3. Raw water treatment downregulates antimicrobial responses post fungal challenge. Abdominal leukocytes were *ex vivo* incubated with zymosan and GFP-ST for 30 min. Following incubation, proportions of ROS-producing cells were measured. Proportions of total ROS producing cells were upregulated when exposed to fungal but not to bacterial mimics (A). Abdominal leukocytes obtained from chickens exposed to raw water displayed higher ROS production in KUL01- cells, when exposed to fungal mimic but no effect was observed to bacterial trigger (B). Abdominal leukocytes were harvested at 0, 4, 12 and 48 h post challenge. NO producing cells were significantly lower at 12 h post fungal challenge, but no effect was observed with bacteria (C). Control are cells not exposed to neither fungal or bacterial challenges. Data represents mean \pm SEM (n=5). A two-way ANOVA and Sidak's multiple comparison test yielded that raw water treatment altered the ROS and NO production. *p<0.05, ***p<0.001.

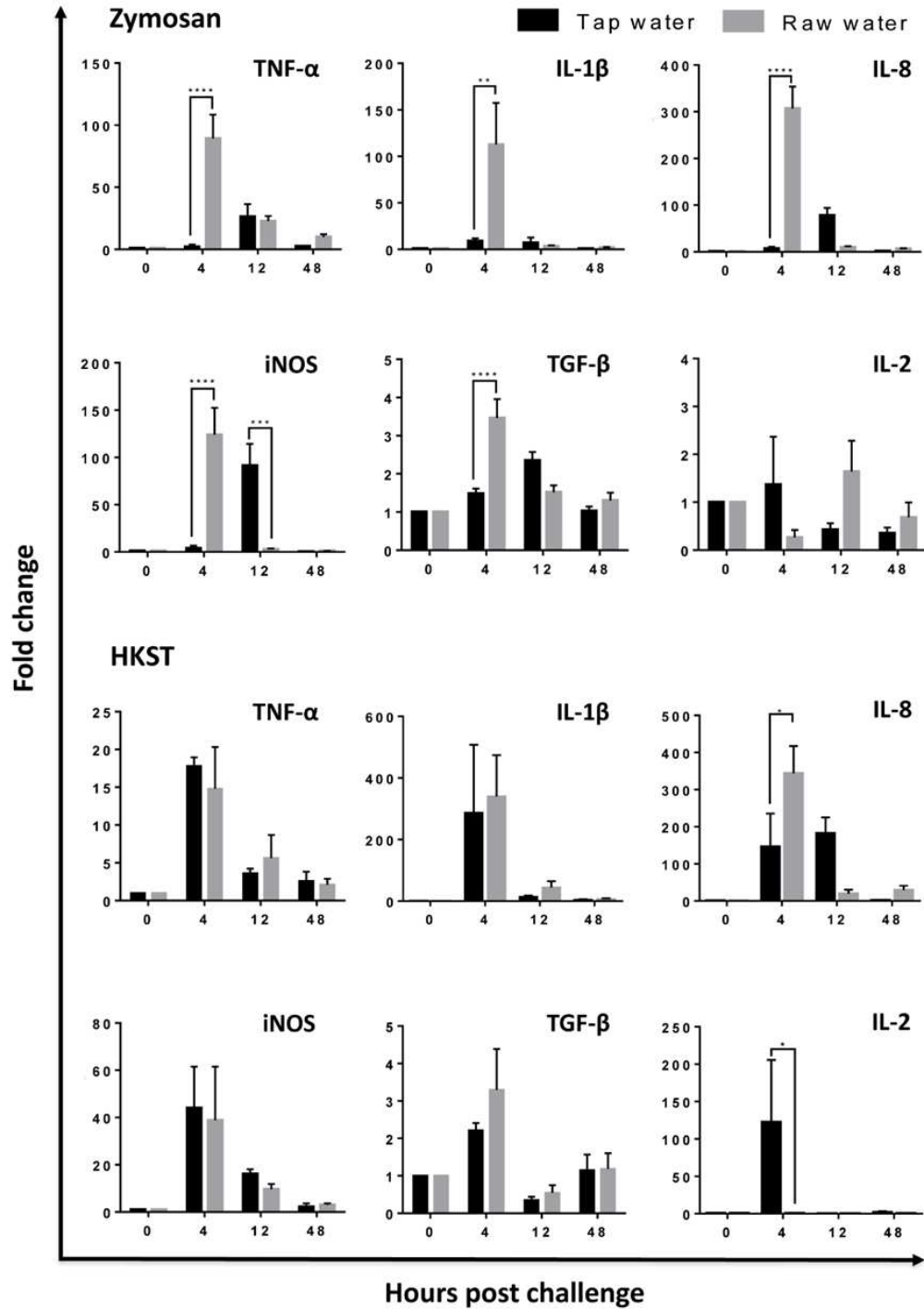


Fig. 5.4. Raw water treatment induces changes in proinflammatory mediators during acute inflammation. Following raw water treatment, chickens were intra-abdominal challenged with zymosan or HKST. Abdominal leukocytes were harvested at 0, 4, 12 and 48 h post challenge. Gene expression was analysed by qPCR. Data represents mean \pm SEM (n=4). Data represents mean \pm SEM (n=5). A two-way ANOVA and Sidak's multiple comparison test yielded that raw water treatment altered the ROS and NO production for each water treatment. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

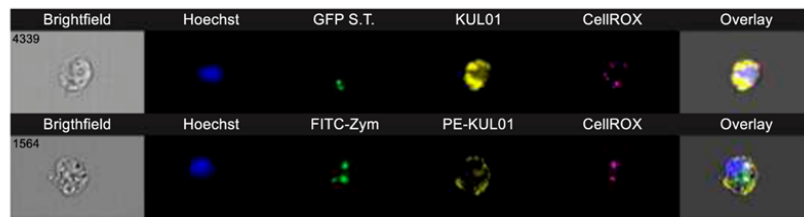
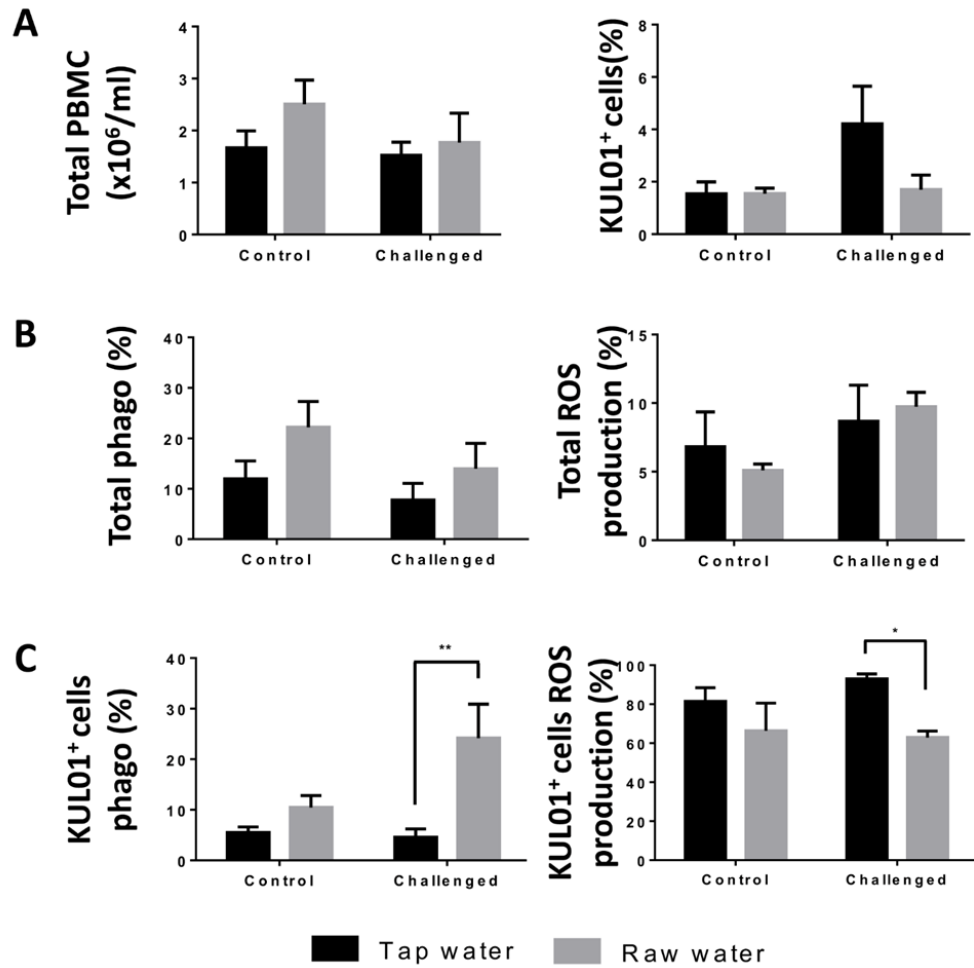


Fig. 5.5. Short-term exposure to raw water induces long-term effect on macrophage performance. Following raw water treatment, intra-abdominal challenge was performed using HKST. After 2 weeks of challenge, blood samples were taken to obtain PBMC. No effect in total PBMC and KUL01⁺ cells proportions were observed (A). Total PBMC were *ex vivo* incubated with GFP-ST and stained with anti-PE-KUL01 and CellROX to evaluate the capacity of KUL01⁺ cells to uptake GFP-ST and produce ROS. Even when total phagocytic capacity and ROS production were not affected by water treatment (B), KUL01⁺ cells obtained from chickens exposed to raw water displayed higher phagocytic capacity with a lower capacity to produce ROS. Data represents mean \pm SEM (n=5). Significant differences were analysed using two-way ANOVA and Sidak's multiple comparison test. *p<0.05, **p<0.01. Representative images are shown.

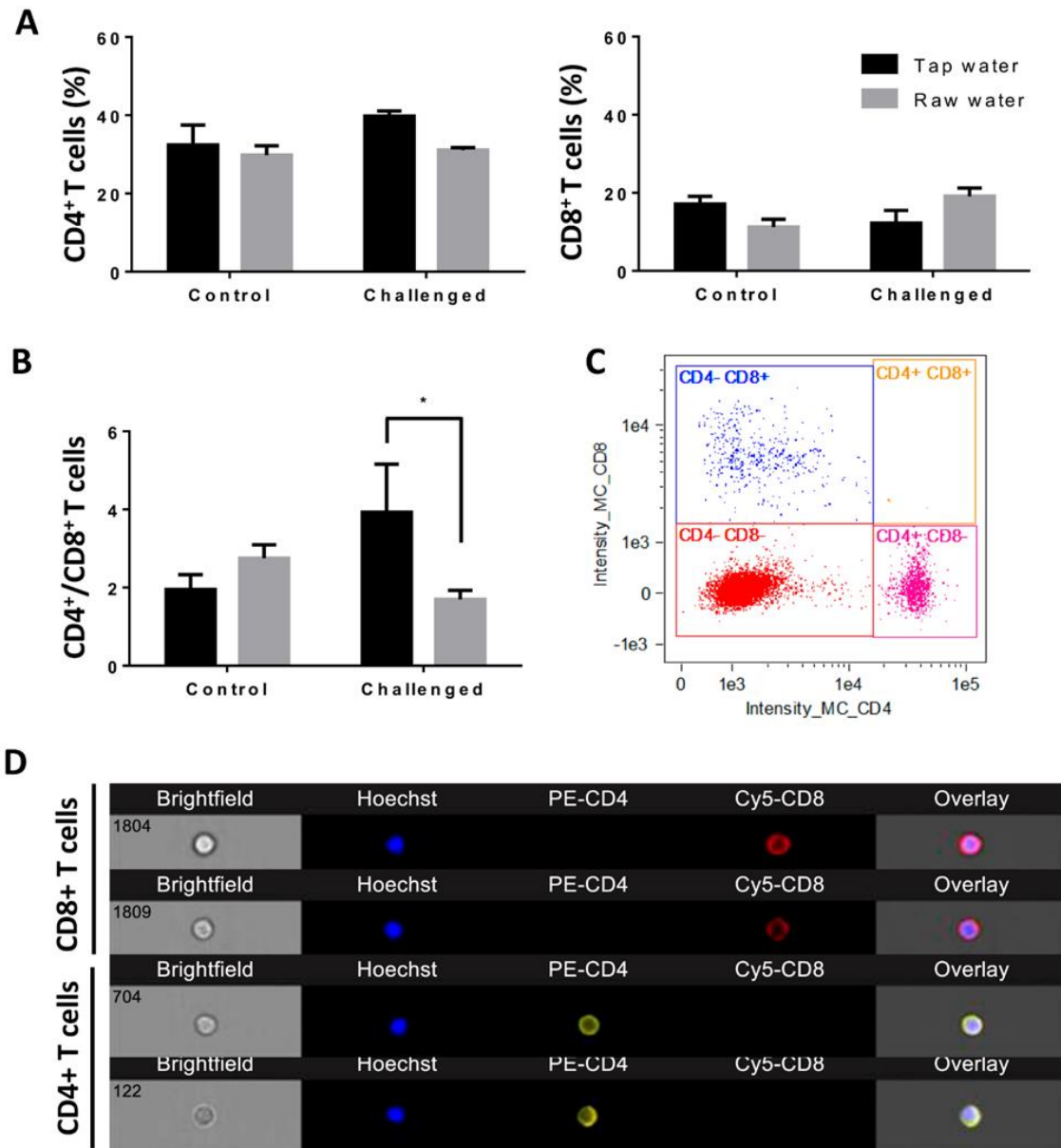


Fig. 5.6. Raw water treatment induces long-term effects in the early adaptive immune responses. Following raw water treatment, intra-abdominal challenge was performed using HKST. After 2 weeks of challenge, blood samples were taken to obtain PBMC through gradient centrifugation using histopaque. Leukocytes were stained with PE-anti-chicken CD4 and Cy5-anti-chicken CD8 to label CD4+ and CD8+ T lymphocytes, and Hoechst 33342 as nuclear staining. Proportions were analysed by imaging flow cytometry. Results revealed that no effect in CD4+ and CD8+ T lymphocyte proportions (A) but CD4+/CD8+ T lymphocyte ratio were lower in animals exposed to raw water. Data represents mean \pm SEM (n=6). Significant differences were analysed using two-way ANOVA and Sidak's multiple comparison test. *p<0.05.

Table 3. Relevant xenobiotics found in raw and spiked water for pig experiments.

Parameter name	Unit	Raw well water		Spiked water	
		Results	Max. Acceptable concentration	Results	Max. Acceptable concentration
Total dissolved solids	ppm	3000	3000	3000	3000
Chloride	mg/L	260	250	260	250
pH	pH	7.85	6.5-8	7.85	6.5-8
Sulphates	ppm	1500	1000	1500	1000
Iron	mg/L	2.6	0.3	2.6	0.3
Calcium	mg/L	230	1000	230	1000
Copper	mg/L	0.0004	0.6	0.0004	0.6
Magnesium	mg/L	78	400	78	400
Manganese	mg/L	0.18	0.05	0.18	0.05
Sodium	mg/L	680	200	680	200
Zinc	mg/L	0.041	25	0.041	25
Faecal coliforms	CFU	0	0	0	0
Ethylbenzene	ug/L	ND	140	7.3	140
Styrene	ug/L	ND	-	0.56	-
Toluene	ug/L	ND	60	33	60
1,2,4-trimethylbenzene	ug/L	ND	-	13	-
1,3,5-trimethylbenzene	ug/L	ND	-	3.5	-
Xylenes (Total)	ug/L	ND	90	43	90

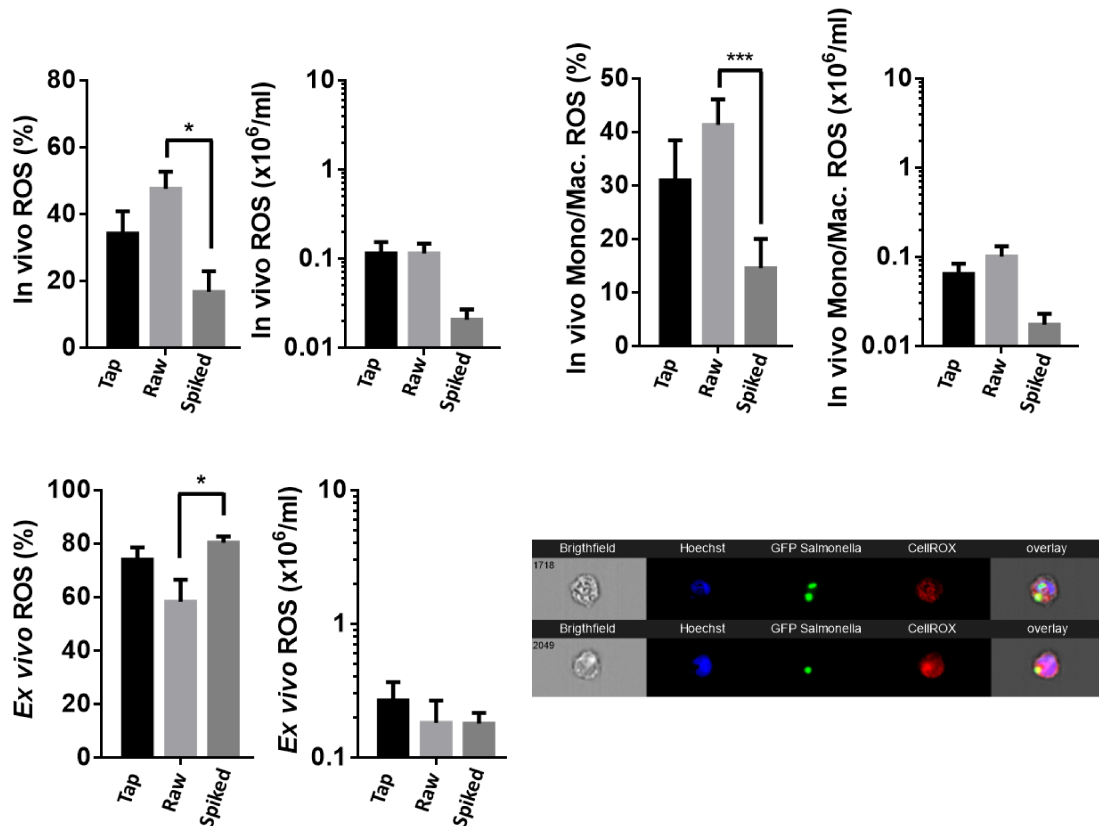


Fig. 5.7. Water contaminants decrease the basal levels of activation state in porcine peritoneal leukocytes, but it is restored following *S. enterica* ser. Typhimurium.

Following short-term exposure (7 days) to different water contaminants sources, I harvested peritoneal leukocytes and measured the basal capacity to produce ROS and after GFP-ST exposure *ex vivo*. Total proportions of ROS producing cells, specifically monocyte/macrophages were decreased in the spiked group. Interestingly, ROS producing cells were restored following exposure to GFP-ST. Although slight decreased in the absolute number of ROS producing cells, these values were not significant.

* $p < 0.05$, *** $p < 0.0001$, $n = 7$.

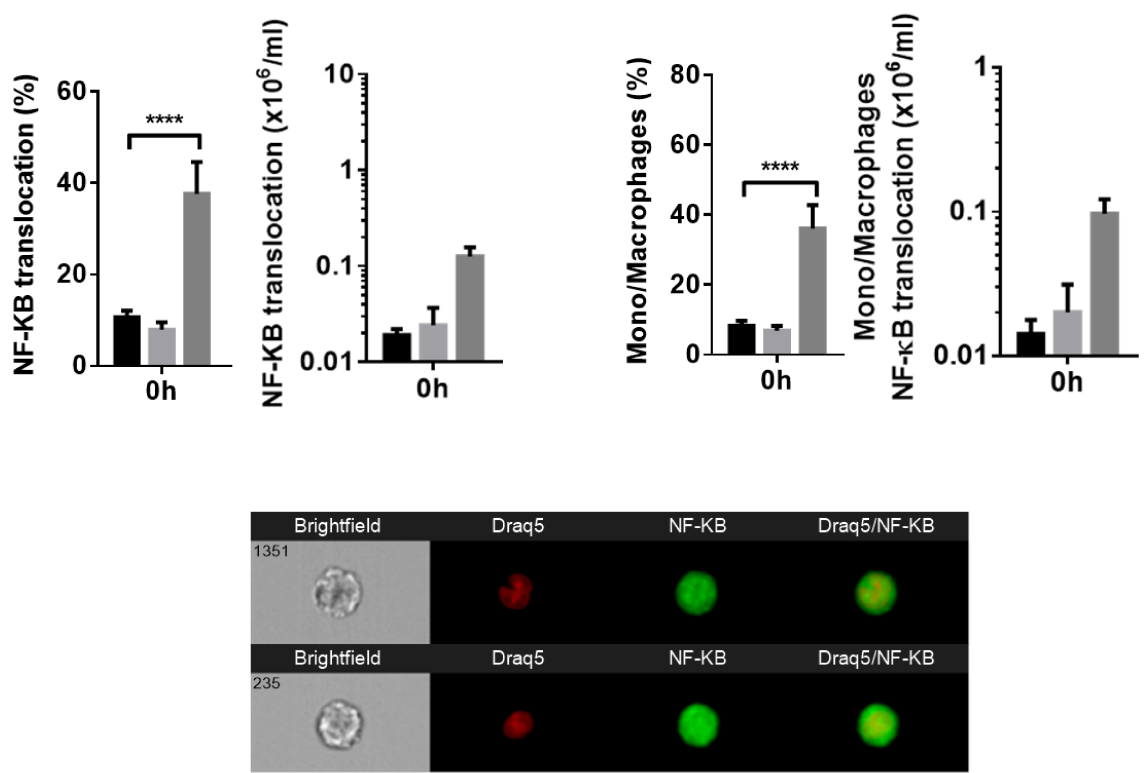


Fig. 5.8. Water contaminants increases basal levels of NF-κB nuclear translocation in porcine peritoneal leukocytes. Following short-term exposure (7 days) to different water contaminants sources, peritoneal leukocytes were harvested and NF-κB nuclear translocation to assess their basal levels. Total proportions of NF-κB nuclear translocation were up-regulated in the spiked group, specifically in the monocyte/macrophage group. Although slightly higher proportions in NFκB nuclear translocation was detected in the absolute number of ROS producing cells, these values were not significant. **** p<0.00001, n=7.

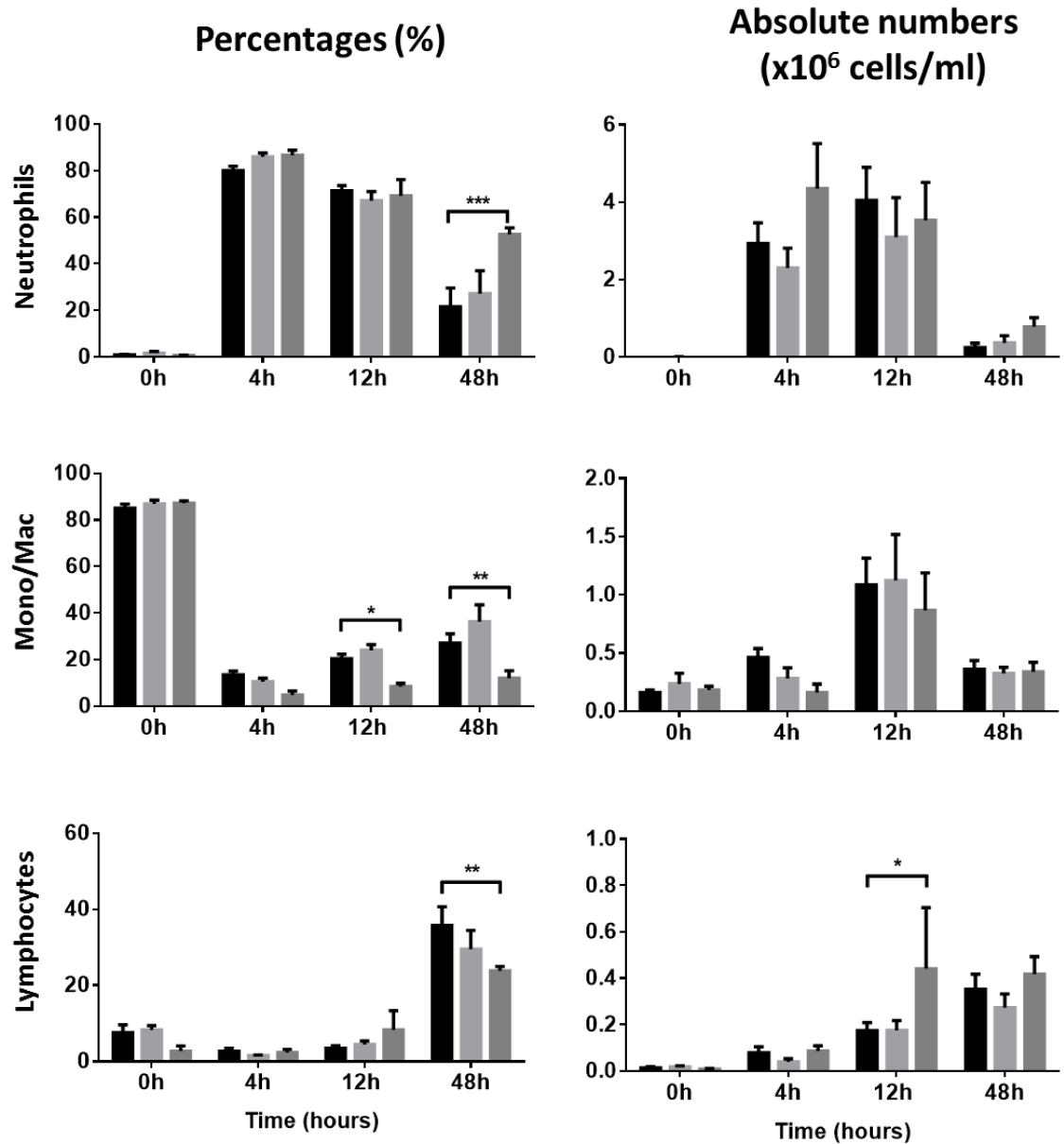


Fig. 5.9. Water contaminants alter the dynamics proportion of porcine peritoneal leukocytes during acute inflammation against HKST. Following short-term exposure (7 days) to different water contaminants sources, pigs were intraperitoneally challenged with HKST to evaluate the dynamics of peritoneal leukocytes during acute inflammation. Although initial stages of leukocyte migration were not affected by water treatments, proportions of neutrophils remained higher at 48 h post challenge. Similarly, proportions of monocyte/macrophages and lymphocytes remained low at this time point. Monocyte/macrophage downregulation was also observed as early as 12 h post challenge. Interestingly, no major changes were observed in the absolute leukocyte numbers at the same time but increased of lymphocyte proportions at 12 h in the spiked group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 7$.

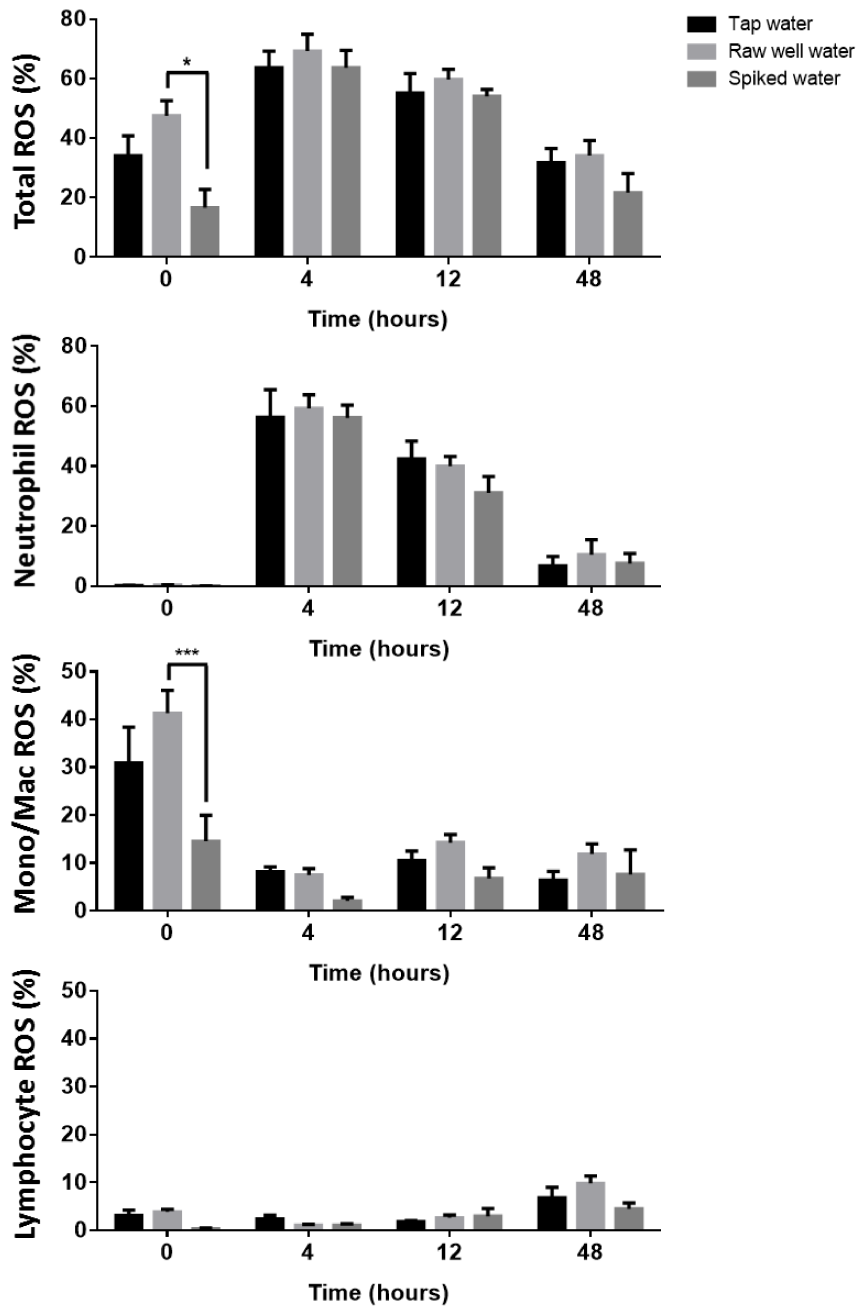


Fig. 5.10. Water contaminants do not induce changes in ROS production levels during acute inflammation against HKST. Following short-term exposure (7 days), pigs were intraperitoneally challenge and cellular components were harvested. ROS producing cells were measured using CellROX dye. Maximum levels of ROS producing cells were detected at 4 h post challenge and progressive decrease at 12 and 48 h. Although slight decrease proportions of ROS producing cells were observed in the monocyte/macrophage group, there was not significant difference.

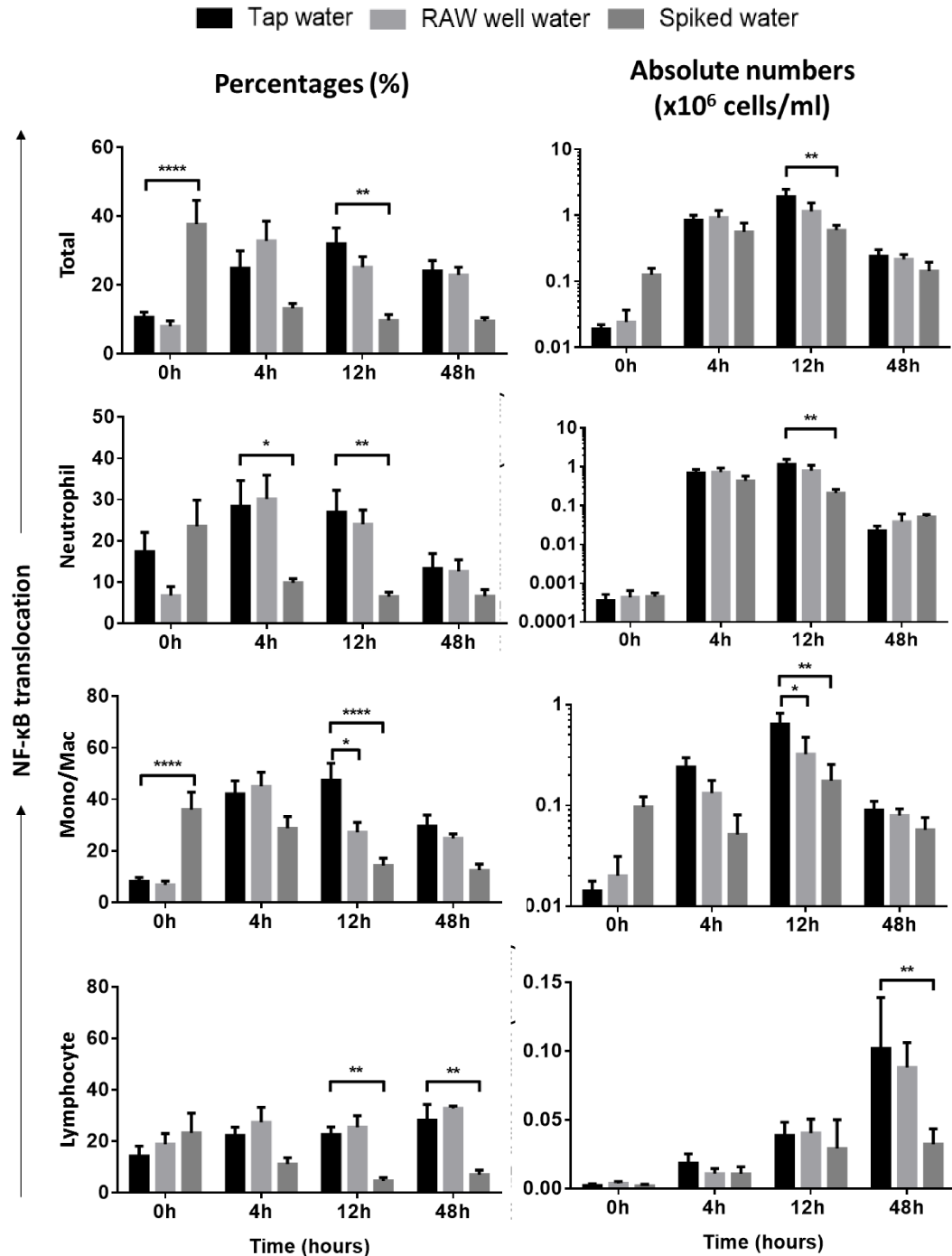


Fig. 5.11. Water contaminants down-regulate NF-κB nuclear translocation during acute inflammation against HKST. Following short-term exposure (7 days), pigs were challenged with HKST and NF-κB nuclear translocation were measured during acute inflammation against HKST. Differential Downregulatory effect on NF-κB nuclear translocation was observed in each leukocyte subpopulation. While early effect on neutrophil was observed, monocyte/macrophage and lymphocyte downregulation were detected in late stages of acute inflammation. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

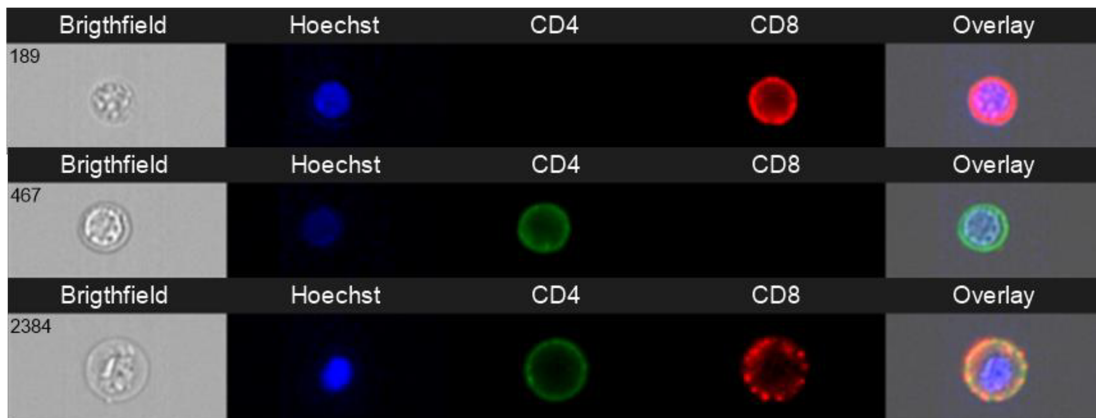
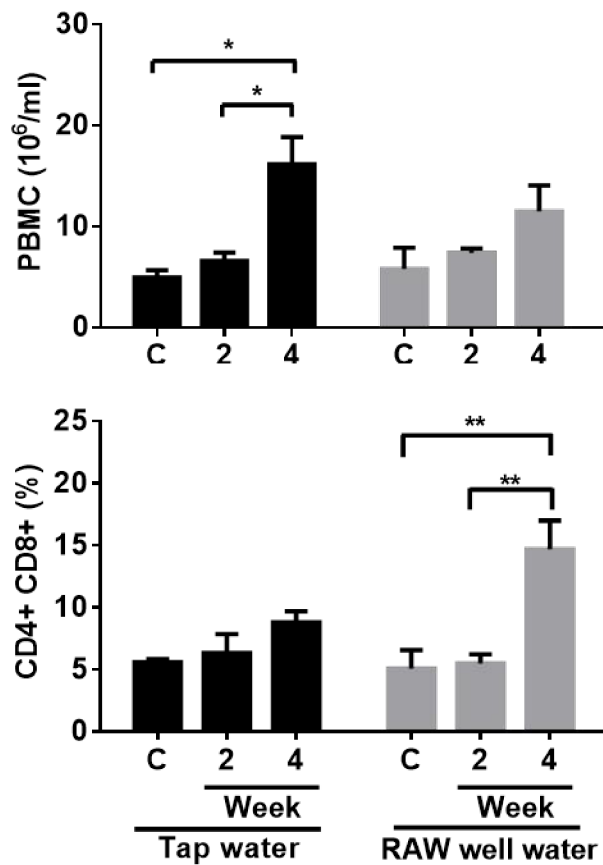


Fig. 5.12. Short-term exposure to water contaminants induces alterations in the peripheral blood mononuclear cells proportions. Following water exposure, pigs were intraperitoneally challenge and blood samples were taken at 2 and 4 weeks. Total PBMC showed a sustained increase at 2 and 4 weeks in the control group, while no increase in total PBMC was observed in the raw water group. Interestingly, slight increase of double positive T lymphocytes was detected in the control group, while a significant increase in this population was observed at 4 weeks post challenge in the raw water group. * $p < 0.05$, ** $p < 0.01$.

Chapter VI

Discussion

6.1. Introduction

Higher vertebrates share multiple mechanisms to effectively display immune responses against pathogens (Buchmann, 2014; Moticka, 2016). Despite this conservation, major differences are described even in closely-related species. In addition to its importance in evolutionary biology, these differences have implications for health and performance in livestock animals and humans. Thus, such specialised processes involving leukocyte dynamics and antimicrobial functions must be closely regulated, modulating the timely return to homeostasis following pathogen control (Iseri and Klasing, 2013).

Over the last decade, our lab has provided significant insights towards characterizing key events during activation of immune responses as well as those during resolving phases of inflammation in multiple species. By comparing animal models at key evolutionary points, such as murine and teleost fish systems, multiple similarities in leukocyte dynamics and activation have been described along with differences in antimicrobial strategies (Havixbeck et al., 2016, 2014; Rieger et al., 2012).

On the other hand, livestock present challenges as strategies that promote the highest performance must be efficiently balanced with those supporting robust antimicrobial defenses. In this context, environmental factors such as water and food consumption, food additives and others, play a significant role in normal development (ELSaïdy et al., 2015). Any disruption of these conditions represents a major source of animal stress leading to serious impact in farm productivity. Due to close interplay between productivity and immunity, such alterations will affect the normal immune performance, even in the absence of health deterioration.

Compared with other models, a limited number of reagents are available for livestock animals. Considering this, researchers are looking into new reliable strategies to assess livestock immune performance. Furthermore, extensive knowledge regarding livestock immunity has been extrapolated from classical animals using *in vitro* assays. Thus, relevant gaps in basic immunology of non-classical animals require to be studied. This will contribute to integrate concepts in evolutionary immunology and their implications in animal/human health. In this context, *in vivo* research offers a closer perspective of immune development using whole living organisms while evaluating the impact of environmental factors. Hence, my first long-term objective was to provide additional depth into the mechanistic processes that induce and regulate acute inflammation in livestock animals, as these are relevant to immunity, performance and energy expenditures. Taking advantage of functional parameters, I characterized key events of an *in vivo* peritoneal challenge model for the assessment of inflammatory responses in broiler chickens and pigs (Chapter III and IV) as representative models of livestock animals. Later, my second objective was to characterize the effects of drinking water quality and how it impacts overall animal immunity (Chapter V). I defined that drinking water quality affects the normal progression of acute inflammation and these effects are detected using molecular and cellular assays. In addition, this drinking water quality has implications for long-term immunity. Altogether, this thesis provides new insights into the comparative development of acute inflammation in poultry and swine, while assessing the effects of water contaminants on the transition from inflammation to long-term immunity. Lastly, this work provides a reliable platform of immunoassays that

allow the evaluation of immune capacity under specific conditions of importance for livestock and human health.

6.2. Summary of findings

The inflammatory process can be divided into two different phases: 1) an early pro-inflammatory state, dominated by an active release of chemotactic mediators and migration of inflammatory leukocytes. This is followed by 2) a pro-resolving phase, characterized by a decrease of infiltrating leukocytes with an increased release of anti-inflammatory mediators along with termination of pro-inflammatory processes. Here, I started characterizing these mechanisms underlying acute inflammation in chickens and pigs. Later, I took advantage of these immune parameters to elucidate the effects of water contaminants on this immune response.

6.2.1. Characterization of immunological capacity of poultry and swine in the abdominal/peritoneal compartment

Firstly, to define inflammatory conditions, it was necessary to estimate the basal immunological status of the peritoneal (or abdominal in avian) compartments. In other models such as mice and teleost, the peritoneal/abdominal cavity provides a sterile compartment that allow studies of *in vivo* inflammation. The peritoneal cavity stores a significant amount of active, resident leukocyte in mice (Ito et al., 2017; Leyboldt et al., 2007; Rao et al., 1994). These cells are used for *in vivo* or *ex vivo* assays, representing a useful marker/read out of basic, homeostatic and inflammatory status. Interestingly, my evaluation of resident leukocytes in poultry and porcine showed contrasting results.

While in swine I observed a large pool of peritoneal leukocytes that can be easily harvested; analysis in poultry showed a lower number of abdominal leukocytes. Thus, the cell harvesting process in poultry was more challenging and requires a certain level of expertise to maximise cell recovery without contamination. It is important to point out that the main source of sample contamination is blood. Blood contamination was observed in slightly higher proportion in chicken samples, while in swine it was almost absent. Although physical features of chickens (smaller abdominal cavity than pigs) might suggest a higher chance of bleeding during intra-abdominal challenge or lavage, absence of some coagulation factors in avian species appears to play a role. Avian and mammals species have been shown to display different coagulation mechanisms since avian species lack coagulation tissue factor (Bigland and Triantaphyllopoulos, 1960; Buzala et al., 2017; Davidson et al., 2003) which explains the presence of bloody samples in chickens. It is also important to highlight that bloody samples affects read-outs of inflammatory analysis; therefore, they must not be considered.

Higher total number of resident leukocytes in swine compared to chickens appeared to be related to differences in systemic distribution or local development of resident leukocytes. Although some studies have revealed that chickens lack or have few resident leukocytes (Genovese et al., 2007; Rose and Hesketh, 1974; Whelan et al., 1997), I found that the chicken abdominal cavity does store a proportion of leukocytes that can be used as biomarkers for immune assessment. Nevertheless, these results provide evidence that poultry and swine immune system display different mechanisms of peritoneal leukocyte development and establishment from immature to mature immunological status. It appears that poultry delays the arrival of resident leukocytes,

while the porcine immune system is faster in this regard. In addition, it remains unclear whether the local resident proliferation process described in murine models (and potentially in swine) is deficient in avian species so that they rely on systemic contribution (peripheral monocytes). Yet, these findings reveal different homeostatic thresholds in the peritoneum/abdominal cavity of swine and poultry that reflect different immunological status in both animal classes.

In mice, most unelicited peritoneal leukocytes are mainly composed of macrophages and lymphocytes (dos Anjos Cassado et al., 2015; Zhang et al., 2010). Peritoneal macrophages play a role as sentinels and are the first responders against potential pathogens, while regulating homeostasis (Bain and Jenkins, 2018; Davies et al., 2013). Slight variations in monocyte/macrophage populations in poultry and swine might be associated with differential development or distribution into abdominal/peritoneal cavity (Chapter III and IV). Remaining proportions were lymphocytes, while absent levels of polymorphonuclear cells in both classes. To the best of my knowledge, this is the first description of differential proportions of unelicited resident leukocyte subpopulations in poultry and swine. As mentioned previously, these proportions of resident macrophages in avian species might be associated with the deficient self-proliferation in the abdominal compartment during first weeks of age. It is known that chickens have deficient immune responses during the first week after hatching and later acquire proper defenses as the animals develop. Nevertheless, resident leukocytes trigger robust responses following pathogenic exposure. Further studies are needed to get a better understanding of the factors driving divergent resident leukocyte development in both species and whether these events occur locally or systemically. Moreover, additional

information is needed to define specific subsets of these leukocyte subpopulations, their contribution to homeostatic regulation and implications in the initiation of acute inflammation in poultry and swine. As in other models, basal levels of resident peritoneal/abdominal leukocytes in poultry and swine can be used as reliable readouts for immunocompromise under multiple stimulus and conditions.

6.2.2. Self-resolving acute inflammatory process displays differential mechanisms in poultry and porcine

Following intrabdominal/intraperitoneal challenges, leukocyte infiltration detected at 4 h in poultry and swine suggest that leukocyte trafficking is highly conserved and concerted following infection (Freire and Van Dyke, 2013; Gilroy and De Maeyer, 2015). Based on the large total numbers detected, leukocyte migration must have started earlier than 4 h post challenge. Evaluation at earlier time points is needed to clarify whether a differential cellular trafficking is observed and if differential homeostatic status plays a role in leukocyte trafficking in poultry and swine. Deeper analysis established that major leukocyte type contributing to leukocyte migration was the polymorphonuclear cell (PMN) similar to other models (Havixbeck et al., 2016; Mocsai, 2013; Nathan, 2006). Although this PMN infiltration displayed similar patterns in both chicken and porcine models during initial stages (4 h), they showed distinct dynamics during the resolving phase of this inflammatory process. These results revealed that the inflammatory process displays unique characteristics depending on the type of challenge, particularly in the resolving phase of acute inflammation. This suggests that poultry and porcine inflammation undergo divergent mechanisms to promote long-term

immune responses following inflammation. Nevertheless, my approach demonstrates that peritoneal challenge model represents a sensitive and reliable tool for assessment of alterations in acute inflammation processes under multiple conditions.

My multiplexed leukocyte differentiation approach resulted as a highly-sensitive and reliable tool for *in vivo* evaluation of leukocyte dynamics, differentiating the three major leukocyte subpopulations in higher vertebrates. This technique allows precise detection in leukocyte development in living entities, representing a useful platform for analysis of multiple biological processes. Yet, I detected some species-specific features. I noticed that overall, the size of porcine leukocytes was larger than their poultry counterparts. It is still unclear to what extent this might have implications in the differential cellular active function between these two animal classes and how this resulted from evolution or selection processes. Despite this fact, major classical features such as granularity, nucleus/cytoplasm ratio and others, described in classical mammals were present in these species with slight variations. Differential nuclear morphology in swine (multi-lobulated) and poultry (bi-lobulated) leukocytes did not seem to affect the functional inflammatory leukocyte function. Literature described this feature to be associated with the protein structure of lamin B receptor (LBR) in multiple species (Hoffmann et al., 2006; Veda, 2011), although its specific action in the context of inflammation is unknown. Taking this into consideration, it is important to be aware of inherent cellular features when analysing samples from avian or swine species. Nevertheless, the efficiency of this technique to precisely differentiate leukocyte subpopulation in divergent species, usage of complimentary tools such as fluorescent dyes, conjugated

antibody markers, etc. will potentiate the analysis of leukocyte subsets/subtypes in multiple species.

6.2.2.1. Characterization of self-resolving inflammatory process in poultry

Local changes during acute inflammation correlates with systemic leukocyte alterations in classical animal models (Havixbeck et al., 2016; Kumar et al., 2004). In my chicken experiments, peripheral dynamics of leukocytes and the hematopoietic compartment (bone marrow) demonstrated systemic leukocyte contribution to local abdominal inflammation. Results found hematopoietic contribution in early phases of inflammation while peripheral circulation sustains leukocyte support later in the acute process. This also suggest that abdominal inflammatory processes promote systemic alterations which can be useful as bioindicators of immune function during initial hours following trigger. This might be advantageous compared to other antibody-based approaches that are not measurable until weeks after challenge.

Contribution of monocyte/macrophages did not seem to have major relevance during initial phases of inflammation (following challenge). However, they gained higher importance as the inflammatory process develops. In fact, different dynamics of monocyte/macrophage and lymphocyte levels are required to achieve homeostasis in poultry (More-Bayona et al., 2017b) that are consistent with my previous observations that avian species display their own mechanistic, cellular and molecular features during the resolving phases. It appeared that homeostatic mediators such as apoptotic cells and ROS production play differential roles in poultry compared to mammals. Thus, I

focused my research on the regulation of these two parameters to resolve avian acute inflammation.

ROS production has antimicrobial and signalling properties. In avian, there has been a long-term debate regarding whether heterophils display ROS production under inflammatory conditions as a mechanism of pathogen clearance (Farnell et al., 2006, 2003; Genovese et al., 2013; Magor et al., 2013; Penniell and Spitznagel, 1975). My findings of ROS production compared to those described in other animal models (Havixbeck et al., 2016, 2014; Rieger et al., 2012) provide evidence that heterophil ROS participate as intracellular signalling molecules rather than antimicrobial function. These results demonstrate that even when heterophils contribute to total ROS production during acute inflammation, their antimicrobial response does not rely on oxidative-dependant function. Instead, heterophils use other non-oxidative dependant mechanisms such as phagocytosis, heterophil extracellular traps (HETs), antimicrobial peptides and others.

Along with downregulation of antimicrobial responses such as ROS production and others, a progressive increment of senescent/apoptotic leukocytes contributes to downregulate inflammatory events (Dal Secco et al., 2004; Gilroy and De Maeyer, 2015; Poon et al., 2014; Soehnlein and Lindbom, 2010; Yoshimura et al., 2010). My findings revealed a gradual increase of apoptotic cells during the acute inflammation and appeared to downregulate functional responses following internalization by inflammatory leukocytes. These revealed a conserved mechanism of inflammation control among vertebrates by apoptosis. The capacity of chicken heterophils to internalize apoptotic cells, a feature observed in mammals (Esmann et al., 2010) but

absent in teleost fish (Rieger et al., 2012) evidenced that the apoptotic cell accumulation plays a role into the downregulation of inflammation and that avian heterophils gained this capacity later in the species divergency from lower vertebrates.

Although avian heterophils displayed phagocytic capacity to internalize apoptotic cells and this downregulates the cellular pro-inflammatory function, heterophil capacity to internalize pathogen-mediated particles decreased at the anti-inflammatory stages. These findings contrast with phagocytic capacity in other models, where these levels remain (Rieger et al., 2012). This shows that even when avian heterophils have acquired capacity to internalize homeostatic particles, they tend to lose this capacity to internalize pro-inflammatory elements as the inflammation progresses. Altogether, this shed light into a unique feature of avian apoptotic cells in the resolution of acute inflammation among mammals and teleost fish immunity.

In addition, I detected lymphocyte-like cells that displayed phagocytic activity throughout acute inflammation. Phagocytic lymphocytes are known to contribute to pathogen control and seem to regulate immune responses and favour long-term immunity in classical mammals. To the best of my knowledge, this is the first description of this type of cells in avian species. Although in low proportions, the contribution of these cells following internalization of pathogens is unknown. Also, their molecular and functional characterization remains uncertain. This might be of relevance since lymphocytes appear to play a role into the resolution of avian inflammation and transit to long-term defenses.

6.2.2.2. Characterization of self-resolving inflammatory process in porcine

Porcine neutrophils are equipped with a full repertoire of enzymes that enable them to actively rely on both oxidative and non-oxidative defenses (Chevaleyre et al., 2016; Sang and Blecha, 2009; Scapinello et al., 2011; Wessely-szponder et al., 2010). My results of ROS production following HKST challenge in swine, comparatively higher than the poultry model, demonstrate ROS as a useful biomarker of antimicrobial responses that can be applied in the assessment of swine immunity. Moreover, it represents a bioindicator of normal progression of acute inflammation. As monocyte/macrophage and neutrophils are the main ROS producers under inflammatory conditions, it adds deeper analysis into other leukocyte functions (e.g. phagocytosis) and interplay between leukocyte types.

My findings of increased macrophage phagocytosis during acute inflammation differed from those in poultry, evidencing a major contribution of macrophage phagocytosis to the resolution of inflammation in swine. Interestingly, proportions of neutrophil phagocytosis remained similar in both pro- and anti-inflammatory stages, while I observed a downregulation in heterophil phagocytosis in the poultry model in the anti-inflammatory phase. Altogether, this suggests that cellular functionality under inflammatory processes undergoes divergent resolving molecular and cellular events in porcine and avian models. Further studies are needed to get a better understanding in how phagocytosis is involved in the development of adaptive immune responses.

Like poultry, presence of small lymphocyte-like cells with phagocytic capacity in porcine, demonstrates the conservation of phagocytic lymphocytes in higher vertebrates as observed in human, mouse and teleost fish (Parra et al., 2013). Phagocytic levels at

resolving phases of inflammation suggest that these phagocytic lymphocytes play an active role in the return to homeostasis. However, further studies are needed to characterize these cells, their mechanistic processes, how they contribute to development of inflammation and the establishment of memory responses in swine. Altogether, I developed a self-resolving *in vivo* inflammatory model using two different pathogen mimics in poultry and porcine. Using this methodology, I characterized the dynamics of cellular events during inflammatory process in poultry and swine. Interestingly, I found some differential mechanisms under acute inflammation between these species highlighting divergent processes to achieve immunity. These encompass events during the resolving phase of acute inflammation. Based on my findings, several cellular (leukocyte proportions and apoptotic cells), antimicrobial (ROS and NO production), molecular (cytokine expression) and others, are useful read-outs for assessment of immunocompromise under certain conditions. Furthermore, these parameters might gain major importance under circumstances of subclinical status of health impairment. Hence, my second objective was to evaluate the effect of underground contaminated water used in small poultry and swine farms taking advantage of parameters characterized in acute inflammation.

6.2.3. Drinking water contaminants induce divergent immune effects in poultry and porcine

Hundreds of by-products waste from industry are introduced into the environment without processing them and will become potentially harmful to human or animals when they are exposed to them. Thus, permanent use of untreated water for animal

consumption has been linked to the increase of production and physiological complications in farms. Consequently, effects of water xenobiotics in animal immune performance need to be addressed. My goal was 1) to define the combinatorial effect on chicken immune defences of multiple drinking water contaminants commonly found in small poultry farms (Southern Alberta, Canada); 2) to determine whether this raw water induces a differential response when animals are exposed to bacterial or fungal pathogens; and 3) to define whether short-term exposure to raw water induces changes in the long-term immunity.

Pollutants found in raw water have been shown to impact the normal animal/human development and performance, mainly in cases of chronic exposure. However, little is known in terms of acute exposure and whether they are encountered in mixtures in aquatic environments. In my experiment, the lack of clinical signs of osmotic diarrhea associated with the presence of multiple chemicals in drinking water shows that low concentration induces subclinical alterations. First, it is possible that these contaminant levels induced alterations in the gut microbial composition. These changes might induce different microbial stimulation to mucosal immunity and leading to major implications in systemic immune development. Alternatively, systemic distribution of water contaminants might exert effects on primordial leukocytes in the hematopoietic tissues developing defective peripheral leukocytes. In this regard, concentrations of total penta chloro-dibenzo-p-dioxin and Octa chloro-dibenzo-p-dioxin, members of a group known as dioxins (Table 2) are associated with multiple disorders and immunocompromise in individuals exposed to them (Dobrzyński et al., 2009; Hites, 2011; Tian et al., 2012).

Higher levels of KUL01⁺ cells in the abdominal cavity of animals exposed to raw water suggested that water contaminants induced a subclinical pro-inflammatory stage that contributes to a higher leukocyte migration/development into the abdominal cavity. This process has been described in other models such as mice, but with single and higher xenobiotic concentration. For instance, studies have reported higher distribution and establishment of monocyte/macrophages in spleen, lung and liver of mice exposed to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), another member of the dioxin family, indicating that TCDD promotes a systemic effect on the distribution of monocyte/macrophages in several tissues through its aryl hydrocarbon receptor (AhR) (Lamb et al., 2016; Vogel et al., 2007, 1997). Mice exposed to these chemicals have shown higher expression of monocyte chemoattractant protein -1 (MCP-1) in spleen, liver, kidney and lung; along with high expression of F4/80, a well-known surface marker of macrophages (Pande et al., 2005; Vogel et al., 2007). My results demonstrated that, in combination with other elements in water, dioxins might be involved in the higher migration/development of macrophages (KUL01⁺) into chicken abdominal cavity. This suggests implications in the tissue homeostasis and organismal level. Furthermore, this evidenced a subtle inflammatory condition that promotes systemic migration of monocyte/macrophages, and as such, might have implications in the immune response against infections. Further studies are needed to identify whether these findings are due to the presence of particular contaminants or a synergistic/additive effect. Nevertheless, this is the first report of increased levels of resident intra-abdominal leukocytes in avian species following exposure to multiple xenobiotics in drinking water.

Effects of water contaminants on ROS production following zymosan exposure suggest that xenobiotics in water have regulatory effects on the basic levels of antimicrobial responses in chicken leukocytes. Studies of Moos et al., 1997, 1994 and Vogel et al., 2007 showed that chemicals/xenobiotics (e.g. dioxins) promoted upregulation of pro-inflammatory cytokines. Here, I observed that in addition to this upregulation, these effects have implications in ROS production. Thus, these results demonstrate that exposure to multiple xenobiotics in low concentration, induces a combinatorial effect on leukocyte distribution in tissues and their activation state upon exposure. Furthermore, this might have major implications in their interaction with other resident and migrating cell types.

Effects of raw water on prolonged heterophil migration after bacterial challenge indicates an unnecessary stress for chicken and implications for recovery from pathogenic infections. This might not only be associated with the progress of inflammatory response but also in delayed development of protective, adaptive long-term immunity. Furthermore, this exacerbated energy expenditure in acute inflammation have potential implications in economic factors on farm such as growth rate, feed conversion and others.

Alongside changes in cellular dynamics, antimicrobial responses were also compromised due to exposure to raw water during acute immune responses.

Downregulation of NO represents a diminished chicken capacity to battle fungal infections. Experiments in rat have demonstrated NO downregulation following chemical toxicity (Cheng et al., 2003). Moreover, this might have implications for intracellular signalling since NO participates in multiple reactions such as release of

Ca²⁺ (Clementi, 1998). It is interesting to note that normal dynamics of NO levels, showed slight differences suggesting that distinct pathogens promote a divergent activation of antimicrobial responses. Lack of this effect under bacterial challenge suggests a detrimental effect in a pathogen-related manner. This suggests that nitric oxide production, and perhaps function, is associated with pathogen type. NO downregulation under zymosan challenge was associated with early downregulation of *iNOS*. Toxicants have shown to inhibit $\text{I}\kappa\text{B}$ phosphorylation that decreases NF- κ B translocation to iNOS promoters in the nucleus. Although I did not evaluate NF- κ B activation in poultry, it appears that this might be the downregulatory pathway detected. Interestingly, early downregulation of *iNOS* expression during acute inflammation support this claim of an effect of water contaminant on nitric oxide production. This differential effect due to raw water was not only observed in the cellular response but also in the pro-inflammatory gene expression. Upregulation of IL8 expression under both bacterial and fungal models, demonstrated that contaminated water induced alteration of common pathways for *IL-8* expression to sustain leukocyte migration during inflammation. Lack of effect on leukocyte migration under fungal challenge, suggests that animals exposed to raw water treatment demanded a major contribution of molecular expression to promote an equal amount of leukocyte trafficking as in normal conditions. Altogether, I revealed that low levels of contaminants in our water treatment promoted cellular and molecular alterations during acute inflammation. Downregulation of *IL-2* caused by contaminated water in the bacterial model might partially explain the lack of lymphocyte development later in the inflammatory process. This might highlight the relevance of lymphocytes during inflammation and their implications in long-term

immunity following water contaminant exposure. To summarize, these results revealed that multiple contaminants in raw water induce differential molecular changes in immune responses against fungal and bacterial mimics (Fig. 6.1) and might have implications for long-term immunity.

A highly efficient innate immune response will induce a robust and protective adaptive immunity. Changes in peripheral monocyte/macrophage function 2 weeks after exposure suggested that multiple contaminants in water promoted long-lasting effects in circulating monocyte/macrophages. Although it is unclear, it appears that these xenobiotics affected development and function of cell progenitors in the hematopoietic compartment, since this is the main store of circulating leukocytes. Similarly, this shows that drinking water quality impacts the initial functional leukocyte response to a secondary challenge as observed following *ex vivo* assays.

CD4⁺ and CD8 T⁺ lymphocytes are primordial mediators of humoral and cytotoxic adaptive immune responses, respectively. Under normal conditions, challenge with extracellular pathogens must induce a strong CD4⁺ T lymphocyte response to promote protective humoral defense. Lower values of CD4⁺/CD8⁺ T lymphocyte ratio along with the effect on circulating monocyte/macrophages demonstrated detrimental effects of water contaminants on their response during acute inflammation and its further progression to acquired immunity.

I also assessed the effect of short-term exposure to underground water contaminants on the immune responses in swine. I used a raw water source commonly used in small pig farms in Alberta, Canada (NW-60-01-01), in a geographical area of heavy metals pollution. Additionally, I included a third experimental group (spiked group), where I

added relevant chemicals frequently encountered in oil spill and other contamination events. Thus, sulfolane (2,3,4,5-tetrahydrothiophene-1,1-dioxide), a widely used chemical for gas treatment and removal of other chemicals from waste streams (Thompson et al., 2013) was added. Levels of contaminants did not induce clinical signs of disease in the animals tested. Like poultry, my approach rather than focus on the individual effect of any of these contaminants, focused on the combinatorial effect on porcine immunity. Absence of changes in resident leukocyte proportions following water exposure suggest that those levels are not playing a role in leukocyte development or trafficking in swine. However, it downregulated their cellular function (ROS levels) under homeostatic stage (prior pathogenic exposure) in the spiked group. Interestingly, levels of ROS significantly increased following *ex vivo* Salmonella exposure.

Altogether, this demonstrates that short-term exposure to water contaminants induces a downregulation in basal homeostatic levels, however, this property can be restored following pathogen-cell interaction. It appears that changes in homeostatic ROS levels are related to alterations in intracellular function, but it does not compromise their antimicrobial performance. This might have implications in the homeostasis maintenance and self-proliferation in the peritoneal cavity and reflects a systemic effect. Further studies are needed to determine whether this systemic effect might represent alteration of normal tissue function.

These findings were associated with NF- κ B activation. NF- κ B is a transcriptional complex that is involved in a series of intracellular events promoting gene expression and cellular activation. I detected no effect on NF- κ B translocation in the tap and raw water groups, however, spiked water promoted an up-regulatory effect. This

demonstrates that even though water contaminants down-regulate ROS production capacity, it upregulates basal cellular activation. It remains unclear which specific pathways are activated through NF- κ B signalling and how this influences the development of immune responses. In addition, this also highlights that reactive oxygen species activation pathways and NF- κ B signalling mechanisms are not tightly linked and water contaminants might down- or up- regulate divergent signalling pathways. Thus, it provides reliable molecular read-outs for assessment of toxicological effects in living organisms.

My *in vivo* challenge assessment of ROS levels correlated with my *ex vivo* observations where the antimicrobial response remained unaffected following exposure to water contaminants. Despite this, ROS production can be used as potential and reliable biomarker of immunotoxicity following water contaminant exposure but lose sensitivity after inflammatory challenge.

Findings of downregulation of NF- κ B activation during acute inflammation in the spiked group evidenced that water contaminants down-regulate a wide range of leukocyte signalling pathways, as this effect was observed in each leukocyte subpopulation. The impact is on neutrophil function in early on in the inflammatory process, while effects on monocyte/macrophages and lymphocytes during the resolution of inflammation, suggests a dual effect of water contaminants on multiple facets of immune response. Since neutrophil contribution during inflammation is led by systemic trafficking from hematopoietic and peripheral compartments to the inflammatory site, these results suggest that water contaminants induce local and systemic alterations during immune defenses. Despite these relevant findings, it is still unclear the exact

mechanisms by which pollutants impact cellular defenses, neither how this might change the development of adaptive responses. As mentioned, these findings are attributed to combinatorial effect of contaminants after short-term oral exposure. Hence, further experiments are needed to determine the individual detrimental effect on immunity.

Unexpectedly, raw water treatment altered the immune parameters of acute inflammation in pigs to a lesser extent than poultry. This might suggest that swine has developed a wider range of regulatory mechanism than avian species to resist the effect of such contaminants. Nevertheless, the down-regulatory effect on NF- κ B activation in macrophages 12 h post *S. enterica* ser. Typhimurium challenge demonstrates similar susceptibility of monocyte/macrophage lineage in both poultry and swine. It remains unknown how these alterations might be involved in the immune responses during acute inflammation and development of long-term immunity (Fig. 6.2).

Analysis of peripheral leukocytes, weeks after *S. enterica* ser. Typhimurium challenge following raw water exposure, demonstrated that these contaminants limited the gradual increase of total peripheral leukocyte counts (PBMC). Although I did not detect specific changes in circulating monocytes, alterations in lymphocyte proportions demonstrated long-lasting effects on lymphocyte development (CD4⁺ CD8⁺ T lymphocytes) which increased after water contaminant exposure. CD4⁺ CD8⁺ T lymphocytes are considered part of memory and regulatory T cells. In normal conditions, proportions of double positive T cells in porcine peripheral blood are around 10-60% of total lymphocytes while in human they reach less than 5% (Pescovitz et al., 1994). Due to its large distribution in circulation, they play a significant regulatory role and are involved in

memory responses in swine. Although it is unclear how these cells develop in the porcine immune system, two theories have been described. First, these cells represent immature lymphocytes that emerge from the thymus and; second, cells that arise from activated CD4⁺ T cells (Zuckermann and Gaskins, 1996; Zuckermann and Husmann, 1996). Nevertheless, these findings might suggest activation of downregulatory mechanisms for development of long-term immunity.

Altogether, findings in both poultry and swine demonstrate that exposure to water contaminants differentially affects immune homeostasis in poultry and swine, while swine seems to be more resistant to raw water contaminants. Following challenge, water contaminants induced a prolonged inflammatory response following Salmonella challenge, detected by higher proportion of PMNs throughout inflammation.

Nevertheless, different functional alterations were observed in both models with systemic long-lasting effects.

Although multiple differences are described in Chapter V regarding effects of water contaminants on acute inflammation, I defined similarities between poultry and swine to respond to Salmonella under water contaminants exposure. Both poultry and swine displayed a prolonged inflammatory response. This is defined by a maintenance of higher levels of PMN cells, while lower levels of monocyte/macrophages and lymphocytes occur later in acute inflammation. As mentioned previously, this has implications in normal development: prolonged immunological stress that leads to expense of energy consumption into immune defenses. Therefore, this prolonged immune response might compromise the normal physiological development of animals exposed to water contaminants. This also might increase susceptibility to secondary

infections in both species. Functional responses were also affected in both species under *Salmonella* challenges. NO production and NF- κ B nuclear translocations in poultry and swine, respectively, displayed alterations compared to their control groups.

Downregulation of these mechanisms have implications for the progress of acute inflammation and its transition to early development of adaptive responses. Indeed, peripheral monocytes and lymphocyte populations were altered in poultry and swine, respectively.

6.3. Future directions

Further studies are needed to add depth into other mechanistic processes during acute inflammation. Although I defined several differential mechanisms in poultry and swine, additional studies are required to assess their implications in the resolution of inflammation to develop long-term immune responses. It is necessary to elucidate the interaction of multiple xenobiotics at different concentrations in the acute and adaptive immunity. B lymphocytes and antibody levels against *S. enterica* ser. Typhimurium must be measured to assess whether altered T lymphocyte populations ultimately induce changes in the antibody-producing cells and antibody production. Since water contaminants are absorbed via the intestinal tract, it is important to analyse the effect of those contaminants on gut microbiota. I predict that interaction water contaminants/microbiota induce changes in microbial population that will promote further alterations in the development of mucosal and systemic immunity. It remains unclear whether there are specific microbial components associated with these changes and if those alterations are transient or permanent in the microbial composition.

Altogether, analysis of gut microbial communities in animals exposed to water contaminants will demonstrate the interplay between mucosal/systemic immunity and the effect of water contaminants to microbial composition.

The underlying mechanisms of avian apoptosis and its contribution to acute inflammation is a topic that needs to be further explored. This will provide better insights into the development of acute inflammation and control in metazoans; and their potential application for livestock health. Furthermore, it is necessary for more studies of phagocytic lymphocyte-like cells in both poultry and swine and their contribution to inflammation and development of long-term responses.

The main objectives of my thesis were to provide a better understanding of acute inflammation in poultry and swine and the application of this knowledge to evaluate the effect of water contaminants in immune function. My research provided new insights into the immunobiology facets of poultry and swine, and regulatory mechanisms following pathogen control. Furthermore, it offers a platform of immunoassays in both poultry and swine for assessment of immune function under specific conditions. This will provide a faster and amenable alternative for evaluation of immunity and livestock health, while allowing the improvement of health care strategies on farm.

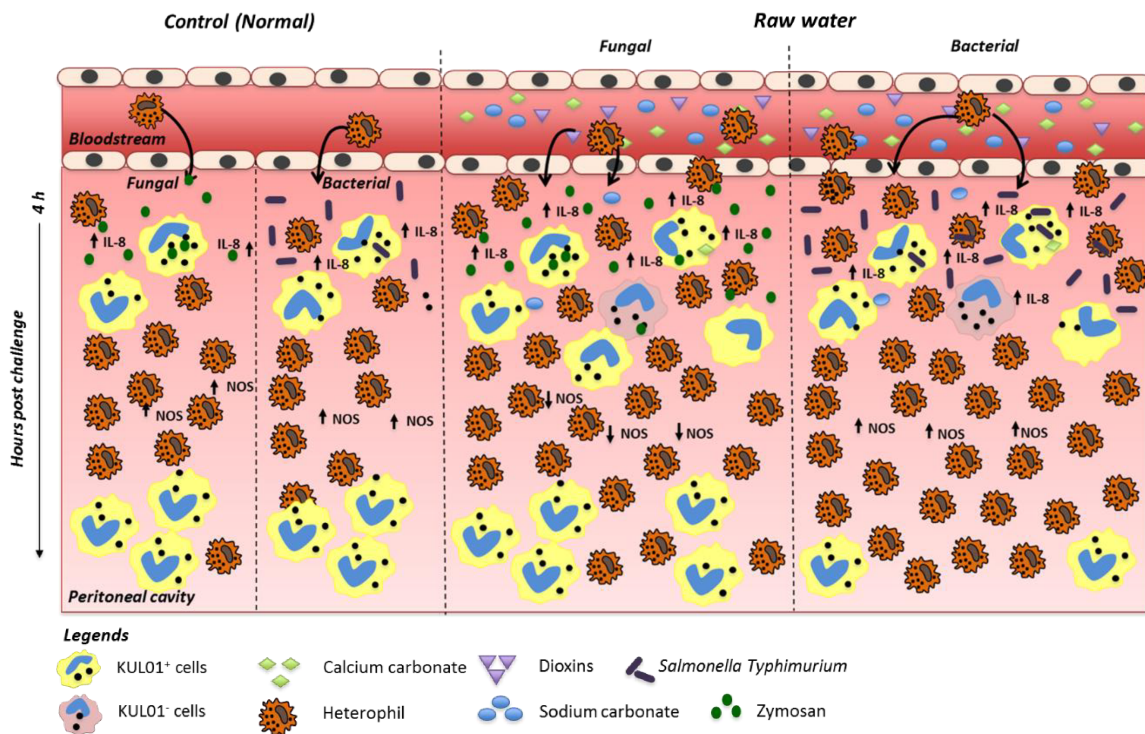


Fig. 6.1. Differential effect of raw water in chicken acute inflammation against fungi and bacterial challenges. Following water exposure, intra-abdominal challenge, promoted differential effects on inflammatory process. Under normal conditions (Control), activated resident macrophages upregulate the release of soluble mediators such as IL-8 and others. Massive heterophil infiltration is paralleled with an active increase of nitric oxide production followed by the appearance of monocyte/macrophage pool, starting the resolving phase of inflammation. Under raw water exposure, I detected differential effects: In fungi model, we observed a massive heterophil infiltration few hours following challenge, attracted by the activated resident macrophage pool along with an upregulated *IL-8*, *TNF- α* and *IL-1 β* expression. At 12 h post challenge, *iNOS* expression was downregulated that led to a decreased NO levels, followed by a progression to a resolution state at 48 h post challenge. In the bacterial model, likewise to what observed to fungi model, a massive heterophil infiltration is lead by an up-regulation of *IL-8*. No down-regulation effect was observed in the cellular and molecular levels of NO at 12 h post-bacterial challenge. However, 48 h post challenge, heterophil proportions remained higher, while monocyte/macrophage pool remained lower, revealing that inflammatory process did not return to homeostasis at this time point.

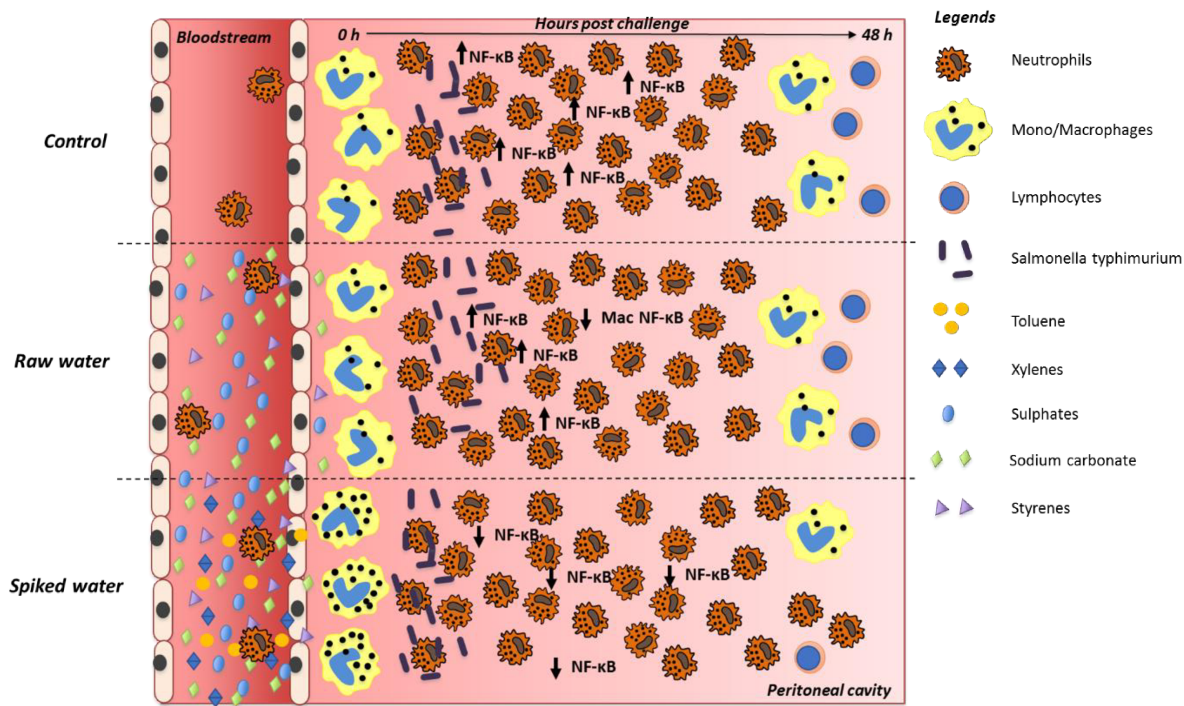


Fig. 6.2. Differential effect of raw and spiked water in porcine acute inflammation against HKST. Following acute exposure to tap, raw and spiked water, key parameters of acute inflammation were measured before and after a challenge with 10^9 CFU of HKST. Under normal conditions (Control), HKST induce a massive leukocyte infiltration of neutrophils, this is consistent with an upregulation of NF- κ B nuclear translocation in inflammatory cells. Later, monocyte/macrophage and lymphocyte pool appearance define the resolving phases of acute inflammation. Under raw water exposure, leukocyte dynamics mirror the normal conditions values, however, I detected a downregulation of NF- κ B nuclear translocation in the monocyte/macrophage group. Under spiked water exposure, it induced an upregulation in the basal levels (before HKST challenge) of cellular activation through NF- κ B, while downregulate levels of ROS production. Following HKST challenge, a prolonged inflammatory process was defined by extended higher levels of neutrophils even at 48 h post challenge. However, these inflammatory leukocytes displayed downregulatory levels of NF- κ B nuclear translocation.

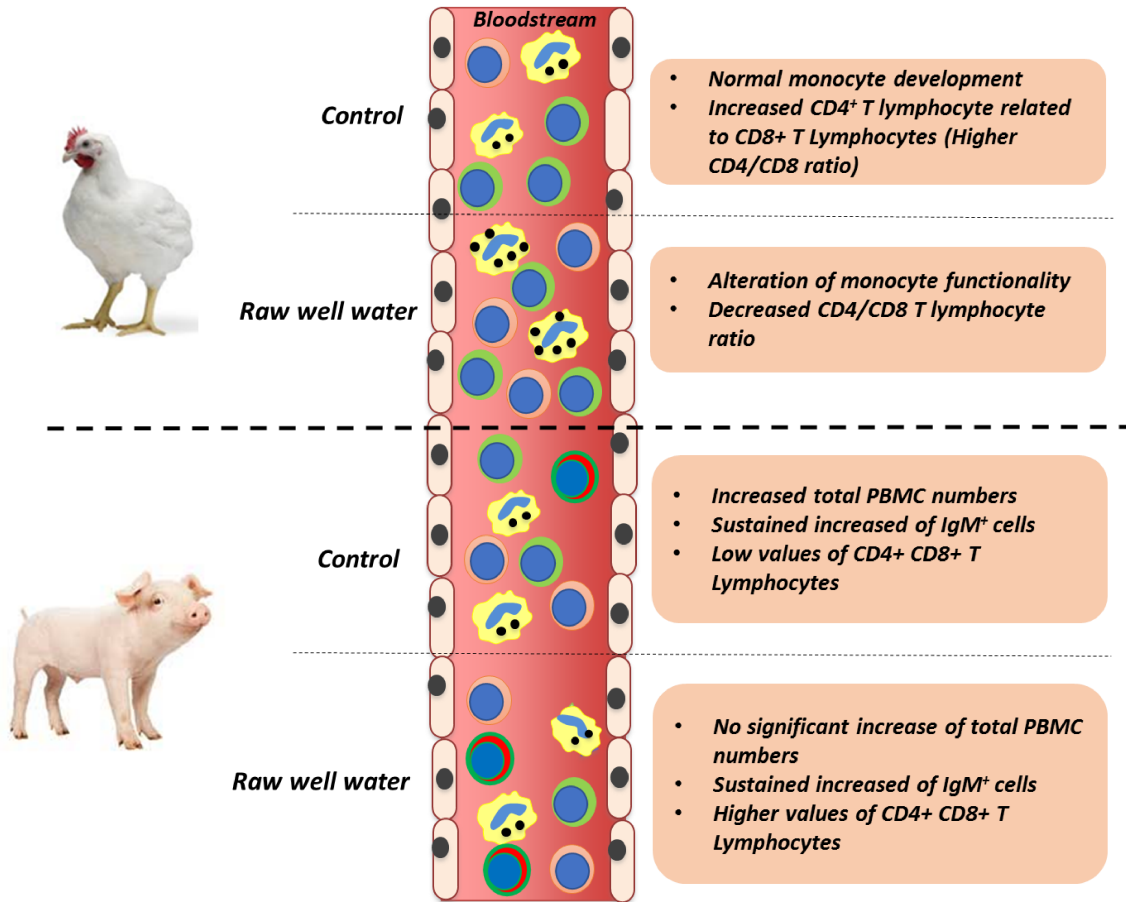


Fig. 6.3. Long-term effect of raw water contaminants on poultry and porcine immunity against *S. enterica* ser. Typhimurium. Short-term exposure to different xenobiotics-containing water sources following by an intrabdominal/intraperitoneal HKST challenge. Two (poultry) and four (porcine) weeks after challenge PBMC was obtained and immune parameters measured. Under normal conditions, a progressive increase of PBMC along with the increase of CD4⁺ T lymphocytes compared to CD8⁺ T cells (higher CD4⁺/CD8⁺ T lymphocyte ratio), with increments of IgM proportions. In poultry, raw water contaminants induced major changes in the monocyte/macrophage (KUL01) functionality and decreased the CD4⁺/CD8⁺ T lymphocyte ratio. In pigs, raw water contaminants, it delayed the increase of total PBMC while had higher proportions of double positive T lymphocytes (CD4⁺ CD8⁺ T lymphocytes).

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