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Role for histone eviction in macrophage activation

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Abstract

Sepsis is life-threatening extreme immune response to an infection, this can often lead to tissue damage, organ failure and death. Despite the advances in modern medicine, sepsis remains a very challenging medical condition. This work aimed to be part of the efforts of the host laboratory to find a therapeutic strategy against sepsis.

Macrophages are major players in the immune response, these cells undergo significant transcriptional changes during infection and inflammation, one of these modifications could be the structural proteins in chromatin called histones. The role of histones, especially in terms of their gene expression regulation, remains an area of profound interest given their potential to influence macrophage activation.

This study aimed to investigate the role of anthracyclines (Epirubicin) in the histone regulation in macrophages during inflammatory responses. The initial hypothesis was that histone gene expression could be regulated by Epirubicin during an infection. To test this theory, it was conducted a serious of experiments, in THP-1 cells and primary mice macrophages with infection stimuli such as LPS and other TLR agonists following Epirubicin treatment. Using advanced experimental techniques, includingELISA and qRT-PCR, it was measured the regulation of mRNA and protein levels of pro-inflammatorycytokines such as TNF-α and Il-1β, and eight specific histone genes in both THP-1 cells and primary mouse macrophages.

Contrary to initial theories, Epirubicin did not show to be a histone up-regulator agent in macrophages, however, an interesting finding was that LPS appeared to be a potential down-regulator of histone expression, especially concerning the histone variant Hist2h2be.

This observation highlights the potential complexities linking inflammatory stimuli to histone regulation. Future perspectives could include a more profound study of histone genes expression during inflammation, by selecting further histone genes, test different infection stimulus and potentially using *in vivo* models and human samples.

Keywords: Sepsis, Macrophages, histones, Epirubicin, Hist2h2be

Resumo alargado

A sepsis é uma condição clínica complexa e potencialmente letal, resultante de uma resposta desregulada do hospedeiro a uma infeção. A sepsis inicia-se frequentemente com uma infeção localizada, que pode ser bacteriana, viral, fúngica ou parasitária. No entanto, o que distingue a sepsis de outras infeções é a forma como o sistema imunitário reage a estes invasores. Em vez de se localizar e eliminar a fonte da infeção, a resposta inflamatória torna-se exagerada e generalizada, afetando tecidos e órgãos saudáveis. Esta resposta exagerada é muitas vezes acompanhada pela libertação excessiva de mediadores inflamatórios no sangue, incluindo citocinas, quimiocinas e fatores de coagulação, estes fatores pro-inflamatórios em quantidades exageradas levam a danos tecidulares e falência de órgãos. Esta doença tem uma taxa de mortalidade muito elevada e ainda não existe uma cura que seja capaz de tratar este problema. Assim, este trabalho tem como finalidade fazer parte de uma estratégia que envolve outros projetos no laboratório, em que se procura encontrar uma nova abordagem para o tratamento desta condição médica.

Os macrófagos são células especializadas do sistema imunitário inato, a sua principal função é detetar, apresentar antigénios, fagocitar e destruir patógenos, como bactérias e vírus. No entanto, a sua atuação não se limita apenas à defesa direta do organismo contra agentes patogénicos, estas células também desempenham um papel vital na modulação da resposta imunitária, produzindo e libertando uma variedade de citocinas e quimiocinas que vão ativar outras células do sistema imunitário.

As antraciclinas representam uma classe de antibióticos anti-tumorais, cujo mecanismo de ação se baseia na intercalação com o DNA e na inibição da Topoisomerase II, uma enzima vital para a replicação do DNA. Este processo interfere no ciclo celular, impedindo a replicação e, consequentemente, a divisão celular. A Epirrubicina, uma análoga da Doxorrubicina, é uma das antraciclinas mais utilizadas em regimes de quimioterapia. Esta apresenta um espectro de atividade anti-tumoral semelhante ao da Doxorrubicina, mas com uma toxicidade cardíaca ligeiramente mais baixa, podendo haver aumento da dosagem e por isso ser mais eficaz nos tratamentos. A Epirrubicina, também pode induzir a formação de radicais livres que acabam por causar danos no DNA e nas membranas celulares, contribuindo para o seu efeito citotóxico.

Num estudo anteriormente realizado, a equipa do laboratório no IGC demostrou que as antraciclinas induzem tolerância à doença face a infeções *in vivo*. Em modelos de ratinhos com sepsis, doses baixas de antraciclinas, como a Epirrubicina, levaram a uma doença menos agressiva e a uma mortalidade reduzida, independentemente da carga patogénica após a infeção. Este estudo também descobriu que o ATM (Ataxia Telangiectasia Mutada), um "sensor" de dano no DNA, é necessário para a tolerância à doença mediada pela Epirrubicina. Além dos papéis nas respostas aos danos no DNA (DDRs), o ATM participa numa complexa rede de vias de sinalização que se ligam com o NF-κB.

Uma descoberta importante em ratinhos com sepsis, tratados com Epirrubicina, e que também foi observada em linhas celulares THP-1 induzidas com estímulos pró-inflamatórios como o LPS, foi a forte supressão da secreção de citocinas, incluindo o TNF. Estes mecanismos de doses baixas de antraciclinas regulam negativamente a transcrição de genes pró-inflamatórios em macrófagos primários de ratinhos, e os danos no DNA causado pelas antraciclinas é o mecanismo para a regulação negativa da transcrição de citocinas e outros mediadores pró-inflamatórios. Neste estudo, foram observadas interações diretas entre a Epirrubicina e fatores associados à cromatina, como subunidades do fator de transcrição pró-inflamatório NF-κB. Devido a estas novas descobertas relacionadas com o mecanismo de ação das antraciclinas na regulação da inflamação, é possível que esta classe de drogas possa contribuir para uma proteção em pacientes com sepsis.

As histonas são proteínas nucleares fundamentais responsáveis pelo empacotamento do DNA em estruturas denominadas nucleossomas. Cada nucleossoma é composto por um octâmetro de histonas,

contendo duas cópias de cada uma das histonas principais: H2A, H2B, H3 e H4, em torno do qual está enrolada uma sequência de DNA. A modulação da estrutura das histonas, através de modificações pós-tradicionais, como acetilação, metilação, fosforilação e ubiquitinação, desempenha um papel importante na regulação da expressão genética. Estas modificações podem alterar a acessibilidade do DNA à maquinaria de transcrição, facilitando ou reprimindo a expressão de genes específicos. No contexto da resposta imunitária, a remodelação da cromatina mediada por histonas é fundamental para a rápida e eficaz ativação ou supressão de genes em resposta a estímulos inflamatórios. Dada a importância das histonas na regulação genética, a possibilidade de compostos como a Epirrubicina poderem influenciar a dinâmica das histonas oferece uma nova dimensão na compreensão das interações entre agentes farmacológicos e respostas pró-inflamatórias.

Para investigações em laboratório, as células THP-1 surgem como uma ferramenta valiosa. Estas células, derivadas de uma leucemia humana, podem ser induzidas a comportarem-se como macrófagos, permitindo estudos detalhados sobre a função e a resposta dos macrófagos a diferentes estímulos inflamatórios.

Em termos de técnicas de avaliação, o ELISA é um método bioquímico que permite detetar e quantificar substâncias específicas, como proteínas enquanto o qRT-PCR é uma ferramenta molecular que permite analisar a expressão de genes através da quantificação d moléculas de mRNA através da deteção de cDNA com o uso de *primers* específicos.

O objetivo principal deste trabalho foi investigar o papel das antraciclinas (apenas usada a Epirrubicina) na regulação das histonas em macrófagos durante uma resposta inflamatória. Para isso foram usadas células de linhagens celulares (THP-1) e macrófagos de ratinhos, estas células sofreram tratamentos com diferentes concentrações de Epirrubicina (0.5μM, 1μM, 2μM) e estimuladas com LPS e outros TLR agonistas. Foram usados controlos sem tratamento com Epi e sem estímulos infeciosos, e outras células com apenas estímulos infeciosos. Na técnica de ELISA foram quantificados os níveis de TNF-α produzidos pelas células, tendo obtido valores concordantes com estudos anteriores; o LPS e outros agonistas levaram à produção de citocinas como o TNF-α e Il-1β em quantidades superiores a 100x os controlos, o que comprova uma forte resposta a estímulos inflamatórios por estas células do sistema imunitário. O qRT-PCR foi usado para quantificar mRNAs de nove genes de histonas.

Durante a análise dos resultados, observou-se que as células tratadas com Epirrubicina não demonstraram níveis significativos de expressão de histonas, e alguns resultados até demonstraram uma redução na regulação destas proteínas o que pôs em causa a hipótese originalmente colocada para este trabalho. Um aspeto interessante encontrado durante estas experiências, foi que as células estimuladas com estímulos inflamatórios como o LPS, levaram a uma regulação negativa de histonas o que levanta a hipótese destes estímulos infeciosos provocarem uma desregulação na produção de histonas. Uma histona particularmente interessante relativamente à regulação negativa por parte do LPS foi a Hist2h2be, esta proteína teve uma clara regulação negativa quando as células foram estimuladas com este estímulo inflamatório. Devido a estes resultados, esta histona poderá vir a ser estudada em projetos posteriores neste laboratório. Algumas teorias relativamente a estas descobertas apontam para que, devido às histonas conferirem uma compactação da cromatina e dificultarem a transcrição de genes, a remoção destas proteínas e a sua regulação negativa, pode ser importante para a produção de citocinas e outras moléculas fundamentais na resposta inflamatória das células, a sua fraca regulação também pode ser devido à prioridade dada pelas células à produção de substâncias pro-inflamatórias em vez da transcrição de genes de histonas. No entanto estas teorias apenas foram colocadas para tentar responder aos desafios encontrados durante este trabalho, para confirmar estas hipóteses serão necessários mais estudos, com parâmetros mais detalhados, para se poder verificar e provar estas teorias.

Palavras-chave: Sepsis, macrófagos, histonas, LPS, Epirrubicina

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Abbreviations

ATM - Ataxia Telangiectasia Mutated
CD4+ - CD4-positive (type of T-cell with CD4 surface protein)
cDNA - Complementary DNA
CHF - Congestive Heart Failure
CSF1 - Colony Stimulating Factor 1
DAMPs - Damage-Associated Molecular Patterns
DDRs - DNA Damage Responses
FGAV – Direção Geral de Alimentação e Veterinária
DMEM - Dulbecco's Modified Eagle Medium (cell culture medium)
DNA - Deoxyribonucleic Acid
DNP - Dinitrophenol
DNR - Do Not Resuscitate
DOX - Doxorubicin (a chemotherapy drug)
E.Coli - Escherichia coli
ELISA - Enzyme-Linked Immunosorbent Assay
Epi - Epirubicin
FADD - Fas-Associated protein with Death Domain
FCT – Fundação para a Ciência e a Tecnologia
FSL-1 - Fibroblast-stimulating lipopeptide-1
HAT - Histone Acetyltransferase
HDACs - Histone Deacetylases
IGC – Instituto Gulbenkian de Ciência
Il-1β - Interleukin-1 beta
kDa - kilodalton (unit of molecular mass)
LPS - Lipopolysaccharide
MHC-II - Major Histocompatibility Complex class II
mRNA - messenger Ribonucleic Acid
NF-κB - Nuclear Factor kappa-light-chain-enhancer of activated B cells
NOD - Nucleotide-binding Oligomerization Domain
NPAT - Nuclear Protein, Ataxia-Telangiectasia locus
PAMPs - Pathogen-Associated Molecular Patterns
PCR - Polymerase Chain Reaction
PGN - Peptidoglycan
PolyIC - Polyinosinic-polycytidylic acid
PRRs - Pattern Recognition Receptors
PTM - Post-Translational Modification
RIPA - RadioImmunoPrecipitation Assay buffer
RNA - Ribonucleic Acid
ROS - Reactive Oxygen Species
RPMI - Roswell Park Memorial Institute (a type of cell culture medium)
SDS - Sodium Dodecyl Sulfate
TBS-T - Tris-Buffered Saline with Tween 20
Th2 - T helper type 2 cell
THP-1 - A human monocytic cell line
TLR - Toll-Like Receptor
TNF - Tumor Necrosis Factor
TNFR - Tumor Necrosis Factor Receptor
TRADD - TNF Receptor-Associated Death Domain
TRAF - TNF Receptor-Associated Factor
WB - Western Blot

1. Introduction

1.1 Sepsis: an overview

Sepsis is a common and serious medical condition characterized by a systemic inflammation and organ failure, resulting from an extreme and dysregulated host response to an infection that leads to a high risk of death (Cecconi *et al.*, 2018). The incidence is high, about 20 million cases per year with a mortality rate of approximately 26% and more than 40% of septic shock, this leads to over 5 million deaths each year, mostly in developing countries (Cecconi *et al*., 2018). The Third International Consensus Definition for Sepsis and Septic Shock in 2016, provided a new definition for septic shock as a more severe form of sepsis, characterized by significant circulatory, cellular, and metabolic anomalies that notably elevate the mortality risk (Rudd *et al*., 2018).

In 2017 the World Health Assembly decided to make Sepsis a health priority and approved a resolution to improve the prevention, diagnosis and management of the disease, however the treatments are costly, and many papers and scientific breakthroughs are originated from rich countries (Rudd *et al.*, 2018).

Sepsis can stem from a broad spectrum of infectious agents, making its clinical manifestations widely diverse and geographically variable. It can initiate both from community-acquired infections or infections contracted during a hospital stay or other healthcare settings (Cecconi *et al*., 2018). Notably, around 80% of sepsis cases treated in hospitals emerge from community environments. The leading source of infection triggering sepsis is the lung (64% of instances), trailed by the abdomen (20%), bloodstream (15%), and renal and genitourinary systems (14%) (Cecconi *et al*., 2018).

A study called Sepsis Occurrence in Acutely Ill Patients (SOAP), reported that the occurrence of sepsis was roughly equal among patients with Gram-positive and Gram-negative bacterial infections. However, there seems to be a shift to Gram-positive bacterial infections, especially those caused by *Staphylococcus aureus*, now potentially outnumbering Gram-negatives (Cecconi *et al*., 2018).

A well-established immune activation pathway in response to infection transpires when the cells of the innate immune system recognize microbial pathogen-associated molecular patterns via pattern-recognition receptors, such as Toll-like receptors (Wyngene *et al*., 2018). This interaction catalyzes the release of both pro-inflammatory and anti-inflammatory agents by activating the transcriptional regulator nuclear factor κB (NF- κB). Cytokines, including tumor necrosis factor α, interleukins 1, 2, 6, and 8, among others, instigate neutrophil-endothelial cell adhesion, activate complement and clotting cascades (Chora *et al*., 2020).

Traditionally, sepsis has been understood as an overwhelming, systemic, pro-inflammatory response to infection, succeeded by an immunosuppressive phase typified by anergy, lymphopenia, and secondary infections (Cavaillon *et al*., 2005). Patients who survive initial sepsis often acquire nosocomial infections with organisms that are not typically pathogenic in immunocompetent hosts, and experience reactivation of latent viruses (Cheng *et al*., 2016). The pathway involving programmed cell death protein 1 and interleukin 7 has been recognized as a significant mechanism underlying the inhibition of T-cell function, hence linked to late sepsis and immunosuppression in early sepsis survivors (Cavaillon *et al*., 2005). These observations have suggested that the early hyperinflammatory state may transition into a subsequent hypo-inflammatory state with considerable immunosuppression (Cavaillon *et al*., 2005).

The exact mechanisms of cell damage and organ dysfunction triggered by sepsis are not fully comprehended and remain a key focus of scientific research. Sepsis often disrupts the systemic blood flow distribution to organ systems through vasodilation and microcirculation disturbances. Advanced microscopic techniques have facilitated the capture of real-time microcirculation images, and newer software packages are in development to quantify dysfunction instantly (Rudd *et al*., 2018). Tissue

ischemia can arise due to a systemic or local mismatch between oxygen delivery and tissue demand. Additionally, mitochondrial dysfunction can lead to a failure of tissue oxygen extraction despite adequate oxygen delivery, a condition known as cytopathic hypoxia. Tissue hypoxia, mitochondrial dysfunction, and apoptosis are considered crucial mediators of sepsis-induced organ dysfunction. Organ dysfunction is a significant predictor of patient outcome, with multiple organ dysfunction associated with a high risk of mortality (Cecconi *et al*., 2018).

Inflammatory mediators also contribute to the coagulopathy often observed in sepsis. Various coagulation pathways are impacted, and manifestations can range from extensive thromboembolism to fibrin deposition in the microvascular bed. One severe complication is disseminated intravascular coagulation, characterized by micro thrombosis and hemorrhage (Cecconi *et al*., 2018).

The search for better diagnostic methods, management strategies and a drug that can alter the progression of the disease is still ongoing; currently the evidence suggests that a better quality of basic care is the most powerful medicine against infections like sepsis, this can be achieved through education and life quality programs (Cecconi *et al*., 2018).

1.2 Macrophages and Sepsis

The immune system is very complex and different types of cells have different roles during an infectious response. Monocytes are one type of innate immune cells, a mononuclear phagocyte found in the bloodstream (Ginhoux *et al*., 2014) Once monocytes attach to endothelial cells and move towards tissues these cells can differentiate into macrophages. This monocyte differentiation is controlled by a combination of hematopoietic factors, such as stem cell factor, interleukin-3, and macrophage-colony stimulating factor (Cavaillon *et al*., 2005). During an infection, monocytes are very important key players in the innate immune system due to their ability to phagocyte pathogens, their antimicrobial activity, and their role as a primary source of inflammation-triggering mediators. As antigen-presenting cells, macrophages also play a crucial role in the adaptive immune response, as the innate immune system is usually thought of as the first barrier against pathogens, comprising various cells and a complex network of pathways (Cheng *et al*., 2016). The macrophages are a type of phagocytic cell that plays a crucial role in defense of the organism. To do so, macrophages rely on the recognition and binding of their pattern recognition receptors (PRRs) to the pathogen-associated molecular patterns (PAMPs) (Cavaillon *et al*., 2005). After binding, a pathway of reactions is triggered leading to phagocytosis and eventual pathogen elimination, but these PRRs also bind to damage-associated molecular patterns (DAMPs) and, at times where this homeostatic function is disrupted, macrophages can be associated with some diseases such as fibrosis, obesity and cancer (Ginhoux *et al*., 2014). Macrophages play a dual role when it comes to cancer development, going from antitumor activity in early stages by mediating pro-inflammatory processes in the tumor microenvironment (M1 macrophages), to tumor-promoting growth and metastasis in established cancer (M2 macrophages) (Cavaillon *et al*., 2005).

This tumor associated macrophages are a heterogeneous population that include resident cells of embryonic origin present at birth and invading monocytes/macrophages recruited during early carcinogenesis by chemokines secreted by tumor cells (Wyngene *et al*., 2018). During the transition from benign growth to invasive cancer, the microenvironment seems to be influenced by cytokines and growth factors that move away from a Th1 - like inflammatory response and instead promote a Th2 - anti-inflammatory immune environment (Wyngene *et al*., 2018). This shift leads to the polarization of macrophages from M1 to M2, which is influenced by various factors, including IL-4 produced by CD4+ T cells and/or tumor cells, as well as growth factors secreted by tumor cells like CSF1 and GM-CSF. This Th2 environment, is also characterized by increased numbers of CD4+ T cells. All these factors

involved in the re-polarizing of resident macrophages or in the recruitment of new monocytes are thought to be subjected to epigenetic changes (Wyngene *et al*., 2018).

Macrophages have an important role fighting infections including sepsis, this medical condition is linked to an excessive production of both pro-inflammatory and anti-inflammatory cytokines. The introduction of LPS (Lipopolysaccharide) or bacteria into experimental animals or humans, leads to almost all tissues be activated and produce proinflammatory cytokines; it is likely that resident macrophages on those tissues are the main producers of these mediators (Wynn *et al*., 2013). For instance, after injecting LPS into mice, peritoneal and alveolar macrophages release tumor necrosis factor (TNF or TNF-α) in *ex vivo* cultures, and TNF-α -positive mononuclear phagocytes can also be found in the lungs post LPS infusion (Wynn *et al.*, 2013).

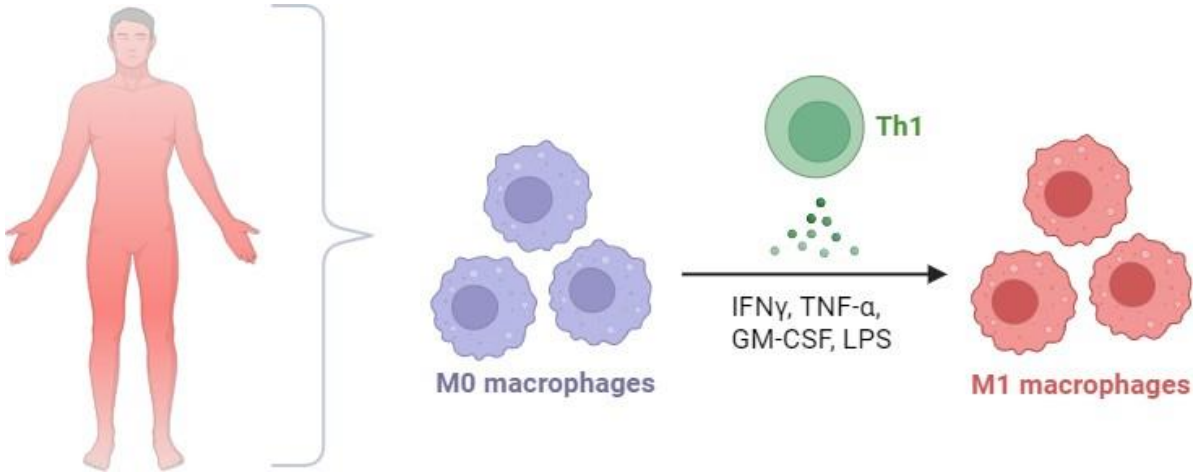


Figure 1.1 – Macrophage activation. Response to pro-inflammatory cytokines, such as TNF-α and infectious stimulus, such as LPS. This figure was created with BioRender.com.

1.3 Similarities between THP-1 cells and Macrophages

THP-1 cells were originally taken from a patient suffering from acute monocytic leukemia. Due to metabolic and morphological similarities they can be differentiated to macrophages when treated with phorbol 12-myristate 13-acetate (PMA) (Li *et al*., 2021). After suffering this transformation, THP-1 cells show several resemblances to human macrophages, like an increased ability to stick to surfaces, the capability to consume foreign particles, and the potential to release cytokines when stimulated. Infection or pro-inflammatory stimulation also leads to THP-1 activation, thus acquiring a macrophage-like phenotype and functions (Li *et al*., 2021).

Both primary macrophages and those derived from THP-1 cells possess the ability to consume and neutralize pathogens such as bacteria, fungi, and viruses, and even apoptotic cells (Chanput *et al*., 2014). These types of cells can respond to PAMPs (pathogen-associated molecular patterns) through Toll-like receptors (TLRs), which starts a release of pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α. When exposed to certain stimuli, both THP-1 cells and macrophages demonstrate an inflammatory reaction, discharging inflammatory cytokines, growth factors, and chemokines (Genin *et al*., 2015).

Like macrophages, THP-1 cells can act as antigen-presenting cells, showcasing antigenic peptides on their surface together with MHC-II molecules to T cells, a vital process in the adaptive immune response (Chanput *et al*., 2014).

Although THP-1 cells provide a beneficial model for studying macrophage biology, they are not a complete replacement for primary macrophages due to their different origins and growth conditions.

THP-1 cells come from a clonal cell line, which means they may not represent the diversity seen in populations of primary macrophages (Chanput *et al*., 2014).

1.4 Infection stimuli and pro-inflammatory cytokines

One of the most intensively described stimuli are Lipopolysaccharides (LPS), these are a major component found in the external membrane of Gram-negative bacteria, including *Escherichia Coli* (*E. coli*) (Rusek *et al*., 2018). This antigen acts as an endotoxin and is recognized by Toll-like receptor 4 (TLR4) present on the surface of macrophages and THP-1 cells, which initiates a series of signals that ultimately results in the secretion of pro-inflammatory mediators, such as IL-1, IL-6, and TNF-α (Wang *et al*., 2008). Previous work showed that the effects of LPS on inflammatory responses are dose-dependent, as a primary contact with monocytes and macrophages with high doses of LPS drives these cells into a tolerance state. Low concentrations of LPS can also lead to innate immune training which results in an increase in the reactivity to a secondary stimulation, compared with previously unstimulated cells. Importantly both states are mediated by histone modifications (Wajant *et al*., 2003).

During bacterial sepsis, PAMPs are responsible for activation of the complement and coagulation systems, contributing to organ failure and death. Peptidoglycans (PGN) are constituents of bacterial cell walls, and PGNs can activate immune responses by interacting with specific pattern recognition receptors (PRRs) such as NOD proteins and TLR2 (Langer *et al*., 2018). Different strains of bacteria, like *E. coli* (PGN Ec) and Staphylococcus aureus (PGN Sa) can have different types of PGNs that cause distinct immune responses when used to stimulate THP-1 cells and macrophages (Langer *et al*., 2018).

Polycytidylic acid (PolyIC), a double-stranded RNA (dsRNA) is one of the most clinical advanced nuclei acid adjuvants used to reproduce a viral infection. PolyIC act trough two distinct dsRNA sensing pathways, TLR3 inside endosomal membrane, and cytosolic receptors like MDA5 and RIG-I, depending on its length and shape (Carson *et al*., 2022). The recognition leads to the production of type I interferons and pro-inflammatory cytokines, contributing to immunity against viruses (Carson *et al*., 2022).

These agonists are widely used in scientific research to stimulate THP-1 cells and macrophages to understand how these cells respond to bacterial or viral pathogens. However, the specific reaction can differ depending on several variables, such as the exact cell type, the stage of cellular differentiation, and the conditions under which the experiment is performed (Chanput *et al.*, 2014).

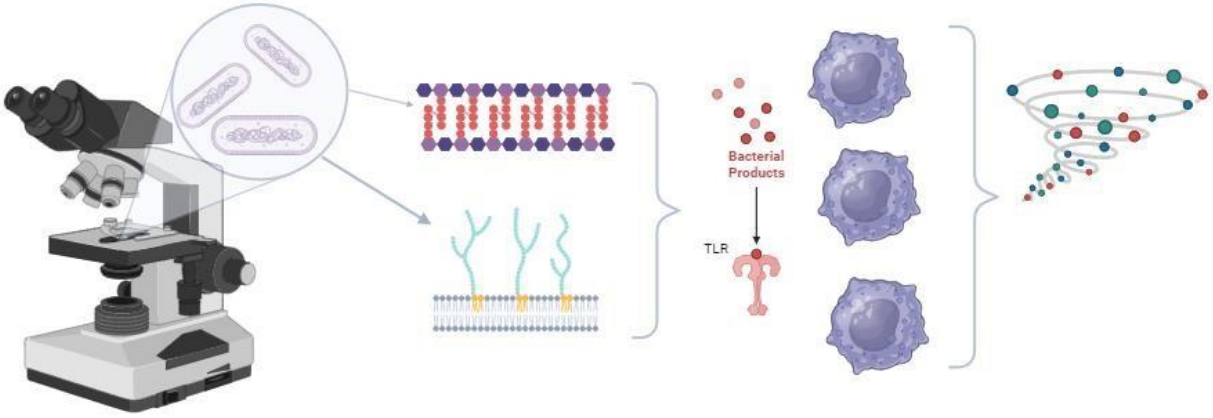


Figure 1.2 – LPS and PGN infection stimuli. Activation of a storm of cytokines through recognition by TLRs and other receptors in innate immune cells. This figure was created with BioRender.com.

1.4.1 Tumor necrosis factor (TNF-α)

Tumor necrosis factor (TNF, also known as TNF-α) is a cytokine produced primarily by activated macrophages with the ability to suppress tumor cell proliferation and induce tumor regression. TNF is a protein with 157 amino acids and is synthesized as a membrane bound protein (pro-TNF) that is released by a TNF enzyme (TACE) mediated cleavage (Wajant *et al*., 2003). On a cellular level TNF was shown to have an important role to activate distinct signaling pathways that regulate cell survival, proliferation, and death (Wajant *et al*., 2003).

The tumor necrosis factor has two receptors, TNF receptor 1 (TNFR-1 also known as p55 receptor) and TNF receptor 2 (TNFR-2 or p75). While TNFR-1 is commonly found across different cell types, TNFR-2 is predominantly present in immune cells. Even though both receptors can interact with TNF, TNFR-1 is typically the key player responsible for TNF's effects across a range of cells. Structurally, TNFR-1 consists of an extracellular domain (ECD), a transmembrane domain (TMD), and an intracellular domain (ICD) (Wajant *et al*., 2003). This receptor, equipped with a death domain (DD), is a significant component of the death receptor group, known for its role in initiating apoptotic processes. In contrast, TNFR-2 does not have a DD, yet it can send a cell death signal, possibly via indirect action through TNFR-1 (Wajant *et al*., 2003). Upon binding by TNF, TNFR-1 forms a homotrimer to recruit the TNFR-associated death domain (TRADD) via mutual binding of their respective DDs. TRADD then acts as a platform, attracting other adaptor proteins, leading to distinct signaling pathways. Among these adaptor proteins are receptor interacting protein (RIP), TNFR-associated factor 2 (TRAF-2), and Fas-associated death domain (FADD) (Wajant *et al*., 2003). These proteins in turn attract crucial molecules to drive intracellular signaling, activating pathways like NF-κB, mitogen-activated protein kinases (MAPK), and mechanisms of cell death (Wajant *et al*., 2003).

When stimulated with LPS, the TNF gene transcription in macrophage cells is increased by a factor of 50 to 200, and the TNF protein secretion can rise by a factor of 10000. TNF is a multifunctional cytokine that is very important in inflammatory processes by stimulating the release of other pro-inflammatory cytokines, chemokines, activating vascular endothelium and promoting the recruitment of leukocytes to inflammatory sites (Wang *et al*., 2008). TNF is also involved in an acute phase reaction, where it induces fever, and metabolic changes; it is also crucial in systemics effects of infection including septic shock (Wang *et al*., 2008).

In response to an infection, certain regions of the chromatin become more accessible, allowing the transcriptional mechanisms to initiate the transcription of genes to produce pro-inflammatory cytokines and chemokines. This chromatin alteration seems to be connected to pos-translational modifications of histones, such as methylation, acetylation and phosphorylation (Wang *et al*., 2008). These modifications can promote or inhibit the binding of transcription factors and other proteins that leads to a gene regulation. In the context of an infection, increased Histone H3 at the promoters and enhancers of cytokine genes, can promote the transcription of TNF and other important cytokines like IL-6 and IL-1β (Roger *et al*., 2010). Also, some enzymes responsible for adding or removing these histone marks, are actively recruited to these gene loci in response to infection stimuli, this leads to an alteration in the chromatin assuring a more efficient gene expression and production of pro-inflammatory cytokines in response to an antigen (Roger *et al*., 2010).

1.5 Histones and Chromatin

Histones are globular proteins present in the nucleus of eukaryotic cells whose goal is to compact DNA in structures known as nucleosomes (Amatori *et al*., 2021). This formation consists of a H3-H4

tetramer and 2 histone H2A-H2B dimers (Marzluff *et al*., 2002) These proteins have a positive charge due to their basic amino acids, allowing them to bind strongly to DNA, which is negatively charged (Amatori *et al*., 2021). The nucleosome is the basic unit of chromatin which, in turn, is made of fibers of loose chromatin (euchromatin) and fibers of compact chromatin (heterochromatin) which differ from one another in their ability to bind to RNA and DNA polymerases, being the euchromatin the one that better allows this anchoring, thus enabling both transcription and replication (Bannister *et al.*, 2011). Besides the mentioned nucleosome density (which translates to chromatin density) being crucial for transcription and replication, epigenetic histone modifications also play a major role. These epigenetic histone modifications are fundamentally acetylation, methylation, phosphorylation and ubiquitylation (Etchegaray *et al*., 2016). The ubiquitylation comprises the binding of ubiquitin to histones, this can lead to inhibition (for example polyubiquitylation of histone H2A) or activation (for example monoubiquitylation of histone H2B at lysine 120 (H2BK120ub)) of gene expression (Bannister *et al.*, 2011). For phosphorylation, it involves the addition of phosphate groups to some amino acid residues such as serine, threonine, or tyrosine and is a process linked to chromatin condensation during mitosis and overall important during the cell cycle (Bannister *et al*., 2011). Methylation is defined by addition of methyl groups to specific amino acid residues, catalyzed by histone methyltransferases (HMTs) (Bannister *et al*., 2011). If the methylation occurs in specific lysine residues on histone H3 such as lysine 4 (K4), occurs gene activation, on the contrary if the methyl group is added to the lysine 9 on histone H3 (H3K9) or lysine 27 on histone H3 (H3K27), it results in gene repression (Bannister *et al*., 2011).

Finally, acetylation is defined as when acetyl groups are added to specific amino acid residues in the histone tails, mediated by histone acetyltransferase (HAT) (Bannister *et al*., 2011). This process is generally associated with gene activation, making the genes more accessible to the transcription machinery and, consequently, increasing their expression (Roger *et al.*, 2010). Furthermore, being a reversible process, and opposed to HAT, there are also 4 subclasses of histone deacetylases (HDACs) whose role is to remove the acetyl group (Roger *et al.*, 2010). Previous studies show the potential of these HDACs and their inhibitors as chemotherapeutic drugs, but other type of chemotherapeutic agent is also drawing attention – anthracyclines (Gonzalez *et al*., 2006).

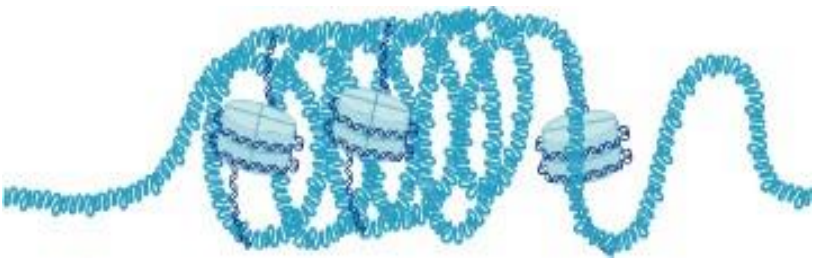


Figure 1.3 – Chromatin structure. Nucleosomes, formed by 8 histone proteins, are depicted in light blue and the DNA wrapped around the nucleosomes is depicted in dark blue. This figure was created with BioRender.com.

1.6 Anthracyclines

Anthracyclines are one of the most effective anti-cancer drugs ever developed (Weiss, 1992). The first anthracyclines created were isolated from *Streptomyces peucetius* in the early 1960s and were named doxorubicin (DOX) and daunorubicin (DNR) (Minotti *et al*., 2004). Both compounds share aglyconic and sugar moieties, the only difference between DOX and DNR is that the side chain of the DOX ends with a primary alcohol, whereas DNR ends with a methyl group (Pang *et al*., 2013). This difference has an important consequence on the spectrum of activity of DOX and DNR, the first one is an essential component in the treatment of breast cancer, childhood solid tumors, soft tissue sarcomas

and aggressive lymphomas (Minotti *et al*., 2004). DNR shows activity in acute lymphoblastic or myeloblastic leukemias. However as with any other chemotherapeutic drugs, both compounds DOX and DNR have shown serious problems in the development of resistance in tumor cells or toxicity in healthy tissues, heart problems, in form of chronic cardiomyopathy and heart failure (CHF) (Minotti *et al*., 2004). Due to the toxicity of this compounds, the maximum recommended dose of DOX and DNR are set at 450 to 600 mg/m2 (Minotti *et al*., 2004).

The next years there was a major effort to discover new anthracyclines in terms of better and more efficient activity and less toxic to healthy tissues. This search led to the discovery of at least 2000 anthracycline analogs, yet only a few of these new compounds reached the stage of clinical trials and development. Among them is the Epirubicin (EPI or Epi), this new compound is a semisynthetic derivate of DOX (as shown in figure 1.4) obtained by an axial-to-equatorial epimerization of the hydroxyl group at C4' in daunosamine (Minotti *et al*., 2004). This change of the position has almost no effect on the mode of action and activity of Epi compared to DOX, however it introduces pharmacokinetic and metabolic significant changes that increase the volume of distribution (Vd) and consequent shorter terminal half-life. Due to these significant changes Epi cumulative doses can be almost doubled those of DOX, resulting in an equal activity but not an increased cardiotoxicity (Minotti *et al*., 2004).

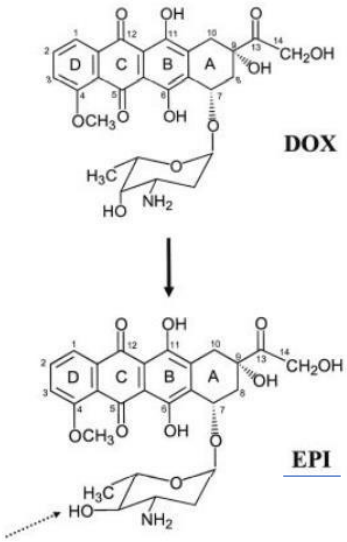


Figure 1.4 – Anthracycline (Epirubicin). Figure adapted from Minotti *et al*., 2004, structure of Epi, compared with doxorubicin (DOX).

The mechanisms of anthracyclines consist in intercalation into DNA and in production of free radicals. Anthracyclines mostly lead to DNA damage, DNA binding and alkylation, DNA cross-linking, DNA strand separation and helicase activity, direct membrane damage, inhibition of topoisomerase II and induction of cell apoptosis (Qiao *et al*., 2020).

Topoisomerases modify the topology of DNA without altering deoxynucleotide structure and sequence, they can cause single-strand or double-strand DNA breaks that are released after changing the torsion status of double helix (Qiao *et al*., 2020). This activity gives an important role to topoisomerases as the supercoiling of the DNA double helix is modulated according to cell cycles and transcriptional activity (Qiao *et al*., 2020).

Anthracyclines act by stabilizing a reaction in which DNA strands are cut and covalently linked to tyrosine residues of topoisomerase II, eventually stopping DNA resealing (Qiao *et al*., 2020). The formation of a complex anthracycline-DNA-topoisomerase II is determined by structure determinants, the planar ring system is important for intercalation into the DNA, as rings B and C overlap with the adjacent base pairs and ring D passes through the intercalation site (Minotti *et al*., 2004). The external moiety of the drug seems to have an important role in the formation and stabilization of the ternary complex, the sugar located in the minor groove is a critical determinant of the activity of anthracyclines as topoisomerase II poisons. Topoisomerase II inhibition increases after removal of amino substituents at C-3' in sugar or of methoxy group at C-4' in ring D. This topoisomerase II- mediated DNA damage is followed by growth arrest in G1 and G2 and programmed cell death. The resistance of tumors to this drug can be explained to altered topoisomerase II gene expression or activity (Yang *et al*., 2013).

Doxorubicin (DOX) and other type anthracyclines, when introduced to cancer cells, can undergo a chemical reaction leading to the formation of a semiquinone, a molecule known to produce reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide (Minotti *et al*., 2004). This ROS-generating cycle is facilitated by certain enzymes, like NAD(P)H-oxidoreductases. During this process, the semiquinone can also interact with the bond between specific rings of DOX, resulting in the formation of 7-deoxyaglycone, a compound that interacts with cellular membranes and induces ROS production near sensitive targets (Minotti *et al*., 2004).

Additionally, DOX's redox cycling can lead to the release of iron from cellular storage. Once DOX interacts with this released iron, it forms complexes that can convert the generated ROS into even more reactive hydroxyl radicals. Due to these capabilities, ROS-induced oxidative damage is often considered a significant action mechanism for anthracyclines in combating tumor cells (Minotti *et al*., 2004).

However, while the capability of DOX to produce free radicals in cells is widely accepted, this is often observed at concentrations higher than what is typically used in clinical trials. In clinical doses, the appearance of certain ROS, like hydrogen peroxide, is usually delayed, suggesting that the free radicals might be produced due to longer-term disruptions in cellular processes rather than as a direct result of the drug activation. An alternative possibility is that current detection methods might not be sensitive enough to identify small amounts of free radicals generated by clinical doses of anthracyclines (Minotti *et al*., 2004).

It is important to understand ROS's roles: they can act as signaling molecules influencing cell cycle and apoptosis or cause oxidative damage to cell components. The DNA damage in cancer cells treated with anthracyclines suggests that direct oxidative damage only takes place when cells are exposed to very high concentrations of the drug (Minotti *et al*., 2004). In clinical concentrations, anthracyclines mainly cause DNA breaks related to their inhibition of a specific enzyme, topoisomerase II. Direct oxidative DNA damage by free radicals, on the other hand, seems to happen only at higher drug levels (Pang *et al*., 2013).

Concerns about the role of lipid peroxidation, another potential damage mechanism by DOX, are similar. While some evidence suggests that DOX might not induce significant lipid peroxidation at clinical concentrations, there is a debate on whether commonly used measurement methods provide a complete picture. Most studies use the Thio-barbituric acid (TBA) assay, which might not be sensitive or specific enough for detailed analysis in culture cells or in vivo experiments. This raises questions about whether anthracyclines might indeed cause lipid peroxidation, the method used fails to detect it accurately (Minotti *et al*., 2004).

As previously mentioned, the main side effects of anthracyclines have many cytotoxic effects with cardiotoxicity being the most prominent. These drugs can cause structural changes in the heart's cardiomyocytes, the genes implicated in this enlargement are the brain natriuretic peptide (BNP) and atrial natriuretic peptide (ANP). High expression levels of these genes lead to cardiac hypertrophy (Minotti *et al*., 2004). Another possibility for cardiotoxicity results from doxorubicin's interference with

cardiac muscle mitochondria. This can modify mitochondrial protein expression, amplifying the redox cycling of doxorubicin and NADH dehydrogenase (Minotti *et al*., 2004). Elevated ROS levels subsequently activate the apoptotic cascade due to the release of cytochrome c from the mitochondria. A total DOX exposure nearing 500 mg/m2 significantly increases the risk of cardiomyopathy, with about 20% of these patients progressing to congestive heart failure. Therefore, cardiotoxicity is primarily attributed to the formation of ROS and iron oxidation (Minotti *et al*., 2004). As DOX influences various biomarkers, evaluating troponins and specific natriuretic peptides (proBNP and DNP) might help in the early detection of its cardiotoxicity. However, attempts to mitigate this toxicity using antioxidants have been unsuccessful, and studies on selective iron chelators have yielded inconsistent results. This suggests other potential mechanisms beyond ROS and iron might be involved in DOX-inducedcardiotoxicity (Minotti *et al*., 2004). Research has also delved into the effects of DOX on the downregulation of GATA binding protein 4 (GATA-4), the activation of p53, and p300 degradation, asthese proteins get impacted, leading to cardiomyocyte apoptosis (Minotti *et al*., 2004). Notably, GATA-4 is a recognized transcription factor essential for postnatal and differentiated cardiomyocytes' survivaland can activate Bcl-xL, an anti-apoptotic gene (Minotti *et al*., 2004). Many patients undergoing chemotherapy experience fatigue and immune suppression. Intriguingly, DOX can boost cytokine production and amplify cytotoxic T lymphocyte responses and enhance natural killer (NK) cell activity.By augmenting these elements of innate and adaptive immunity, DOX can inflict substantial cardiac damage (Minotti *et al*., 2004).

In conclusion, while DOX and other anthracyclines can produce ROS leading to oxidative damage, the actual role and impact of this process at clinical concentrations remain a subject of ongoing discussion and investigation. There are some studies that suggest also toxicity of the anthracyclines in the liver, brain, kidneys and cause infertility. New studies should focus on altering anthracyclines to be more target-selective and new drug delivery systems (Minotti *et al*., 2004).

DNA damage is therefore assumed to be one of the main activities of the anthracyclines contributing to their anti-tumor activities in clinical concentrations. Another consequence of the anthracyclines, histone eviction (which refers to the removal or disruption of histones from the DNA), (discussed below), may also be relevant for chemotherapy (Chora *et al*., 2020).

1.7 Role of histone eviction in macrophage activation

Macrophages, as primary cells of the innate immune system, suffer significant transcriptional reprogramming upon encountering several stimuli, this reprogramming involves extended alterations in chromatin, which ultimately dictates the transcriptional path of the macrophage. Histone eviction constitutes one of such modifications that could be critical to macrophage activation. The consequences of histone eviction are typically an upregulation of gene expression due to increased accessibility of transcription factors and RNA polymerase II to those regions.

Previous studies showed that during macrophage activation by inflammatory stimuli (like LPS), nucleosome remodeling occurs at enhancer regions of certain pro-inflammatory genes leading to displacement of histones. This process is essential for the binding of transcription factors and subsequent expression of these pro-inflammatory genes.

Work by Giaimo *et al*., 2019 showed that the removal of H2A.Z from promoters of certain genes was necessary for macrophage activation and expression of genes involved in the inflammatory response.

Histone eviction and subsequent gene expression changes can have significant therapeutic implications. A study by Fanucchi *et al*., 2019 used a model of lung inflammation to show that disrupting the eviction of histone H3 led to changes in macrophage gene expression and reduced inflammation.

While these studies provide a strong foundation, further research is needed to fully elucidate the mechanisms and consequences of histone eviction in macrophage activation.

1.8 Anthracyclines and histone eviction

The anthracyclines are a class of drugs recognized for their DNA damage capabilities, and by inhibiting Topoisomerase II enzymes or intercalating into DNA, as explained before. Historically, their anticancer attributes were credited to their DNA-damaging roles, which initiates DNA damage responses (DDRs), these can stop cell cycle and induce cell death. While these drugs have been around since the 1960s, recent studies have questioned whether DNA damage is their primary anticancer mechanism (Minotti *et al*., 2004). New studies suggest that chromatin damage without DNA strand-breaks, caused by histone eviction from the DNA, might have a prominent role in the chemotherapeutic effects of anthracyclines, which can now be placed in a new category of epigenetic drugs (Chora *et al*., 2021).

The chromatin damage is characterized by histone eviction from nucleosomes, this can be a result of anthracyclines intercalating into the DNA or direct drug interactions with histones or chromatin associated factors. A study conducted by the same team of this dissertation in IGC, found that anthracyclines suppress certain cytokines and chemokines by binding to the p65 subunit of the pro-inflammatory transcription factor NF-κB at its recognized promoters (Chora *et al*., 2021).. Given this recent discovery, the known roles of the anthracyclines need to be revised, including their role in inflammation and sepsis protection. While DNA damage is seen as the primary mechanism of action of anthracyclines in sepsis tolerance (discussed below), it does not explain the entire protective phenotype (Chora *et al*., 2021).

Understanding chromatin damage events and induced downstream signaling will better explain how anthracyclines protect against sepsis. A key observation is that after histone eviction, there is likely a replenishment of new histones onto nucleosomes, as suggested by increased histone gene expression in the heart and liver of DOX-treated mice. The host laboratory has also observed histone mRNA induction in RNA seq data of primary mouse macrophages stimulated with LPS (unpublished). To maintain the link between distinct sets of PTMs and genomic locations, newly synthesized histones, or histones evicted from chromatin, acquire modifications, any inconsistency in the deposition downstream of anthracycline mediated eviction can change the transcriptional programs (Chora *et al*., 2021).

Moreover, the increased requirement for metabolites to be added to histones might modify cellular metabolic pathways, the histone eviction can potentially alter gene expression of a wide variety of targets, and re-direct cellular metabolites. Due to vast evidence for epigenetic and metabolic dysfunctions in sepsis, protective mechanisms that relate chromatin to metabolic changes are expected to play a crucial role in future treatments (Chora *et al*., 2021).

1.9 Anthracyclines against Sepsis

In a previous study the team in the host laboratory at IGC, has shown that anthracyclines induce disease tolerance to infection *in vivo*. In mouse models of sepsis, low dosages of this class of drugs such as Epirubicin led to less severe disease and decreased mortality independently of the pathogen load after the induced infection (Figueiredo et al., 2013). This study also found that ATM (Ataxia Telangiectasia Mutated), a master DNA damage sensor, is required for Epirubicin- mediated disease tolerance. In

addition to roles in DDRs, ATM participates in a complex network of signaling pathways that intersect with NF-κB (Figueiredo et al., 2013).

One central finding in Epirubicin-treated septic mice which was also observed in the monocytic cell line THP-1 challenged with pro-inflammatory stimuli such as LPS, was the strong suppression of cytokine secretion, including TNF (Figueiredo et al., 2013) This mechanism of how low doses of anthracyclines down-regulate the pro-inflammatory transcriptional program in primary mouse macrophages, and if the DNA damage cause by anthracyclines is the mechanism for the transcriptional downregulation of cytokines and other pro-inflammatory mediators, was the basis for a recent study published by the host laboratory (Figueiredo et al., 2013) In this study, direct drug interactions were observed between Epi and chromatin associated factors, such as subunits of the pro-inflammatory transcription factor NF- κB. Therefore, a new role for anthracyclines in the regulation of inflammation was recently uncovered and may contribute to the protection in sepsis (Chora *et al*., 2021).

The possibility that Epi may be protective from sepsis also in humans and may be used in adjuvant therapy in addition to the current treatments, still awaits clinical trials. For now, clinical trial is being conducted by the host laboratory to test the safety of Epirubicin in sepsis patients when used in low doses: NCT05033808.

2. Aims

As mentioned in the introduction chapter, the host laboratory has a preliminary observation by RNA seq that the mRNAs of histones are induced after treatment with Epirubicin (Epi) in pro-inflammatory conditions that lead to macrophage activation (unpublished results). These results were obtained in cell models of sepsis, more specifically in LPS-activated primary mouse macrophages, where Epi-treated macrophages were compared with untreated. Because of these observations, we hypothesized that histone gene expression in regulated by Epi in inflammatory conditions, likely after histone eviction by Epi.

The main aim of this work was, therefore, to test if histone gene expression is regulated by Epi during inflammatory responses. More specific aims were:

- Test if histone mRNA expression is regulated by LPS or another inflammatory stimulus that activates macrophages, and if so, which TLRs are likely to be involved;
- Test if Epi leads to induction of histone mRNAs when macrophages are activated with inflammatory stimuli;
- Test if different histones have different regulation due to Epi or inflammation in macrophages;
- Test if changes in mRNAs of histones because of inflammatory stimuli or Epi treatments are accompanied by changes in the levels of histone proteins in macrophages.

In summary, the aims of this project are based on the knowledge that anthracyclines lead to histone eviction and the previous observation that histones are induced by Epi in activated macrophages. The general aim of this work, and particularly the specific aims, will contribute to the understanding of the role for histone eviction in macrophage activation.

3. Methods

All re-used materials were washed, decontaminated and sterilized at the IGC washing room facility. Materials and key resources can be found in Supplementary data.

3.1 Cell culture

3.1.1 Cell freezing in liquid Nitrogen

The cells were removed from a plate or a flask in their culture medium. The cell suspension was then spined for 5 minutes at 1200rpm, and the medium was removed. The cell pellet was washed with 1xPBS and centrifuged again.

The cell pallet was resuspended in 1mL of freezing medium. The cells were transferred to cryovials and made sure that screw top lid was well closed. The cryovials were left for at least 2 days at -80°C in a cryogenic cooler with 100% isopropyl alcohol before they were transferred to liquid N_2 storage boxes.

3.2 THP-1 cell culture

Frozen THP-1 cells were briefly thawed in a 37°C water bath and then transferred to a sterile 15mL tube containing 9mL of pre-warmed C10 medium. The tubes were centrifuged at 300 x g for 5 minutes, and then the pellet was resuspended in fresh C10 medium, and the cells were transferred to a T-25 flask.

The THP-1 cells were maintained in C10 medium, and the culture medium was refreshed every 2-3 days or when the cell concentration reached the upper limit of the culture flask, as observed under the inverted microscope. The cell flasks were maintained in an incubator at 37°C and with 5% of CO_2. All cell culture work was performed inside a culture wood in a cell culture room.

For the experiments, the cells were counted in a Neubauer chamber and then seeded in culture plates in C10 medium and treated as detailed below (Supplementary data 2).

3.2.1 Mice and primary cell cultures

Animal care and experimental procedures were conducted in accordance with Portuguese guidelines and regulations after approved by the Instituto Gulbenkian de Ciência and DGAV. The mice used were males with 8–12 weeks old of the *Mus Musculus* strain C57BL/6J produced by the Animal house at the Instituto Gulbenkian de Ciência and under the ethics approved project with reference A011.2019. These mice were bred and maintained under specific pathogen-free (SPF) conditions at the IGC with 12h light/12h dark cycle, humidity 50-60%, ambient temperature 22 ± 2°C and food and water ad libitum. After euthanasia by CO_2 inhalation, the mouse skin was sterilized with 70% ethanol and femurs and tibia of hind limbs were removed, stripped of muscle and washed in RPMI 1640 medium.

Bone marrow cells were flushed from cut bones using an insulin syringe with a 30G needle into 10mL of macrophage RPMI medium. Cells were then pelleted by centrifugation at 450g for 5 minutes and the cell pellet resuspended in 10mL of macrophage RPMI medium.

Cells were counted in a Neubauer chamber and plated at a density of 3 x 10^6 cells (including red blood cells) per 10mL of macrophage RPMI medium supplemented with 30% of L929-conditioned

medium from mouse macrophage-stimulating factor-producing L929 cells (produced previously by the host laboratory).

After three days, an equal volume of fresh macrophage RPMI medium with 30% of L929-conditioned medium was added to the cells. After four additional incubation days, the medium was replaced by 10mL of fresh macrophage RPMI medium with 30% of L929- conditioned medium. After 24h to 48h, cells were scraped from plates, counted in a Neubauer chamber and seeded in culture plates in C10 medium (Supplementary data 2).

Preparation of L929-conditioned medium containing macrophage growth factor (produced previously by the host laboratory).

Mouse macrophage colony stimulating factor (M-CSF)-producing L929 cells (https://www.atcc.org/products/ccl-1) were cultured in T175 flasks, in 40mL of DMEM medium and grown to confluency. The culture medium was left unchanged for 5 days, for production of M-CSF (macrophage growth factor). Cells were then centrifuged at 290g for 5 minutes and the supernatant was collected, and filter sterilized for future use in medium for mouse macrophage differentiation.

3.2.2 Treatment with Anthracyclines (Epirubicin) and inflammatory stimuli (4h and 24h)

These treatments (Table 3.1) were performed in THP-1 cells and in mouse macrophage cells:

Table 3.1 - Pro-inflammatory agonists.

Agonists	TLR
Fixed Bacteria Gram-negative, *E. Coli*	TLR4
Lipopolysaccharide (*E. Coli* 055: B5), LPS	TLR4
Peptidoglycan (*E. Coli*), PGN	TLR2
Synthetic analog of double-stranded RNA (dsRNA), high molecular weight, PolylC HMW	TLR3
Synthetic analog of double-stranded RNA (dsRNA), low molecular weight PolyIC LMW	TLR3
FSL-1 (Pam2CGDPKHPKSF), a synthetic lipoprotein derived from *Mycoplasma Salivarium*	TLR2/6

For the treatments with Epirubicin (Epi), which was used from a stock solution at 1724µM in PBS 1x, the concentrations were 0.5µM, 1µM and 2µM. The dilutions were performed serially in culture medium.

THP-1 cells

THP-1 cells were plated in 24 well plates with 300000 cells per well in 150µL of C10 medium; Epi treatment was added in 50µL of C10 and incubated for 1h; 50µL of C10 medium alone was added to untreated cells; after that, it was added the TLR pro-inflammatory stimuli agonists in 50µL of C10; 50µL of C10 medium alone was added to unstimulated cells; the plates were incubated for 4h (Plate 1)and 24h (Plate 2).

At the end of the incubation time the cells were collected and transferred into Eppendorf tubes and spined at 10000rpm, at 4°C, for 5 minutes. The supernatants (SNs) were collected into new tubes and stored at -80°C for future analysis and the cell pellets were resuspended in 100µL of RLT buffer (QIAGEN) and stored at -80°C for future RNA extraction (Supplementary data 2).

Mouse macrophages

Mouse macrophages were plated in 12 well plates with 550000 cells per well in 1mL of DMEM medium and incubated overnight for cells to adhere to the plates. In the next day, 400µL of medium were removed from each well and Epi treatment was added in 100µL of medium and incubated for 1h; 100µL of DMEM medium alone was added to untreated cells. After that time, it was added the TLR pro-inflammatory stimuli agonists in 50µL of DMEM; 50µL of DMEM medium alone was added to unstimulated cells. The plates were incubated for 4h (Plate 1) and 24h (Plate 2).

At the end of the incubation time the supernatants (SNs) were removed from the cells and were collected into new tubes and stored at -80℃ for future analysis. For future RNA extraction, the cells remained in the plates and were washed with 400µL of 1xPBS, carefully to avoid detaching the cells. The 1xPBS was removed and 250µL of RLT buffer (QIAGEN) was added to each well and plates stored at -80℃. For protein extraction, the cells remained in the plates and were washed with 400µL of 1xPBS, carefully to avoid detaching the cells. The 1xPBS was removed and 250µL of RIPA lysis buffer was added to each well and plates stored at -80℃ (Supplementary data 2).

3.3 ELISA TNF-α

The ELISA technique was used on the supernatants of THP-1 cells and mouse macrophages at 4h and 24h using the Biolegend kits and protocol (Supplementary data 2). On the day before performing the ELISA, the capture antibody was diluted in 1X Coating Buffer A, and 50µL of this solution was added to each well in a 96-well plate. The plate was then incubated overnight at 4°C.

On the following day, all reagents were allowed to reach room temperature, and triplicates of the samples were prepared with a dilution of 5x in RPMI media. Duplicates of the standard dilutions were also prepared for the calibration curve. Next, the plate was washed three times with 50µL of Wash Buffer in each well, and the excess liquid was dried with paper.

To block non-specific binding and reduce impurities, 100µL of 1X Blocking Buffer A was added to each well and incubated at room temperature for 1 hour with agitation.

After 1 hour incubation, the plate was washed again three times with Wash Buffer. Then, 50µL of the samples and standards were added to the appropriate wells, and the plate was sealed and incubatedat room temperature for 2 hours with agitation.

After the incubation, the plate was washed three times with Wash Buffer again, and 50µL of the diluted Detection Antibody solution was added to each well and incubated at room temperature for 1 hour with agitation. The plate was washed again three times with Wash Buffer, and 50µL of the diluted Avidin-HRP solution was added to each well, followed by incubation at room temperature for 30 minutes with agitation.

Subsequently, the plate was washed again three times, and 50µL of Substrate F Solution was added to each well, then incubated in the dark for 15 minutes. To stop the reaction, 50µL of H_2SO_4 (2 N H_2SO_4, prepared by the host laboratory) was added to each well. Positive wells appeared yellow, resulting in different shades of yellow depending on the concentration of cytokines in the well.

Finally, the absorbance at 450 nm was measured in a plate reader (Tecan Infinite 200). A linear based calibration curve was adjusted to the data from the standard dilutions and the concentrations of TNF-α for each sample were calculated using the equation corresponding to that curve.

3.4 RNA Extraction and quantification

For the extraction and quantification of RNA, only the samples with the 4h inflammatory stimuli were used with the aim of detecting early regulatory events following proinflammatory stimulation. After removing the supernatant and leaving the cells with RLT Buffer overnight stored at -80°C (in Eppendorf tubes or in plates), RNA extraction was performed using the QIAGEN kit and protocol (Supplementary data 2).

The samples were placed in Eppendorf tubes (cells were already in Eppendorf tubes or were transferred from the plates), and 450µL of 70% ethanol was added to each tube, mixing thoroughly using a micropipette.

Next, 700µL of each sample, including the precipitate, was transferred to a RNeasy Mini spin column placed inside a 2mL collection tube. The column was then centrifuged at 10000xg for 15 seconds, and the supernatant was discarded.

For DNase digestion, the following steps were performed: 350µL of Buffer RW1 was added to each RNeasy column, and it was centrifuged again under the same conditions. The supernatant was discarded and 10µL of DNase I solution (RNase-Free DNase Set, QIAGEN) was added to 70µL of Buffer RDD and gently mixed. Then 80µL of the DNase I mix was added directly to the RNeasy column, and it was incubated at room temperature for 15 minutes.

Subsequently, 350µL of Buffer RW1 was added to the column, centrifuged, and the supernatant was discarded. Then, 500µL of Buffer RPE was added to the column, and it was centrifuged again under the same conditions.

Next, 500µL of Buffer RPE was added, and the column was centrifuged at 10000xg for 2 minutes, followed by discarding the supernatant.

The column was placed in a new 2mL collection tube and centrifuged for 2 minutes at maximum speed to dry the membrane. The RNeasy column was then placed in pre-labeled new 1.5mL Eppendorf tubes, and 30µL of RNase-free water was added directly to the column's membrane. The tube was centrifuged for 2 minutes at 10000xg. As a result of the process, 30µL of RNA was obtained in the 1.5mL Eppendorf tube and stored at -80°C.

3.4.1 RNA quantification

To verify if the RNA extraction was successful, the RNA quantification was performed using the Nanodrop spectrophotometer equipment. (ThermoFisher Scientific, IGC common equipment). To analyze the RNA samples, it was necessary to open the Nanodrop software and choose the "Nucleic Acids" option.

Before starting the measurement of the actual sample, it was necessary to run a "blank" sample, for which we used distilled water. The following steps were followed: open the metal arm of the spectrophotometer, and pipette 1 µL of the blank sample onto the pedestal, then close the arm. In the program, it was chosen the "Blank" option, once the blank sample is measured, the "Measure" button will become active, allowing the user to measure the desired sample. After finishing each measurement (including the blank), the pedestal was cleaned with a tissue.

During the readings, it is important to analyze the peaks and graphs obtained for each sample to check for any contaminations or impurities. Pure RNA typically shows absorbance values between 2.0 and 2.2. This range can indicate the quality and purity of the RNA sample.

3.4.2 cDNA Synthesis

For cDNA synthesis, the Xpert cDNA Synthesis Supermix kit was used. After quantifying the RNA, the concentrations of RNA to be used for each sample were calculated so that the starting concentration of RNA for each cDNA synthesis reaction was of 500ng and 1000ng depending on the quantification. The RNAs were diluted with RNase-free water up to 16µL using new, pre-labeled 1.5mL Eppendorf tubes. Next, 4µL of Xpert cDNA Synthesis Supermix (5x) was added to each Eppendorf containing the RNA solution, making a total volume of 20 µL in each tube.

The Eppendorf tubes containing the RNA and Supermix were incubated at 37°C in a water bath for 15 minutes, followed by an additional incubation at 10°C in a thermoblock for 10 minutes.

After the incubation, the cDNA samples were stored at -80°C for later use in PCR. It is important to note that no quality control was performed during this step because quality controls were planned to be used during the PCR technique. The cDNA samples obtained from this synthesis will be used as templates for PCR, where quality controls are typically incorporated to assess the integrity of the cDNA and the success of the synthesis reaction.

3.5 Quantitative Real Time PCR

For the quantitative real time PCR technique, after cDNA synthesis, it was necessary to dilute the cDNA samples with 60µL of RNase-free water. This step was only required once for each cDNA sample.

For this analysis, a prior plate map was prepared, containing the samples and controls of the experimental assay in triplicates (3 technical replicates for each sample); also included controls for the PCR, such as the No Template Control (NTC, consisting of RNase-free water) and control wells with dilutions of one of the samples to test for the linearity of the amplification for each primer pair. 384-well plates were used, and for each PCR, mixes were prepared for each gene to be analyzed. The volumes for each well were 1µL of Forward primer,1µL of Reverse Primer (specific for each gene), 5µL of SYBR Green, and 2µL of RNase-free water. These calculations were then adjusted for each mix depending on the number of wells. The primers were designed using PrimerBank (Supplementary data 14 for human histone genes, and Supplementary data 15 for mice histone genes). In each well, 9µL of the mix for each gene to be analyzed was addedand 1µL of cDNA sample.

After placing all the samples and replicates into their respective wells, the plate was sealed with a PCR seal and run on the qPCR equipment (QuantStudio 7 Flex Thermofisher Applied Biosystems, Common equipment, IGC) for 1 hour and 45 minutes. The amplification protocol included an initial denaturation of 5 minutes at 95°C; the PCR ran for 40 cycles, each with 15 seconds of denaturation at 95°C and 1 minute annealing/ extension at 60°C. For quality control of the specificity of the primer pairs, a melting curve was performed after the amplification cycles were completed, the housekeeping gene used as reference was GAPDH.

After the run time, the obtained results needed to be analyzed using specific formulas and calculations:

$$\Delta C_T(test) = C_T\ (target, test) - C_T(ref, test)$$

$$\Delta C_T\ (calibrator) = C_T(target, calibrator) - C_T(ref, calibrator)$$

$$\Delta\Delta C_T = \Delta C_T(test) - \Delta C_T(calibrator)$$

$2^{-\Delta\Delta CT}$ = Normalized expression ratio

Protein extracts

Cells in plates resuspended in RIPA lysis buffer (supplementary data 4), (previously prepared by the host laboratory) were thawed on ice and transferred to cold Eppendorf tubes. Cell lysates were then centrifuged at 14 000rpm, 4ºC for 15 minutes. The supernatants were collected into new cold Eppendorf tubes and quantified.

3.6 Protein quantification – Bradford assay

This quantification was prepared in a 96 well plate map including 6 standards wells, a well with each sample, a blank control of water, all in duplicate (Supplementary data 5).

The protein extract was thawed on ice. Next, the standards (Stock BSA solution is at 10mg/mL) were prepared using serial dilutions as explained in Supplementary data.

For each standard well, 160µL of standard solution was pipetted, and for each blank well, 160µL of H_2O was added, both in duplicate.

For each sample well, 160µL of H_2O and 1 µL of the protein sample were added.

Next, 40µL of the Quick Start Bradford reagent was added to each well and mixed gently. The plate was then incubated in the dark for 5 minutes.

After the incubation, the plate was placed into a Tecan Infinite 200 plate reader, and the absorbance was measured at 595nm. The quantification of each sample was performed after plotting the data of the standards into a linear calibration curve.

3.7 Western Blot

3.7.1 Gel preparation for Western blot

To start the Western Blot technique, it was necessary to prepare the acrylamide gels, for which the glass plates were arranged in a plastic chamber with the purpose of holding the gels while polymerizing.

The separating gel and the stacking gels were then prepared. For the separating gel, the protein size and the percentage of acrylamide in the gel were considered. First, a test was performed with the Actin protein, which has an approximate size of 42kDa, and the Histone proteins have sizes between 11-17 kDa, depending on the type of Histone. Therefore, a 15% percentage of acrylamide was used for 2 gels of 1.0 mm thickness (see supplementary data 6 and 7).

After preparing the gels, the separating gel was poured into the chamber glass until it reached around 2/3 of the chamber height.

Next, distilled water was added over the separating gel to allow the formation of asmooth line interface on top of the gel. The gel was allowed to polymerize, which typically takes about1 hour or until it was visibly solidified. After that, the excess water was discarded.

Then, the comb was carefully placed on top of the separating gel, and the gel stacking solution was added gently around the comb to avoid forming bubbles. The gel was allowed to polymerize for about 30-40 minutes.

Once the gels were ready, the glass plates containing the gels and the comb were removed from the plastic chamber and wrapped in damp paper to prevent them from drying out. The gels were then stored at 4°C until they were ready to be used for the Western Blot technique.

3.7.2 Sample dilution and Protein denaturing

The protein samples were maintained cold and diluted with the appropriate volume of distilled water to the concentration of 15µg of protein in 10µL of volume. Then, 10µL of cold 2x Laemmli buffer (previously prepared by the host laboratory: 4% SDS (w/v), 20% (v/v) glycerol, 0.004% (w/v) bromophenol blue, 0.125M Tris-Cl, pH 6.8), freshly supplemented with 5 % of 2-mercaptoethanol, were added to all samples.

The proteins were denatured by incubating for 5 minutes at 98ºC in the thermoblock, then the samples were spined down and let cool down for a few minutes. Any precipitates that formed were not loaded in the gel.

3.7.3 SDS-PAGE Gel running

The gel chamber was assembled and filled with SDS Running buffer (Supplementary data 8). The proteinladder (Amersham ECL Rainbow Marker – Full Range #RPN800E) was loaded in one of the wells (8µL) and the samples (20µL) in the remaining wells using capillary tips.

The run was started at 80V until the samples reached the separating gel and then at 100V maintaining constant voltage.

3.7.4 Blotting (wet) -Nitrocellulose membrane

The appropriate transfer buffer was prepared (Supplementary data 9**)**. Filter paper and membrane were cut at the approximate size of the gels. Containers were filled with buffer to soak filter paper, membrane and sponges and an additional big container (Styrofoam box) was filled with ice to fit the transfer cassette.

The transfer sandwich was opened, and everything was kept soaked with transfer buffer. The transfer cassette black part was placed down and laid one sponge and three filter paper sheets. Carefully, the stacking gel was cut off, the separating gel was placed over the filter paper and the membrane on top of the gel.

Three filter paper sheets were placed over the membrane and used a serological pipette to roll over the sandwich to get air bubbles out. Lastly the sponge was added and closed the transfer cassette.

The cassette was placed in the transfer container (black part to black) with buffer until the top. Next the transfer container was closed and was placed in a Styrofoam box with ice to keep it cold.

Then it was necessary to run the transfer at constant 300mA (max 200V) for 90 minutes. After the transfer was completed, the cassette was removed from the buffer and opened. The gel and the filter papers were discarded, and the membrane was carefully removed and marked if required (in case there are more than one membrane to be distinguished). The membrane parts that exceeded the limits of the gel were cut.

3.7.5 Blocking and Primary Antibody

The membrane was incubated in the blocking solution for 1 hour, shaking at room temperature in a small plastic container. The primary antibody mix was prepared in blocking solution at 1:2000 dilution. The membrane was then incubated with the respective primary antibody - Anti-β-actin from mouse, (Santa Cruz sc-58673) - overnight, shaking at 4ºC (Supplementary data 12).

3.7.6 Washing and Secondary Antibody

On the following day the primary antibody was collected into one falcon tube and stored for future use at -20 ºC. The membrane was washed three times with TBS-T (Supplementary data 11) and shaking for 10 minutes each time. The secondary antibody (anti-mouse horseradish peroxidade (HRP) - conjugated, Abcam ab6823) was added mixed in blocking solution at 1:4000 dilution and incubated for 1 hour at RT. After the 1 hour shaking the secondary antibody was collected and stored at -20 ºC for future use and the membrane was washed again 3 times for 10 minutes with TBS-T.

3.7.7 Detection / Developing

The detection solution, consisting of a substrate for HRP, (ECL prime WB detection GE Healthcare #RPN2232) was prepared mixing solution A and B at a ratio of 1:1 (5mL per blot). Then the membranes were placed in a clean container and the detection solution was pipetted over the membranes so that the whole surface of the membrane is covered.

With tweezers the membranes were adjusted so that they were completely soaked and then were incubated for approx. 5 minutes. The excess detection solution was dried with a paper and the membrane was placed in the detection tray from the GE Amersham 680 Imager (Common equipment, IGC) with the protein side facing up. It was used the Auto function to acquire images.

3.8 Quantification and statistical analysis

Data are expressed as mean values ± standard deviation. Excel was used for data analysis and for the graphical representation of the ELISA results. Statistical analysis was performed with GraphPad Prism 10.0 (GraphPad Software) and this program was also used for the graphical representation of the qRT-PCR results. The unpaired nonparametric Mann–Whitney test was used for comparison two treatment conditions and the following symbols were used in figures to indicate statistical significance with a confidence level of 95%: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

This statistical analysis was used on TNF-α data in each experiment and is shown in one of the graphs in the results as an example. It was also used statistical analyses on some histones; however, it is not shown in the results, as our analysis was not strictly dependent on the statistical significance of the results.

All raw data is deposited in IGC's internal server and is available upon request and for future studies. Details of all the experiments performed can be found in IGC's electronic Lab Book.

4 Results

4.1 TNF and Histone regulation by Anthracyclines and inflammatory stimuli in THP-1 cells

4.1.1 TNF secretion following treatment with Epirubicin and stimulation with TLR agonists

Tumor necrosis factor (TNF-α or TNF) is a very important cytokine produced essentially by macrophages in response to an infection, mostly through TLR4 stimulation, but also, less significantly, by TLR2 stimulation. To test if the THP-1 cells could respond to pro-inflammatory stimuli by producing inflammatory cytokines such as TNF, similarly to macrophages, it was performed the ELISA technique on the supernatants of the cultures of THP-1 cells to measure the TNF production to different infection stimuli (4.1 A). TNF-α production was also quantified after treatment with Epirubicin (Epi) to determine if the behavior of this batch of THP-1 cells was in line with the expected in the presence of Epi (4.1 B): previously, the host lab has published that treatment with Epi leads to dose-dependent repression of TNF after TLR4-mediated inflammatory stimulation (Chora et al., 2020).

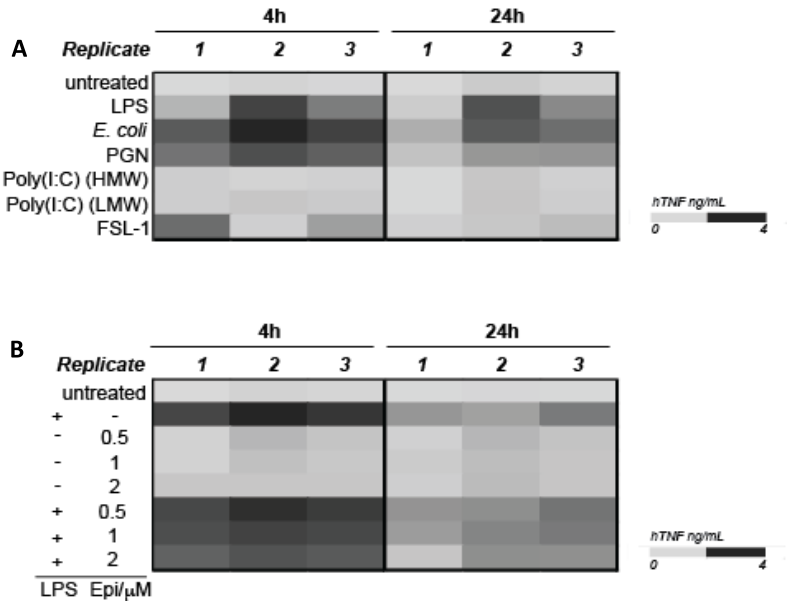


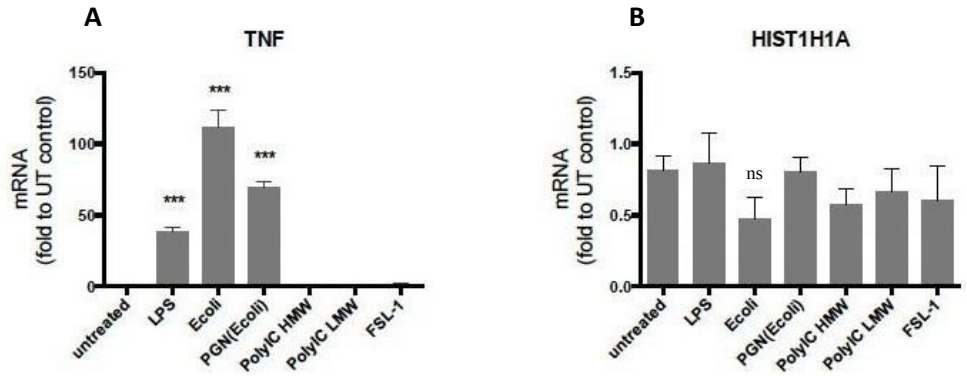
Figure 4.1 – TNF secretion by THP-1 cells following stimulation with TLR agonists and treated with Epirubicin (Epi). Figure **A** shows a heatmap of the TNF secretion quantified in the supernatants of cells untreated or stimulated with pro-inflammatory agents for a 4h and 24h period. Three replicates, corresponding to three independent experiments, are shown and the agonists used were: LPS, *E. coli* (TLR4 stimulants); PGN (TLR2 stimulant); PolyIC of High and Low molecular weight (TLR3) and FSL-1 (TLRs 2 and 6). The figure **B** heatmap shows TNF secretion quantified in the supernatants of cells untreated or treated with various concentrations of Epi for 4h and 24h period in the presence or absence of LPS. Three independent experiments were performed (replicates 1 to 3), with averages of technical replicates for each sample (three technical replicates for each condition corresponded to different wells in the assay plates).

The significant production of TNF by THP-1 cells treated with LPS and *E. coli* in all replicates assayed shows a robust response to these TLR4 agonists, as expected from this monocytic cell line (Fig. 4.1A). Also as expected from the literature, in the 24h period, there is a significant decrease in production of TNF in both treatments; this suggests an upregulation of this cytokine in the first hours of infection, and a downregulation over time due to negative feedback mechanisms to avoid excessive inflammation damages, whereas in the supernatants protein degradation occurs over time therefore compromising detection, and some possible cell death may also occur caused by the persistence of the inflammatory stimuli. Both time periods do not show any expression of TNF in the unstimulated cells. The cells stimulated with PolyIC have shown no expression of TNF, because this agonist is recognized by TLR-3 receptors, which do not signal intracellularly towards pathways that lead to production of this cytokine. There is also an increase, although less pronounced, in the secretion of TNF with PGN and FSL-1, with some variability between results in the replicates, but the general trend was towards low induction of this cytokine. These results show that TLR2 can also signal towards transcriptional induction of TNF.

In the chart B (Fig.4.1B) there is also an increased production of TNF in cells treated with LPS in the 4h range period. Epi was capable of repress TNF depending on the concentration used, as previously observed (Chora et al., 2020).

4.1.2 Regulation of histone mRNA following treatment with Epirubicin and stimulation with TLR agonists

After testing the TNF expression in ELISA to verify the capacity of activation of the THP-1 cells, it was conducted different quantitative RT-PCT (qRT-PCRs) to measure Histone expression. From a previous RNAseq experiment in the host laboratory, data was suggestive of induction of histones by Epi during inflammation (in LPS-stimulated cultures of primary mouse macrophages). As histone regulation by anthracyclines (the family of drugs of Epi) or by other DNA-targeting anti-tumor drugs is unknown, we decided to further investigate this observation. We decided to test if histones are regulated by any inflammatory stimulus (4.2) and by Epi in the presence of inflammation (LPS, as observed before in the RNAseq) or independently of inflammatory stimuli (4.3, 4.4 and 4.5). THP-1 was the selected cell line for its resemblance with macrophages (see above) and the expression of selected histones was tested by qRT-PCR. Independent experiments were performed and results for each are shown below.



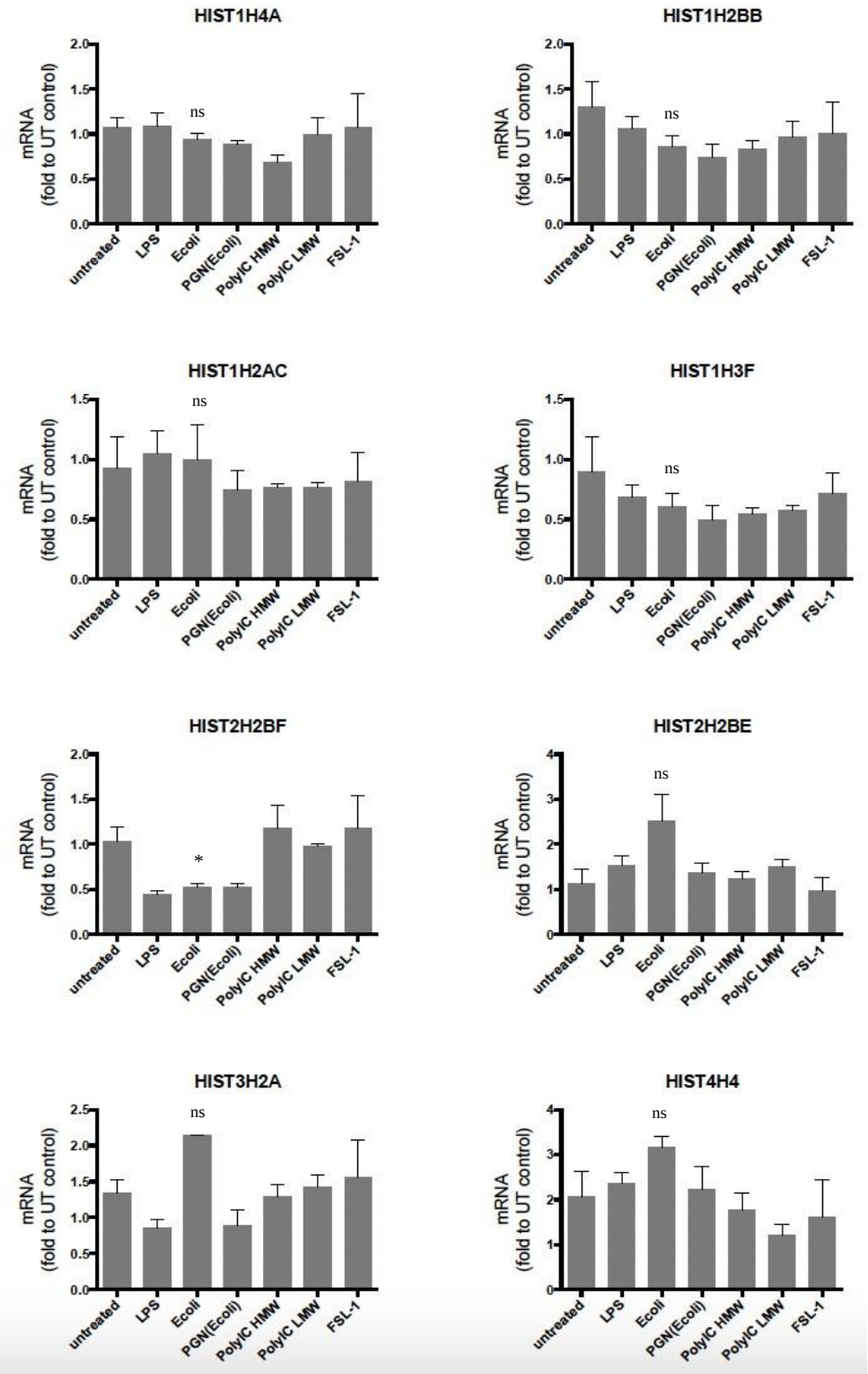
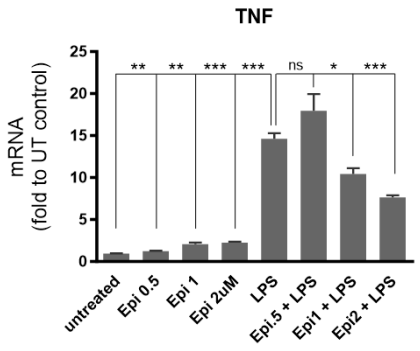


Figure 4.2 – Expression of TNF and different types of histones after stimulation with TLR agonists in THP-1 cells. The qRT-PCR analysis was used to determine the relative levels of mRNA after 4h of stimulation with selected TLR agonists compared withthe mRNA levels of untreated cells. The agonists used were: LPS, *E. coli* (TLR4 stimulants); PGN (TLR2 stimulant); PolyIC of High and Low molecular weight (TLR3) and FSL-1 (TLRs 2 and 6). A, relative levels of TNF mRNA; B, relative levels ofhistones mRNAs. Averages for technical triplicates are shown, the unpaired nonparametric Mann–Whitney test was used for comparison of two selected conditions and the following symbols indicate statistical significance with a confidence level of 95%: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. In this case, the selected conditions that were compared statistically for all histone genes were untreated vs E. coli.

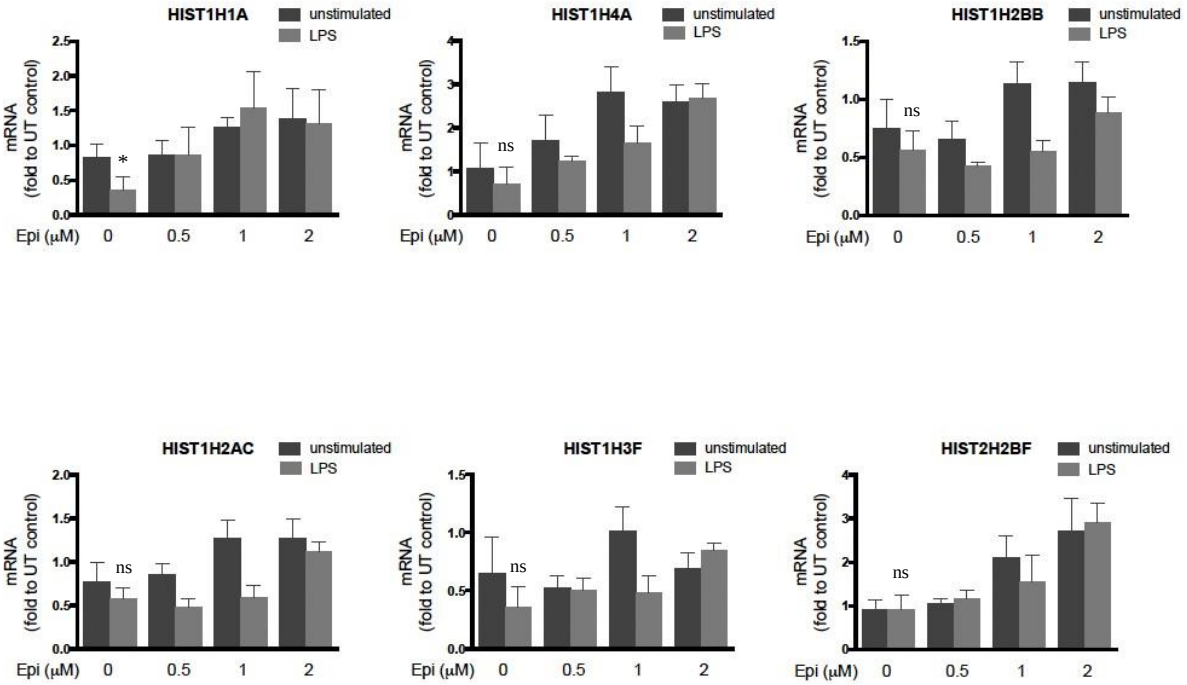
The qRT-PCR of the TNF expression confirms what was seen in the ELISA technique: the fold value of 40 time higher than the control (in the LPS and PGN treatments) shows the elevated expression of this cytokine, which is especially increased in the cells treated with *E. coli*, that showed fold value of 100x more than the control. However, the overall histone expression does not seem to have significant changes in the levels of expression following TLR stimulation (less than 2-fold change in value). HIST2H2BE, HIST3H2A and HIST4H4 show an increased level in expression with *E. coli* stimulus, that suggest the possibility of some regulation of expression of these Histones with a component of *E. coli.*

After testing histone regulation in THP-1 cells by TLR agonists, the effects of Epirubicin were tested. For that, cells were treated with 3 different concentrations of Epi (0.5µM,1 µM and 2 µM) and 100 ng/mL of LPS stimuli was added one hour after the pre-incubation with Epi and used to stimulate cells for 4 hours.

A



B



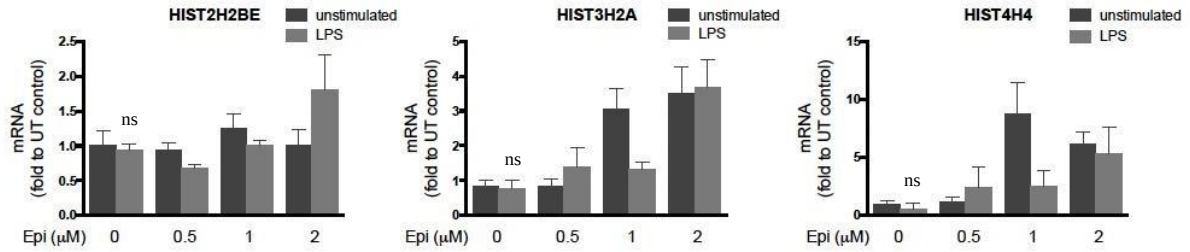
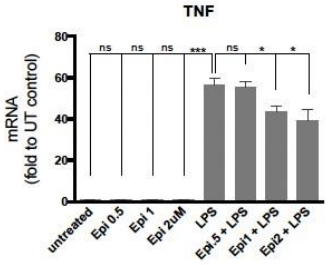


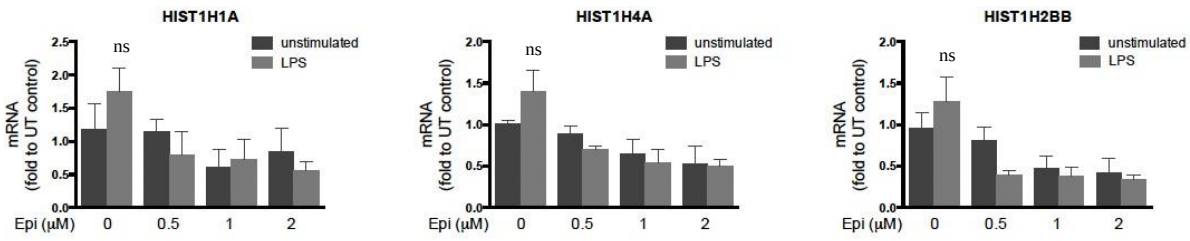
Figure 4.3 – TNF and different types of histones expression treated with Epirubicin (Epi) and stimulated with LPS in THP-1 cells (replicate 1). qRT-PCR analysis was used to determine the relative levels of mRNA (compared with untreated cells, UT) aftervarious concentrations of Epi treatments for 1 hour that were followed by 4h of stimulation with LPS. A, relative levels of TNFmRNA; B, relative levels of histones mRNAs. Averages for technical triplicates are shown, the unpaired nonparametric Mann–Whitney test was used for comparison of two selected conditions and the following symbols indicate statistical significance with a confidence level of 95%: *p < 0.05; **p < 0.01; ***p < 0.001.

In chart A (Fig. 4.3), all samples stimulated with LPS show an elevated expression of TNF (fold change to control >5 times higher), but the expression was not regulated by Epi (as already known from published results). This result was in line with the observations from the ELISAs shown above. In charts B (Fig.4.3), only HIST1H4A, HIST2H2BF, HIST3H2A, and HIST4H4 showed a significant change in the fold to UT control (>2) when cells were treated with 2 μM of Epi, mostly independent of LPS stimulation. HIST3H2A expression in Epi concentrations 1 and 2 μM was higher than 3-fold compared to UT control without LPS stimulation. HIST4H4 was the histone where the regulation was more pronounced: showed an expression of more than 5-fold to UT control in the cells treated with Epi (1 and 2 μM) and treated with Epi 2 μM in the presence of LPS.

A



B



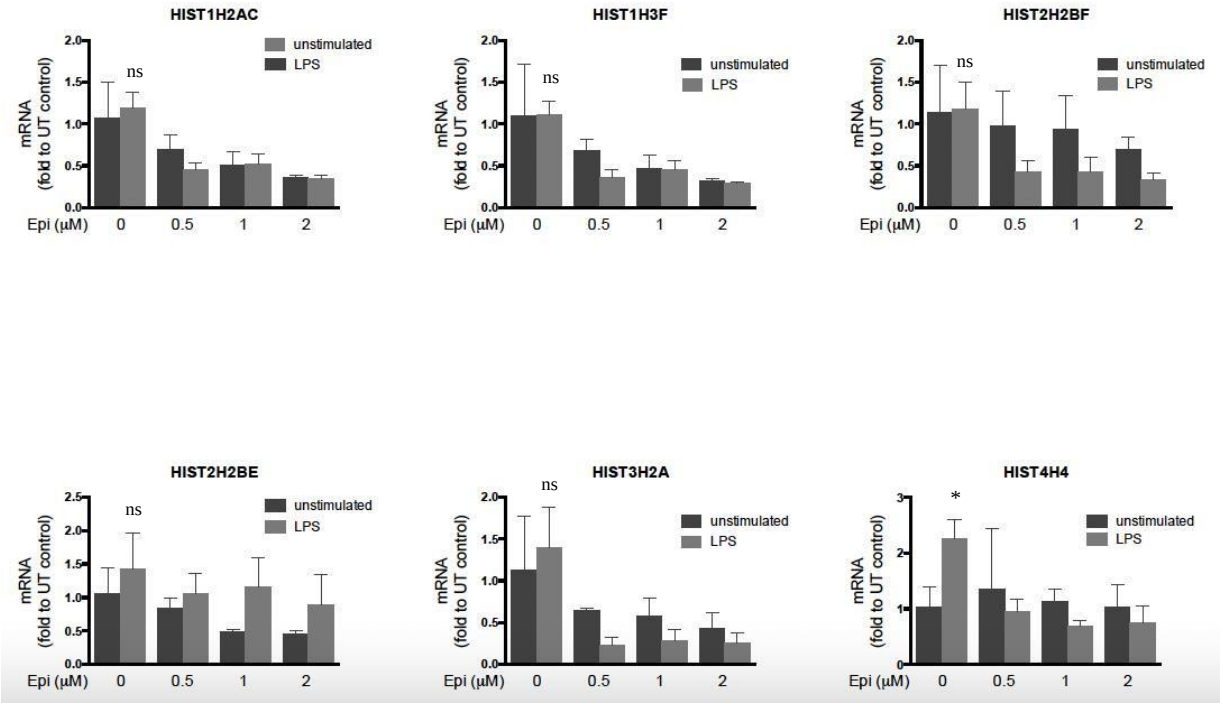
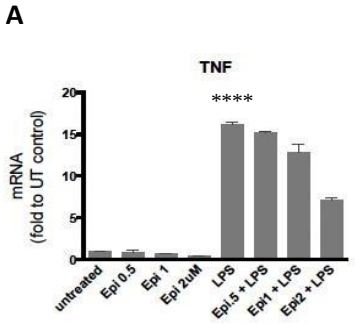


Figure 4.4 – TNF and different types of histones expression treated with Epirubicin (Epi) and stimulated with LPS in THP-1 cells (replicate 2). qRT-PCR analysis was used to determine the relative levels of mRNA (compared with untreated cells, UT) aftervarious concentrations of Epi treatments for 1 hour that were followed by 4h of stimulation with LPS**.** A, relative levels of TNFmRNA; B, relative levels of histones mRNAs. Averages for technical triplicates are shown, the unpaired nonparametric Mann–Whitney test was used for comparison of two selected conditions and the following symbols indicate statistical significance with a confidence level of 95%: *p < 0.05; **p < 0.01; ***p < 0.001. In this case, the selected conditions that were compared statistically for all histone genes were unstimulated vs LPS treatment.

As already observed in the previous experiment, TNF in chart A (Fig. 4.4) shows very significant values of expression, of more than 40-fold to UT control and there is a downregulation of expression of this cytokine in higher concentration of Epi. Importantly, there was no TNF expression in cells unstimulated or in cells treated with Epi alone. These results are well in agreement with the ELISA results and with previous published data. In charts B (Fig.4.4), all histones expression had low values of fold change (<2), which suggested that there was no significant regulation. Most of Histones had between 0.5-1.5-fold change in expression after Epi treatments or LPS stimulation.



B

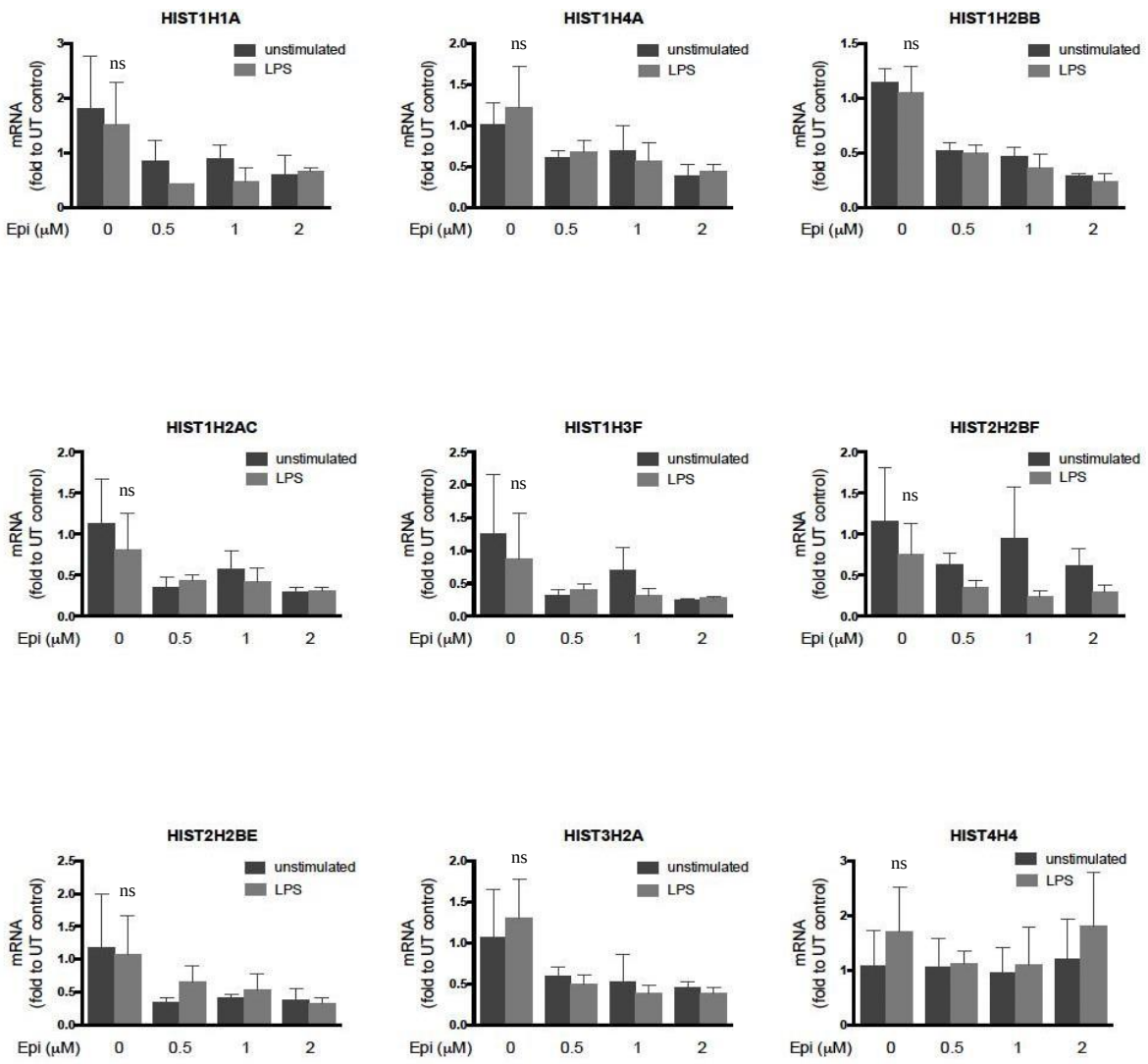


Figure 4.5 – TNF and different types of histones expression treated with Epirubicin (Epi) and stimulated with LPS in THP-1 cells (replicate 3). The qRT-PCR analysis was used to determine the relative levels of mRNA (compared with untreated cells, UT)after various concentrations of Epi treatments for 1 hour that were followed by 4h of stimulation with LPS. A, relative levels of TNF mRNA; B, relative levels of histones mRNAs, the unpaired nonparametric Mann–Whitney test was used for comparison of two selected conditions and the following symbols indicate statistical significance with a confidence level of 95%: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. In this case, the selected conditions that were compared statistically for all histone genes were unstimulated vs LPS treatment.

As observed in the previous figures, in chart A (Fig.4.5), TNF shows significant values of induction of expression in all conditions stimulated with LPS: up to 15-fold increase when compared to UT control. As also observed in the previous experiments and in line with the ELISA results, the LPS induction of TNF was lowered by Epi treatments: a slight decrease with Epi 0.5 µM, followed by a more pronounced decrease with Epi 1 µM and down to approximately 5-fold to UT control with Epi concentration 2µM. In B charts (Fig. 4.5), it showed either a downregulation of Histones expression in Epi treatments or no regulation. There was also no significant regulation by LPS.

4.2 TNF and Histone regulation by Anthracyclines and inflammatory stimuli in primary mouse macrophages

To further test histone regulation by infection and Epi, in a context possibly providing more biological significance, we decided to extend the study to primary mice macrophages. Wild type animals were sacrificed for bone marrow isolation and differentiation experiments into macrophages were conducted; a series of experiments with independently differentiated macrophages were performed to test TNF and histone regulation.

4.2.1 TNF secretion following treatment with Epirubicin and stimulation with TLR agonists

The ELISA technique was conducted with the macrophage's supernatants, in a similar way to the THP-1 analysis but using a kit for the detection of mouse TNF instead of detection of human TNF. By testing the TNF production in response to LPS (and other TLR4/2 agonists), it is also possible to assess if an efficient macrophage differentiation was achieved. The following figures (Figs. 4.6 to 4.9) show the ELISA heatmap results of independent experiments.

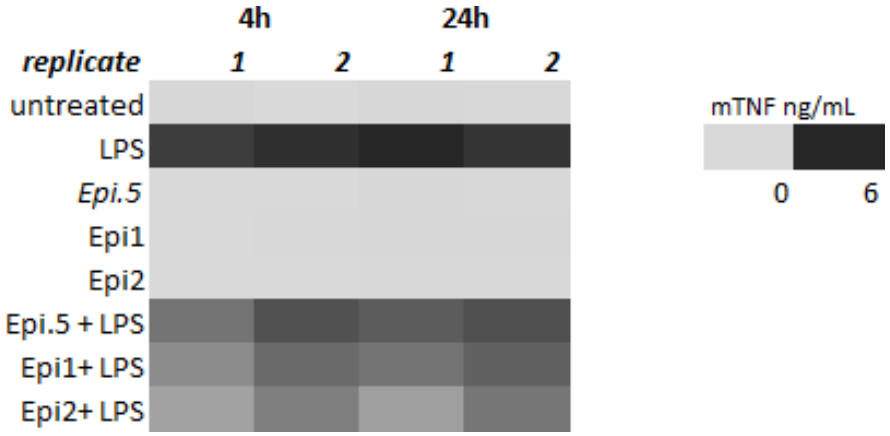


Figure 4.6 – TNF secretion of macrophages treated with Epirubicin (Epi 0.5 µM, 1 µM, 2 µM) in the presence or absence of LPS in macrophages (replicates 1 and 2). ELISA heatmap of the TNF secretion quantified in the supernatants of macrophages untreated ortreated with various concentrations of Epi for 4h and 24h period in the presence or absence of LPS. Averages of technical replicates are shown, corresponding to different wells in the assay plates.

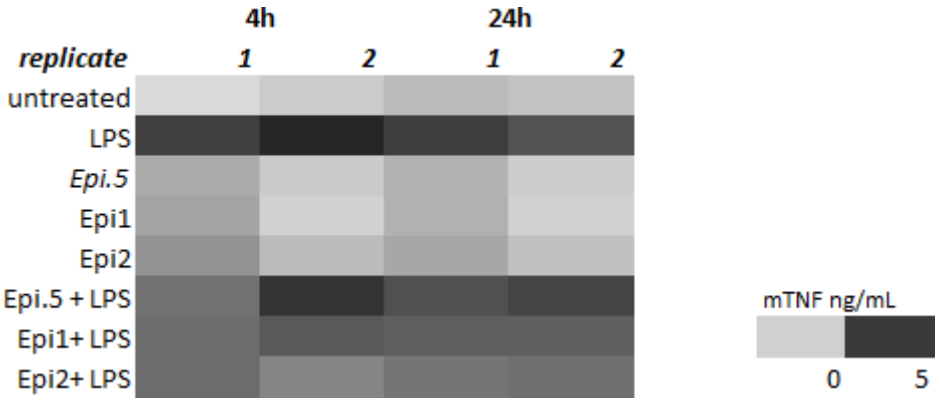


Figure 4.7 – TNF secretion of macrophages treated with Epirubicin (Epi 0.5 µM, 1 µM, 2 µM) in the presence or absence of LPS (replicates 3 and 4). ELISA heatmap of the TNF secretion quantified in the supernatants of macrophages

untreated or treated with various concentrations of Epi for 4h and 24h period in the presence or absence of LPS. Averages of technical replicates are shown, corresponding to different wells in the assay plates.

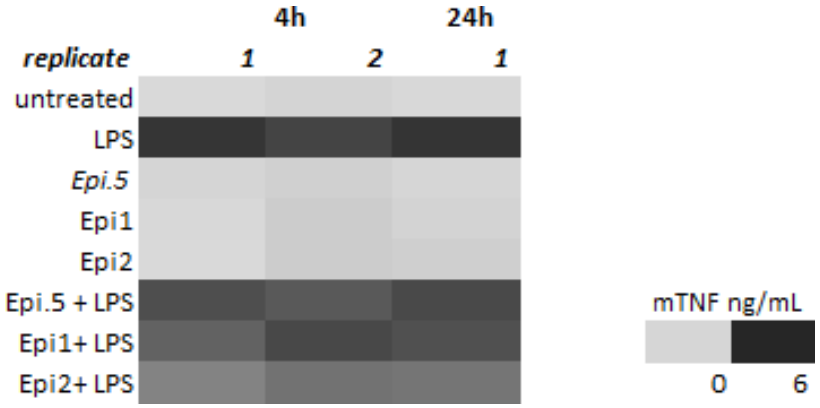


Figure 4.8 – TNF secretion of macrophages treated with Epirubicin (Epi 0.5 µM, 1 µM, 2 µM) in the presence or absence of LPS in macrophages (replicates 5 and 6). ELISA heatmap of the TNF secretion quantified in the supernatants of macrophages untreated ortreated with various concentrations of Epi for 4h and 24h period in the presence or absence of LPS. To note that macrophagesin replicate 6 were only treated for 4 hours. Averages of technical replicates are shown, corresponding to different wells in theassay plates.

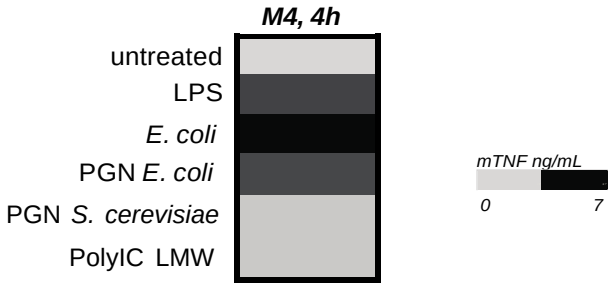


Figure 4.9 – TNF secretion of macrophages treated with TLR agonists in macrophages (replicate 4). ELISA heatmap of the TNF secretionquantified in the supernatants of macrophages untreated or stimulated with pro-inflammatory agents for a 4h. The agonists usedwere: LPS, *E. coli* (TLR4 stimulants); PGN from *E. coli* and *S. cerevisiae* (TLR2 stimulant); and PolyIC of Low molecular weight. Only one replicate is shown.

The secretion of TNF in primary macrophages shared similar results as the THP-1 cells. All experiences had elevated levels of secretion of TNF when cells were stimulated with LPS and compared with unstimulated, with or without the Epi treatment. In addition to LPS, other TLR4 and 2 agonists also led to increase in secretion of TNF; on the contrary, PolyIC did not induce secretion of TNF, because this agonist is recognized by TLR3 receptors, which do not signal intracellularly towards pathways that lead to production of this cytokine (Figure 4.9). In both time periods, the unstimulated cells, including the cells that were treated with Epi but without any infection, showed no TNF secretion.The macrophages stimulated with LPS showed, as expected, considerable increase in TNF secretion, both at 4 and 24 hours. Macrophages treated with Epi plus LPS showed significant levels of TNF production on both 4h and 24 period, but this secretion was lower than in cells without Epi. As with THP-1 cells above, and as previously published, Epi led to a dose-dependent decrease in TNF secretionafter LPS stimulation.

4.2.2 Regulation of histone mRNA following treatment with Epirubicin and stimulation with TLR agonists

After testing the TNF-α expression by ELISA, histone mRNA expression in primary mice macrophages was tested by qRT-PCR. Because the results from THP-1 cells did not seem to corroborate the previous RNAseq observations from the host laboratory suggesting induction of histones by Epi during inflammation, we decided to test in primary macrophages, where the results had been obtained. The main inflammatory stimulus used was LPS, as this was the same stimulus as used before in the RNAseq.

Independent experiments, referring to macrophages isolated from distinct animals, were performed and results for each are shown below, with averages for technical replicates for each sample (triplicates within each condition correspond to different wells in the assay plates).

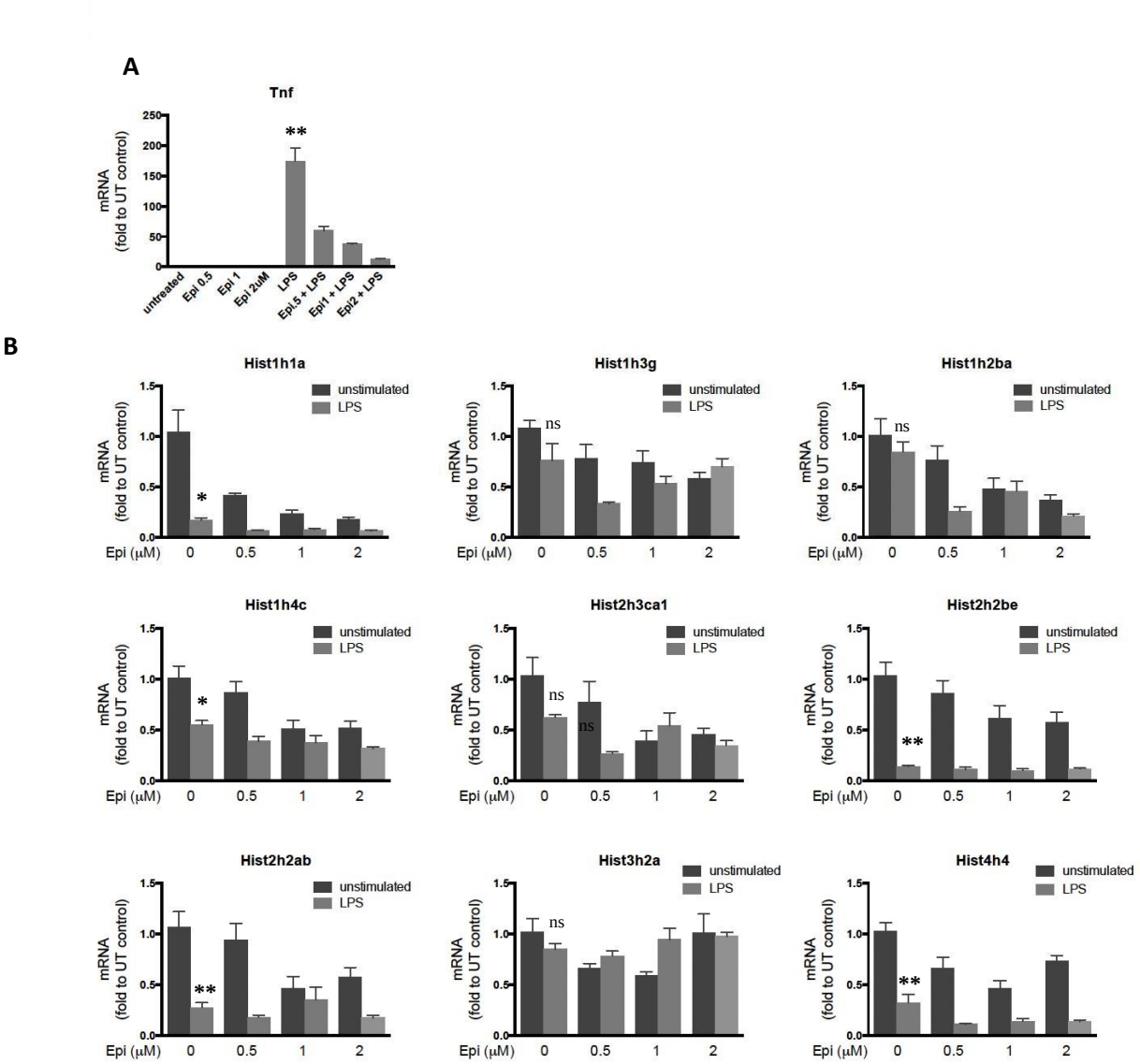


Figure 4.10 - TNF and different types of histones expression treated with Epirubicin (Epi) and stimulated with LPS in macrophages (replicate 2). The qRT-PCR analysis was used to determine the relative levels of mRNA (compared with untreated cells, UT)after various concentrations of Epi treatments for 1 hour that were followed by 4h of stimulation with LPS**.** A, relative levels of TNF mRNA; B, relative levels of histones mRNAs, the unpaired nonparametric Mann–Whitney test was used for comparison of two selected conditions, the following symbols indicate statistical significance with a confidence level of 95%: *p < 0.05; **p < 0.01; ***p < 0.001. In this case, the selected conditions that were compared statistically for all histone genes were unstimulated vs LPS treatment.

As expected from publications and from the results in THP-1 and the ELISA results with macrophages, in chart A (Fig. 4.10), there is a strong upregulation of TNF in cells stimulated by LPS (>150 times fold change value). When LPS-stimulated cells were treated with Epi, this pro-inflammatory cytokine showed still a significant expression, but this expression was decreased (between >10 and >50 times fold to UT control) due to the reported effects of Epi. In charts B (Fig. 4.10), there were no changes in histones regulation that were more pronounced than a fold change of equal or less than 1.

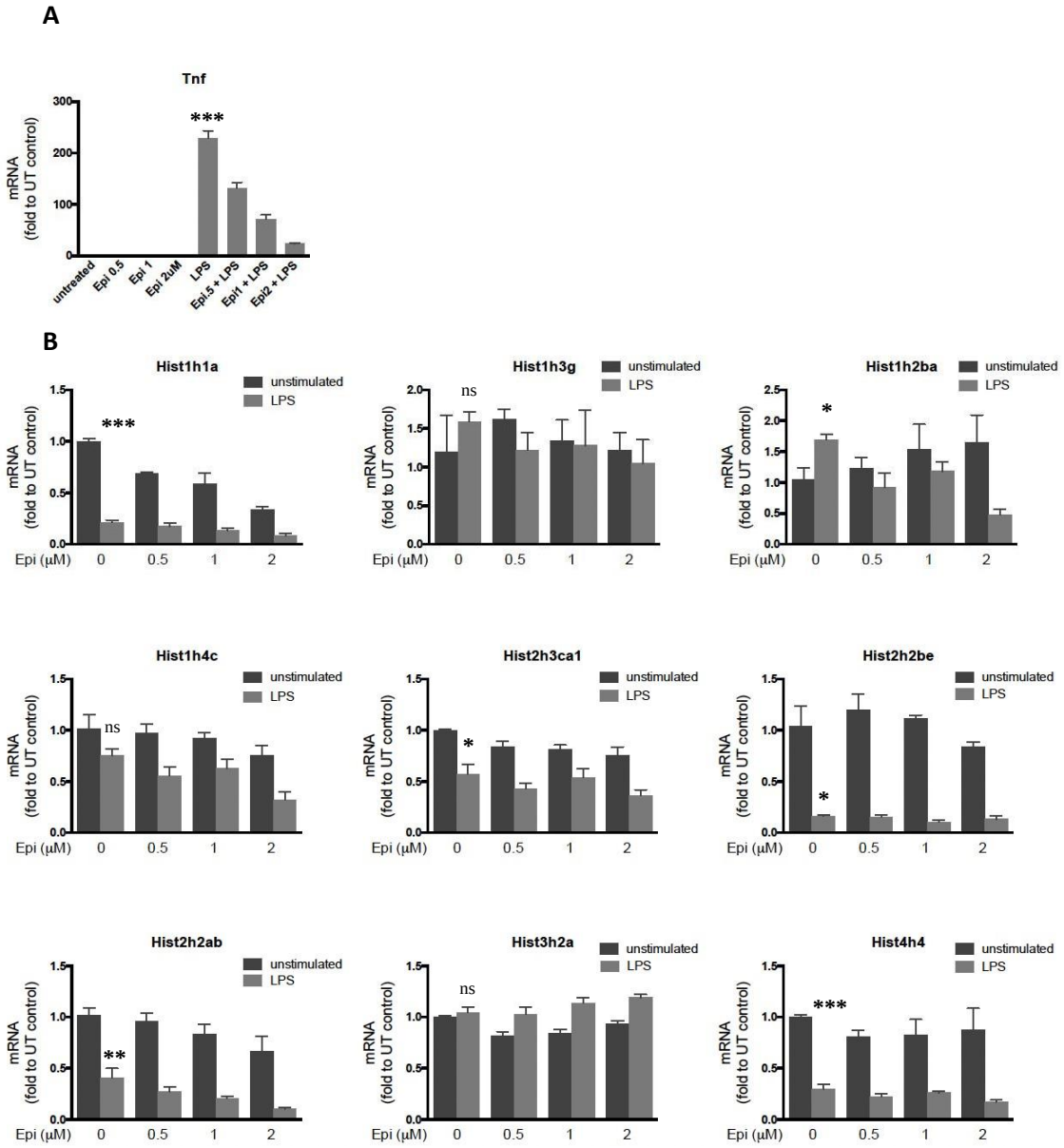


Figure 4.11 - TNF and different types of histones expression treated with Epirubicin (Epi) and stimulated with LPS in macrophages (replicate 3). The qRT-PCR analysis was used to determine the relative levels of mRNA (compared with untreated cells, UT)after various concentrations of Epi treatments for 1 hour that were followed by 4h of stimulation with LPS**.** A, relative levels of TNF mRNA; B, relative levels of histones mRNAs, the unpaired nonparametric Mann–Whitney test was used for comparison of two selected conditions, the following symbols indicate statistical significance with a confidence level of 95%: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. In this case, the selected conditions that were compared statistically for all histone genes were unstimulated vs LPS treatment.

Similarly, to the results in the previous figure, in chart A (Fig. 4.11), there is a strong upregulation of TNF in macrophages stimulated with LPS (>200 times fold change value). When LPS-stimulated macrophages were treated with Epi, there was a well-described decrease in TNF secretion that was Epi dose dependent. In charts B (Fig.4.11), there were no changes in histones regulation that were more pronounced than a fold change of 1 except for Hist1h3g and Hist1h2ba that have values of expression that increased more than 1, but still less than 2-fold change, after Epi treatments. This suggest that there is not a significant change in regulation with cells treated with Epi with or without LPS stimuli.

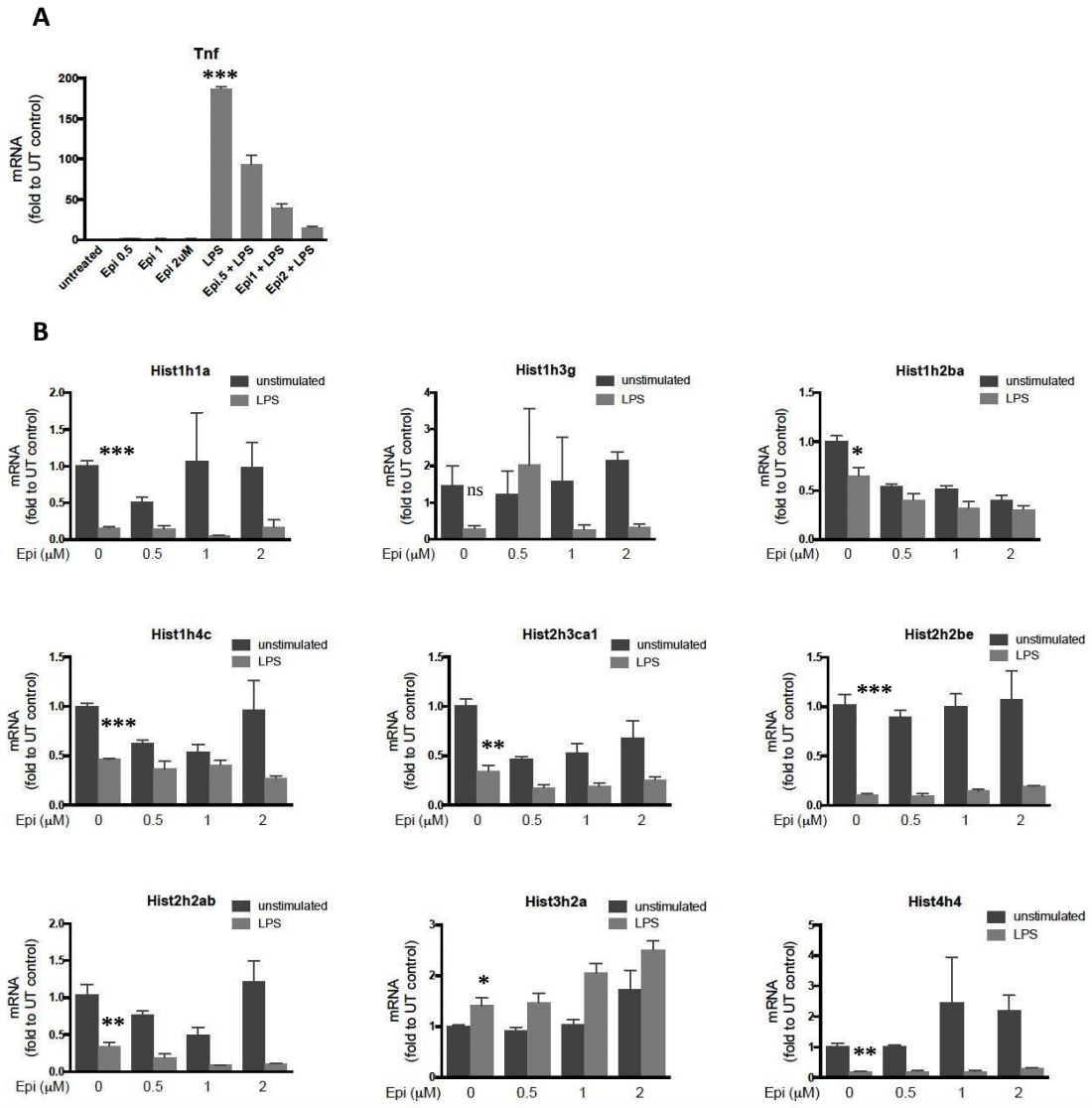


Figure 4.12 - TNF and different types of histones expression treated with Epirubicin (Epi) and stimulated with LPS in macrophages (replicate 4). The qRT-PCR analysis was used to determine the relative levels of mRNA (compared with untreated cells, UT)after various concentrations of Epi treatments for 1 hour that were followed by 4h of stimulation with LPS**.** A, relative levels of TNF mRNA; B, relative levels of histones mRNAs, the unpaired nonparametric Mann–Whitney test was used for comparison of two selected conditions, the following symbols indicate statistical significance with a confidence level of 95%: *p < 0.05; **p < 0.01; ***p < 0.001 In this case, the selected conditions that were compared statistically for all histone genes were unstimulated vs LPS treatment.

Similarly, to the results in the previous figures, chart A (Fig. 4.12) shows considerable upregulation of TNF in macrophages stimulated with LPS (>150 times fold change), which was repressed by treatment with Epi as before. The charts B (Fig. 4.12) do not show changes in expression that exceed fold change >1.5.

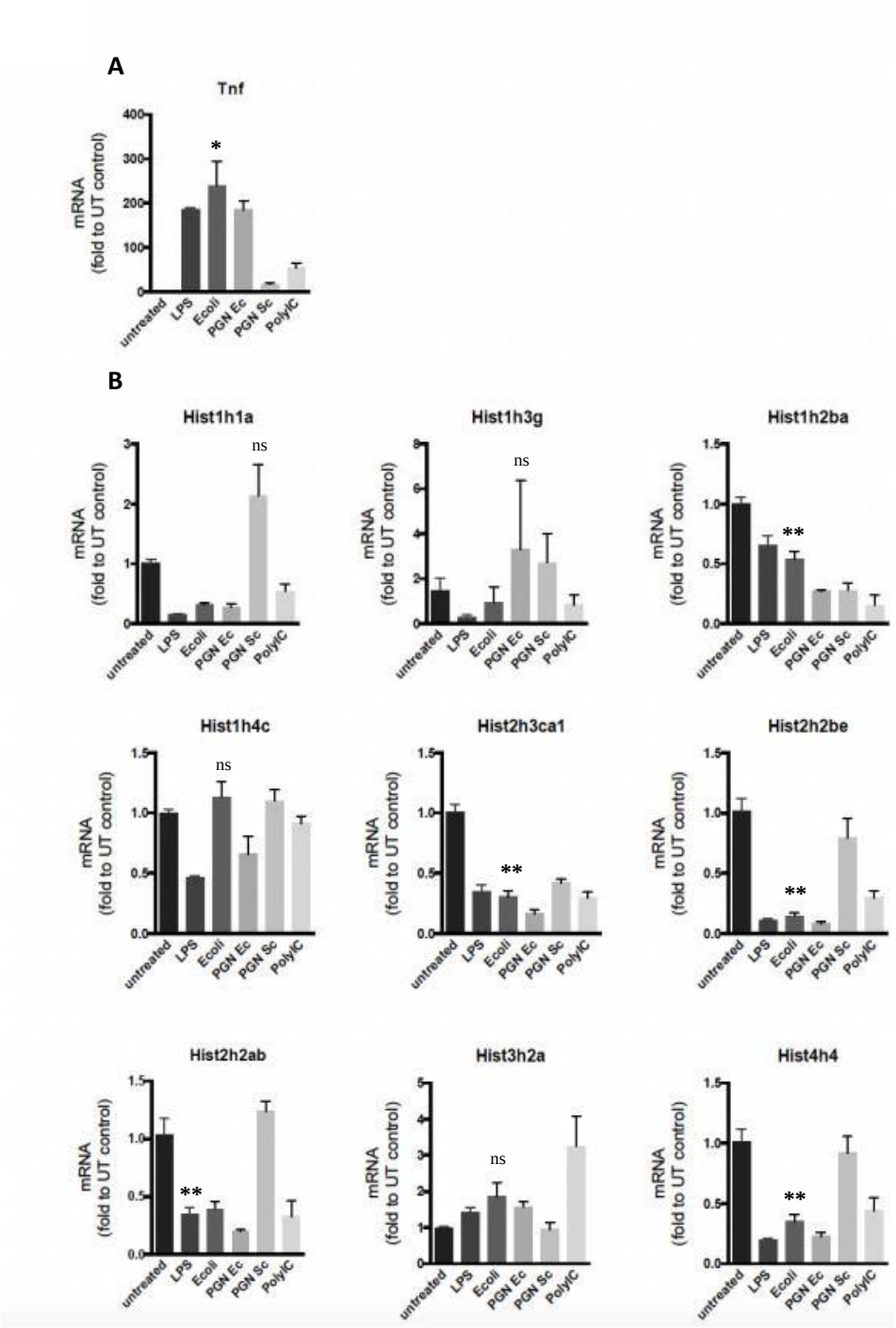


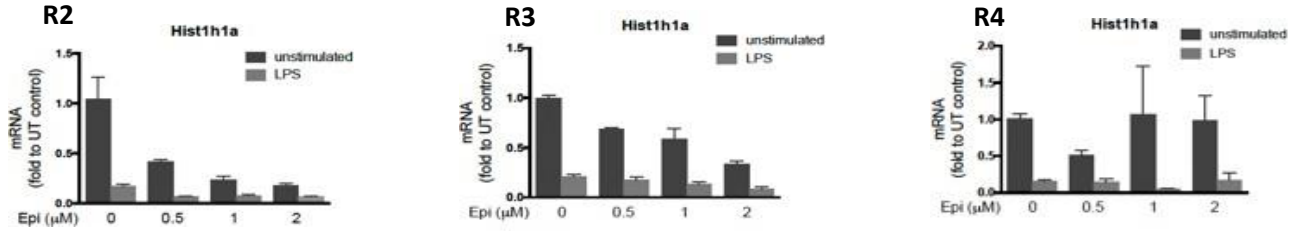
Figure 4.13 - Expression of TNF and different types of histones after stimulation with TLR agonists in macrophages (replicate 4). The qRT-PCR analysis was used to determine the relative levels of mRNA after 4h of stimulation with selected TLR agonists compared with the mRNA levels of untreated cells. The agonists used were: LPS, *E. coli* (TLR4 stimulants); PGN from *E. coli*and from *S. cerevisiae* (TLR2 stimulant); and PolyIC of Low molecular weight (TLR3). A, relative levels of TNF mRNA; B, relative levels of histones mRNAs, the unpaired nonparametric Mann–Whitney test was used for comparison of two selected conditions, the following symbols indicate statistical significance with a confidence level of 95%: *p < 0.05; **p < 0.01; ***p < 0.001. In this case, the selected conditions that were compared statistically for all histone genes were untreated vs E. coli.

In A (Fig. 4.13), there is a strong up-regulation of TNF with LPS, *E. coli* and PGN stimulated cells compared with untreated (150 times higher fold change). In B (Fig 4.13) Hist1h1a has fold change higher than 2 in cells stimulated with PGN *Sc*, and no regulation in the other stimuli. Also, with up- regulation was values Hist3h2a with PolyIC stimuli, which showed fold change values higher than 3 compared with untreated. Overall, histones mostly showed a downregulation in the expression of the Histones for the different stimuli of the experiments.

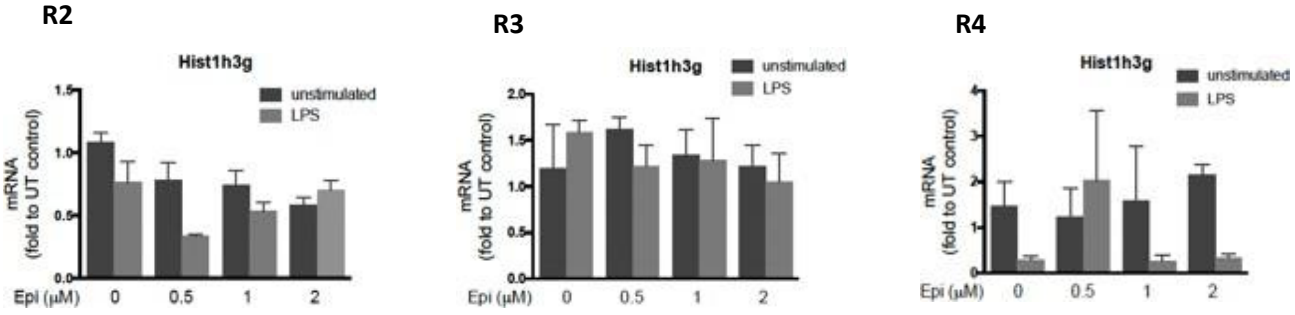
4.2.3 Regulation of histone mRNA by anthracyclines: comparison of the different tested histones

The results shown so far do not suggest that anthracyclines up-regulate the mRNA levels of the histones tested, as had been hypothesized in this project. We now present the same data already shown above but organized to facilitate the comparison between experiments, where each row shows one of the tested histone mRNAs and the three independent experiments performed can be compared.

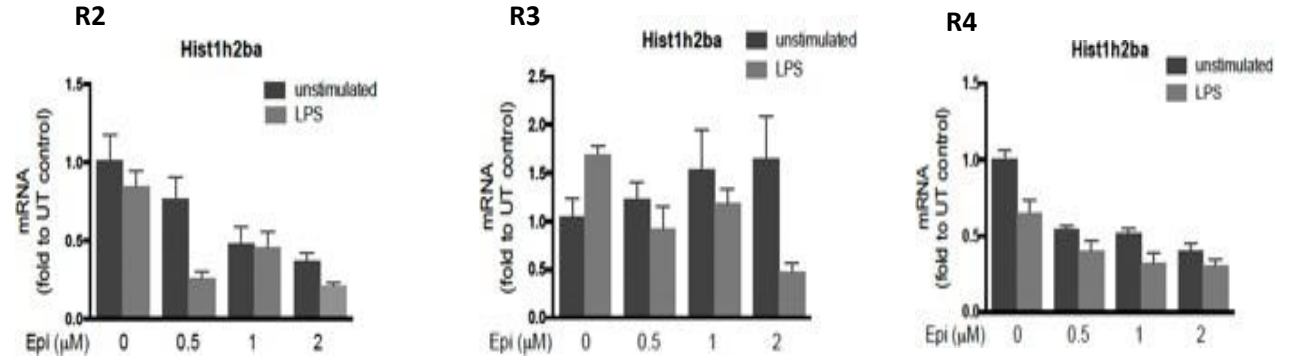
A. Hist1h1a



B. Hist1h3g

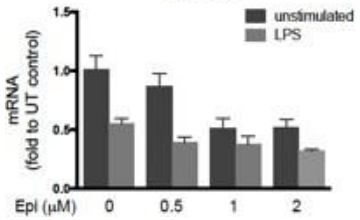


C. Hist1h2ba

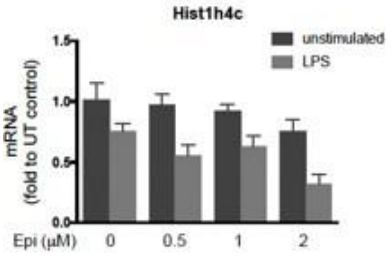


D. Hist1h4c

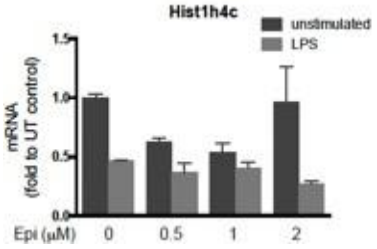
R2



R3

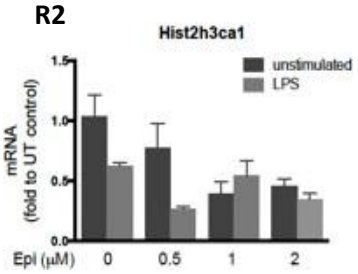


R4

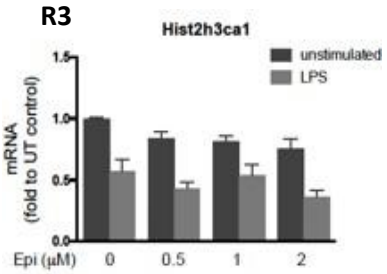


E. Hist2h3ca1

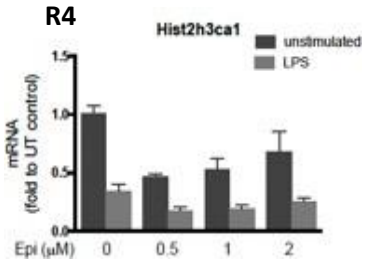
R2



R3

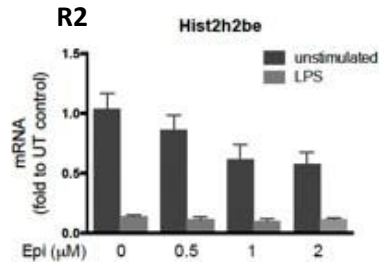


R4



F. Hist2h2be

R2



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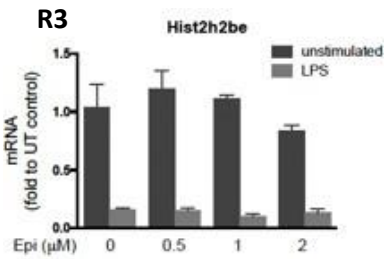
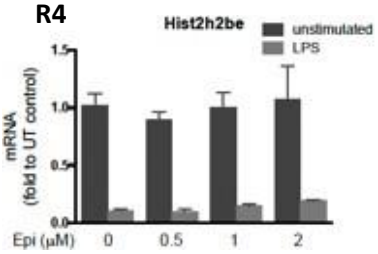
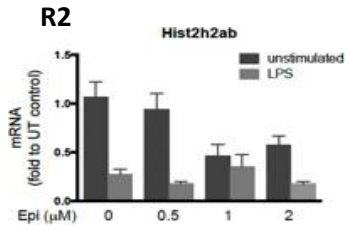


R4

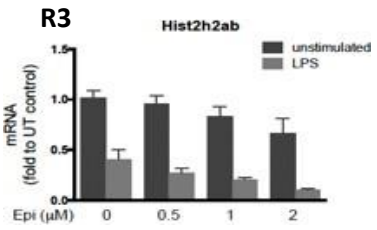


G. Hist2h2ab

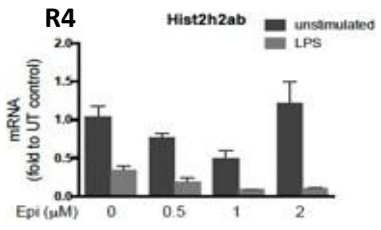
R2



R3

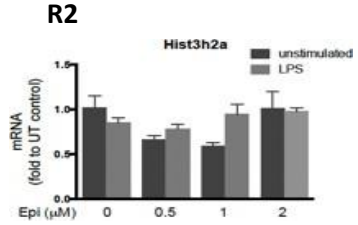


R4

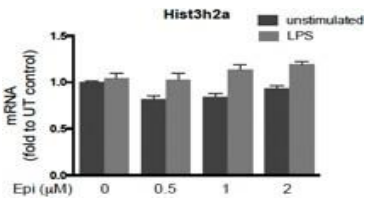


H. Hist3h2a

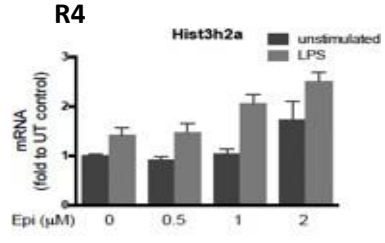
R2



R3



R4



I. Hist4h4

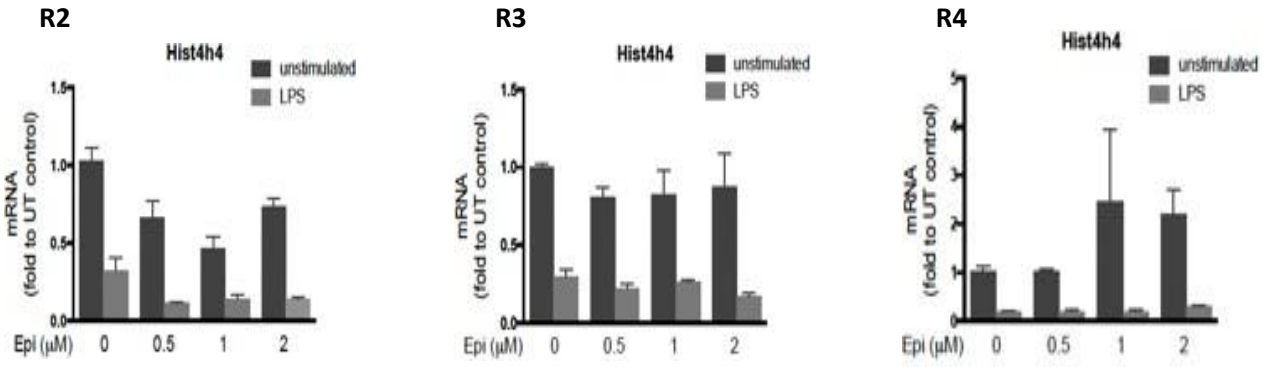


Figure 4.14 – Comparison figure of Histone regulation in all replicates in macrophages. This figure shows a comparison all histones expression in qRT-PCR used in this study (same results as shown above) in all experiments of primary mouse macrophages.In **A**, Hist1h1 show a downregulation in cells stimulated with LPS across all 3 experiments. In **B,** Hist1h3g does not show any significant up or downregulation in both cells stimulated and unstimulated. In **C**, Hist1h2ba share the same base results as previous histone. In **D**, Hist1h4c do not show any significant regulation. In **E**, Hist2h3ca1show a slight downregulation inthis histone in cells stimulated with LPS. In **F**, Hist2h2be show a clear downregulation in cells stimulated with LPS. In **G**, Hist2h2ab have a significant downregulation with cells stimulated with LPS. In **H**, Hist3h2a is more regulated in cells stimulated with LPS than unstimulated cells. In **I**, Hist4h4 a very significant downregulation in cells stimulated with LPS.

As already mentioned, the results show a high response of the primary macrophages to the pro-inflammatory stimulation with LPS as it was observed with the strong upregulation of TNF (more than 150 times in all three experiments).

The mRNA expression of some histones is probably being regulated independently of the cell cycle especially after stimulation with LPS, even if the tested histones are not conventionally known as RI (replication independent).

After analyzing the overall results (Fig 4.14), there is no indication or confirmation that Anthracyclines (only tested for Epi) can lead to an increase in histone expression. If anything, the results showed a pattern of a downregulation of histones stimulated with LPS. The main regulator of histones in the experiments was infection, by LPS, although TLR agonists such as *E. coli* (which includes LPS) and PGN, have also unregulated some histones.

4.2.4 Protein levels of anthracycline-treated extracts

Macrophages are key mediators of the immune response; they can suffer significant changes in protein expression in response to different infection stimuli. One good example is TNF protein, which is considerably induced during inflammation. The increase in TNF protein secreted levels after stimuli was assayed by ELISA above, both in THP-1 cells and in primary mouse macrophages; TNF protein secretion was lowered after incubation with Epi, as also shown in the ELISA results. We then focused on the regulation of histones in inflammation and by Epi. After testing if mRNA expression levels of histones are dependent on inflammatory stimuli and of Epi treatments, we are also interested in testing at the protein level. In the future, the protein amounts of selected histones will be tested by western blot (WB), and perhaps the histone post-translational modifications of those histones. For that, we have prepared and quantified the protein samples extracted from the cells and performed a WB for a known gene to have another confirmation of the cells' integrity.

The protein that was chosen was the β-actin, a cytoskeletal protein that plays a fundamental role in the cell structure, movement, and intracellular signaling.

The results bellow show β-actin expression in macrophages in the various conditions tested: incubation with various concentrations of Epi (0.5µM, 1µM and 2µM); and in the presence or absence of LPS stimulation.

The inclusion of untreated controls for both LPS-stimulated and unstimulated conditions ensures a comprehensive understanding of the baseline expression, providing a point of reference for all experimental treatments. The figure 4.17 shows the WB analysis concerning β-actin protein levels.

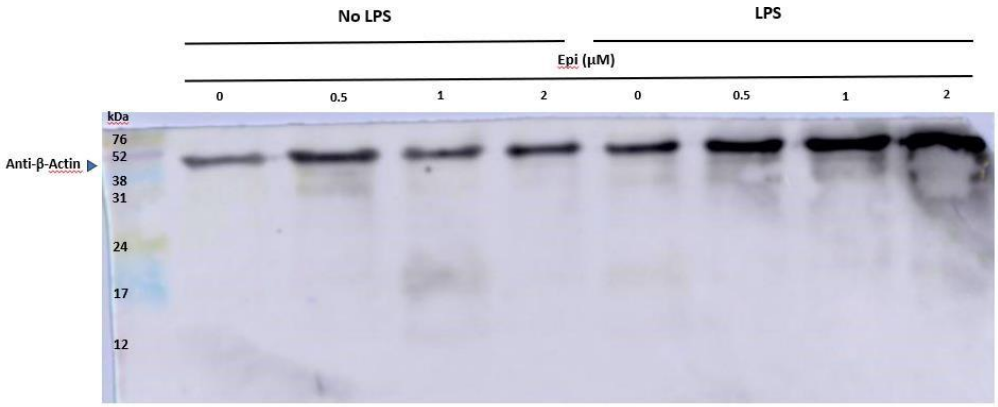


Figure 4.15 - Western blot analysis of β-actin expression in mouse macrophages. The conditions tested were unstimulated cells with and without various concentrations of Epi (0.5µM, 1µM and 2µM) and the cells with LPS stimulation and Epi. The incubation time was 4h. The anti-β-actin antibody (Santa Cruz sc-58673) was used at 1:2000 dilution and the detection antibody (Abcam ab6823) was used at 1:4000 dilution. A molecular marker was used in the first lane to indicate the protein size, in each lane, 15µg of total protein were loaded according to the extracts quantifications by Bradford. The image shown was obtained using the automatic option of the software in the Imager equipment and corresponds to an exposure of less than 1 minute.

The distinct bands observed at approximately 42 kDa correspond to the β-actin protein (Fig. 4.15). The relative intensity of the bands provided insights into the expression levels of β-actin in different conditions. Despite the quantification efforts, there seems to be differences in loading between the conditions tested; this could be due to mistakes during the Bradford assay or during the application of the samples in the gel, mostly due to pipetting errors. These differences in loading should be taken into consideration when other antibodies are used to detect other proteins, such as histones for example. For future quantifications of histones and histone modifications at the protein level by WB, it should be calculated the areas and intensity of those bands relative to the areas and intensity of the bands in the β-actin gel WB shown in this figure.

5 Discussion

5.1 TNF-α expression in THP-1 cells and primary mice macrophages

The role of TNF-α, a pro-inflammatory cytokine, in immune responses is well-established, especially within the context of its production by macrophages in response to stimuli such as lipopolysaccharide (LPS) (Baxter et al., 2020). The study of the TNF-α expression (qRT-PCR) in our experiments was important to analyze the cell activation status of the THP-1 cells and the primary macrophages after stimulation with infection stimulus such as LPS; it was also tested for other cytokines such as IL-1β (not shown in the results, but it was also highly regulated by cells stimulated with LPS).

5.1.1 TNF-α expression analyses in THP1-cells

The THP-1 cells were submitted to 4 experiments measuring the TNF-α expression by qRT-PCR and the secretion of this cytokine by ELISA. In one experiment (Figs. 4.1 and 4.2) the cells were stimulated with several inflammatory agonists (LPS, *E. coli*, PGN, PolyIC HMW, PolyIC LMW and FSL-1), whereas in the other 3 experiments (Figs. 4.1, 4.3, 4.4 and 4.5) the THP-1 cells were stimulated with LPS and treated with 3 different concentrations of Epi (0.5μM, 1μM, 2μM). LPS is a strong infection stimulus that binds to the TLR4 cell receptors at the surface of THP-1 cells and macrophages, inducing a signaling cascade that leads to the transcriptional upregulation of inflammatory cytokines including TNF-α.

The results observed with cells only stimulated with LPS, showed a strong upregulation of TNF-α mRNA: in all three experiments had fold change values of more than 10 times the controls by qRT-PCR (Figs. 4.3, 4.4 and 4.5), with one of the experiments even increasing 50 times (Fig. 4.4). These results support previous literature and serve as a positive control on the activation status if the stimulated THP-1 cells used (Schildberger et al., 2013).

In cells stimulated with LPS and treated with Epi there was also a strong regulation of TNF-α mRNA. However, there is a pattern of a downregulation of TNF when the concentrations of Epi were increased: starting at 0.5μM of Epi, there was no significant difference compared to cells only stimulated with LPS, but at higher concentrations (1μM and 2μM) there is a diminished TNF-α expression, and the regulation of this cytokine across all three experiments decreased almost half between concentrations. These results show that Epi can inhibit the expression of TNF-α in a dose-dependent way, as previously shown by other works from the host laboratory. This observation may be important in treatment of sepsis patients, as decreasing the production of pro-inflammatory cytokines can help regulate the immune response to the infection, especially in cases of exacerbated responses, as occurs in sepsis (Figueiredo *et al.*, 2013). As already mentioned, a clinical trial is being conducted by the host laboratory to test the safety of Epi in sepsis patients (NCT05033808).

In the experiment with TLR agonists, the results showed that there was a clear upregulation of TNF-α mRNA with cells stimulated with LPS (as confirmed with the other three experiments), PGN, and the higher expression was with the cells stimulated with *E. coli* (Fig. 4.2) (Kawai *et al.*, 2010). Cells stimulated with PolyIC high and low molecular weight and FSL-1 did not show any regulation of TNF-α. PolyIC is usually recognized by TLR3 cell receptors, and it is more likely to regulate other type of cytokines like type I interferon, a class of mediators which are produced in response to viral infections (Alexopoulou et al., 2001). FSL-1 primary receptors are TLR 2 and TLR6 and in THP-1 cells there was no TNF production in response to this stimulus, this can happen due the fact that THP-1 cells only mimic macrophage cells and can have defects in TLR2 signaling pathways, or the FSL-1 doses were not the

ideal to induce the TNF-α expression (Takeuchi *et al*., 2002). The ELISA results were in line with the qRT-PCR results.

The unstimulated cells serving as controls showed no regulation of TNF-α in THP-1 cells. These results showed that in absence of an inflammatory stimuli the cells stay in non-activated state, and without an external stimulus like LPS there is no activation of pathways that produce pro-inflammatory cytokines like TNF-α. Also untreated cells did not show any expression of TNF-α, these controls are important to verify if Epi without any stimuli could induce the TNF-α, and the results showed that this cytokine depends on an infection stimulus like LPS. From these results from qRT-PCR and ELISA, we were confident that the THP-1 cells used were an appropriate cell model to test inflammatory responses in vitro (Chanput *et al*., 2014).

5.1.2 TNF-α expression analysis in macrophages

After testing the expression and secretion of TNF-α in THP-1 stimulated cells, we conducted the same experiment in primary mouse macrophages. We also performed four experiments using the same parameters as above and recorded mRNA expression for TNF and histones (Figs. 4.10, 4.11, 4.12 and 4.13), as well as ELISA protein levels for TNF (Figs. 4.6, 4.7, 4.8 and 4.9). The results in primary macrophages were identical to THP-1 cells, however the extent of the immune response to the same stimulus in macrophages were much stronger compared to the THP-1 cells. These macrophages were highly responsive to the pro-inflammatory stimulation by LPS, as observed from the strong up regulation of TNF-α mRNA expression more than 150x in all the four experiments, much higher than the 15 times of THP-1 cells (Ginhoux *et al*., 2014). This suggests a much more potent immune response to infection stimuli in primary cells than the THP-1 cells. The ELISA results also showed more secretion in macrophages than in THP-1 cells, comparing unstimulated to LPS-stimulated cells.

The macrophages stimulated with LPS and treated with Epi also showed higher values of expression of TNF-α than the THP-1 cells. Higher doses of Epi had the same results as shown before for the THP-1 cells and this proves that this class of drugs leads to downregulation in pro-inflammatory cytokines such as TNF-α, as shown before by the host laboratory (Figueiredo *et al*., 2013). Like THP-1 cells, macrophages stimulated with TLR agonists showed a strong upregulation of TNF-α in LPS, PGN and *E. coli* stimuli (Beutler *et al*., 2003).

As seen before with THP-1 cells, there was no regulation of TNF-α mRNA or secretion in unstimulated cells, and in macrophages only treated with Epi. This showed that the macrophages used were not previously stimulated and activated and therefore could be used in our ex-vivo experiments of pro-inflammatory activation.

5.1.3 Comparative analyses

Both experiments with THP-1 cells and primary mouse macrophages showed similar results: cells stimulated with LPS showed an upregulation of TNF-α, however macrophages showed a much bigger immune response to infection stimulus. Unstimulated cells and treated with Epi without any infection stimuli did not show any expression of TNF-α, showing that Epi does not induce inflammatory responses in the doses used in our experiments. Also, for both THP-1 and macrophages, the protein ELISA results corroborated the mRNA qRT-PCR results.

In both experiments, higher doses of Epi were able to downregulate this cytokine production, this can be due to the ability of this class of drugs to intercalate with DNA, the topoisomerase II inhibition, or the direct interaction with NF-κB, which leads to downregulation of NF-κB transcriptional target genes such as TNF-α. These findings go in line with previous studies (Chora *et al*., 2020), the ability of

Epi to downregulate the expression of pro-inflammatory cytokines could reveal and important protector against sepsis (Chora *et al*., 2020).

Both cell types were considered adequate for the study of histone regulation by anthracyclines in the context of inflammation because nor THP-1 nor macrophages showed any prior immune activation when the cells were left without inflammatory stimuli or drug treatment.

5.2 Histone expression in THP-1 cells and primary mice macrophages

The chromatin structure is very important in regulating cell processes such as replication, genes transcriptional regulation and overall genomic maintenance and stability. Through these processes, chromatin mediates essential functions including cell cycle, gene expression, DNA repair, and cell death (Struhl *et al*., 2013).

Eukaryotic chromatin consists of DNA, histones and other proteins, and the basic unit are nucleosomes, this units are composed of 147 DNA base pairs coiled around a histone octamer core (Liu et al., 2016). These octamers consist of four heterodimers of core histone proteins, namely H2A, H2B, H3 and H4, a fifth histone, H1 binds to internucleosomal DNA ("linker DNA") to secure more complex chromatin structures (Bannister *et al*., 2011).

In humans, there are 73 identified histone genes, including 16 genes for H2A, 22 for H2B, 14 for H3, 15 for H4, and 6 genes for H1. These genes are typically grouped together in specific regions of the chromosome and are referred to as histone clusters; these multiple genes copies in tandem repeats allows for a rapid synthesis of histone proteins during DNA replication, ensuring the chromatin structure and DNA integrity for cell division and daughter cells. Most of this histone genes within these clusters are replication dependent, this means that they are expressed during the S phase of the cell cycle when the DNA is replicated, in a process also named chromatin replication (Marzluff *et al*., 2002).

The primary cluster, containing of around 80% (55 genes) of these genes, is located at the HIST1 locus on chromosome 6, histone cluster 2 and 3 (HIST2 and HIST3 loci) are located on chromosome 1, and Hist4h4 gene, which codes for the H4 core histone is found on chromosome 12 cluster 4 (DeRan e*t al*., 2007).

To study the histone regulation in THP-1 cells and macrophages, we decided to select 8 histones genes, at least one of each cluster of the three chromosomes in three experiments (replicates 2,3 and 4), the location of the chosen histones can be seen the figure below (figure 5.1). These histones were selected since there were primers for the qRT-PCR already tested and to cover all four histone clusters and all types of histones; in the mice we used orthologs of the same histones. Histone upregulation by Epi, following LPS stimulation of primary mouse macrophages, was previously suggested by an RNAseq experiment performed in the host laboratory, as mentioned before (Bannister *et al*., 2011).

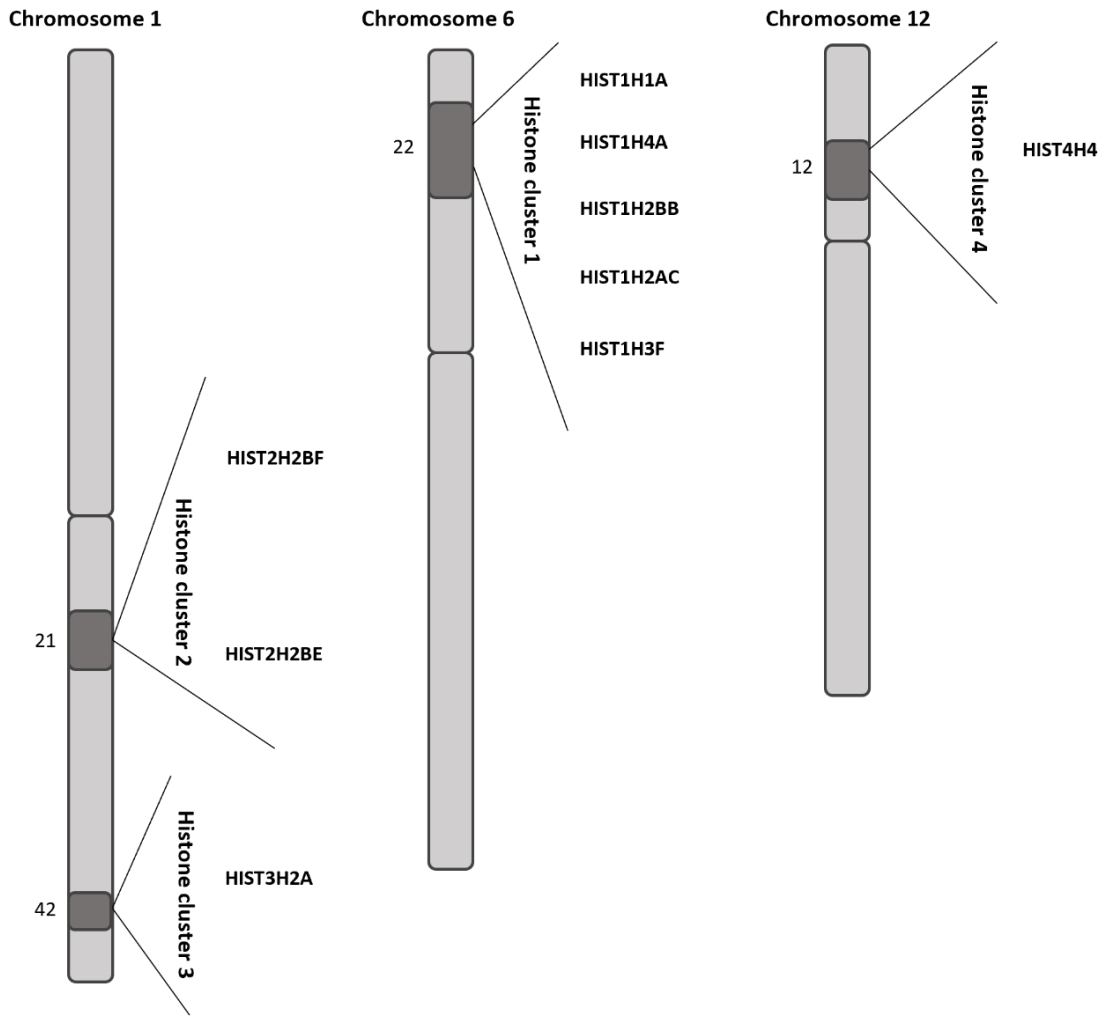


Figure 5.1 – Histone clusters in human chromosomes. This figure was adapted from Amatori *et al*., 2021. For each gene cluster (HIST1 to HIST4), the number of identified genes in that cluster is shown on the left of the chromosome. In chromosome 1, we used HIST2H2BF and HIST2H2BE of the histone cluster 2, and HIST3H2A of the histone cluster 3. For the chromosome 6 we used HIST1H1A, HIST1H4A, HIST1H2BB, HIST1H2AC, and HIST1H3F of the histone cluster 1. In chromosome 12 we used HIST4H4 of the histone cluster 4.

5.2.1 Histone expression analysis in THP-1 cells treated with Epi and LPS stimulation

The primary aim of this work was to test if histone gene expression is regulated by Epi during inflammatory responses. To test this hypothesis, we used THP-1 cells and primary mouse macrophages.

We started by testing if TLR stimuli, other than LPS, also induced histone expression (Fig. 4.2). We observed that histone mRNA expression was not significantly regulated following TLR stimulation, including by LPS, with most of the histones mRNA levels changing less than 2-fold. HIST2H2BE, HIST3H2A and HIST4H4 may be upregulated by *E. coli* treatment, even if only slightly and at this point it is not possible to identify which components of *E. coli* may lead to this putative upregulation (Bhatt et al., 2012).

In THP-1 cells we performed 3 experiments to test Histones regulation by Epi during inflammation (Figs. 4.2, 4.3 and 4.4). The results in qRT-PCR for the experiment 2 showed a selective histone regulation, among the tested histones, only HIST1H4A, HIST2H2BF, HIST3H2A, and HIST4H4 demonstrated significant regulation (>2-fold change compared to untreated control) in the presence of

2 µM Epi. The HIST3H2A was considerably sensitive to Epi treatments, with expression levels showing more than a 3-fold increase even without LPS stimulation (Sanchez-Gonzalez et al., 2006). The most regulated histone was HIST4H4, this protein was the most responsive histone, with its expression increasing more than 5-fold with Epi treatments (1µM and 2 µM). This suggests that Epi at this concentration may selectively target specific histones genes for upregulation, even in the absence of LPS stimulation (Sanchez-Gonzalez et al., 2006).

However, the results observed in experiment 2 were not reproduced in the other experiments. In experiments 3 and 4 revealed a slight downregulation but generally the histone expression levels tended to be stable, with fold changes spanning between 0.5 to 1.5. This narrow range indicates minimal to no significant regulation of histone expression in response to Epi treatments or LPS stimulation and does not support the hypothesis of upregulation of histones by Epi.

The differences observed across the three experiments highlight the inherent variability in cellular responses, including in cell lines. This could be attributed to subtle differences in experimental conditions, cellular states, division cycle or other uncontrolled variables.

It is possible that the impact on histone expression might be influenced by factors such as the cellular state of THP-1 cells. When cells are in G1 and therefore not replicating DNA and chromatin for division, it could be that Epi induces transcription of some histones, as observed in part of the experiments. In this case, some histones previously defined as replication coupled may have some degree of regulation independent of replication. When cells are duplicating their DNA, mostly in S, regulation by Epi may not be as prevalent as regulation by the cell cycle, which is very pronounced and fast, leading to induction of mRNA levels many folds. Therefore, the induction of histone transcription by Epi may not be detected in some stages of the cell cycle. To test this, the cells would have to be synchronized in specific stages of the cell cycle, for example by serum starvation to avoid entry into S phase. Cells would be tested during serum starvation and after releasing from starvation and entry into S phase to compare regulation of histones by Epi.

Also, mRNA levels are determined by transcription and mRNA decay. As such, future experiments should test the stability of each histone mRNA. We can test mRNA stability indirectly by determining the mRNA half-life after transcription inhibition using Actinomycin D, as we can assume that changes in mRNA levels reflect mRNA degradation. All these experiments were considered, but before we decided to proceed the work by also testing the hypothesis in macrophages which are a more synchronous cell population because of being primary cells and following a well-defined short differentiation protocol in the presence of macrophage colony stimulating factor (M-CSF). We have also not tested histones at the protein level, which would also be important and that will be done in macrophages.

5.2.2 Histone expression analysis in macrophages

Contrary to the TNF-α mRNA that was strongly upregulated by LPS stimulation with and without Epi treatment in macrophages (as discussed above), almost all histone charts showed downregulation of histone mRNA expression in cells stimulated with LPS, with or without Epi, and only Hist3h2a had a slight induction of expression with this inflammatory stimulus.

As with THP-1, in macrophages there was no indication or confirmation that Epi led to an increased histone expression, if anything, histone expression was repressed to various extents by Epi. Increased Epi concentrations led to a downregulation of histones mRNAs: cells treated with 1µM and 2µM of Epi showed across the majority of experiments a repressed histone expression, independently of being unstimulated or stimulated with LPS.

The immediate cellular response to LPS involves the upregulation of pro-inflammatory cytokines, chemokines, and other effector molecules (Beutler et al., 2003). This rapid and pronounced transcriptional response requires significant chromatin transformation to facilitate the access of transcriptional machinery to the respective gene promoters (Liu et al., 2014). Such remodeling can involve histone modifications like acetylation, methylation, and phosphorylation that can promote or repress gene transcription. For instance, histone acetylation is often associated with gene activation, making chromatin regions more accessible (Liu et al., 2014). Specific chromatin remodeling and histone modifications could temporarily suppress histone gene expression in response to LPS, making DNA regions containing histone genes become less accessible to transcription factors and other transcriptional regulators, leading to their reduced expression. Regulation of transcription factor (activators and repressors) binding to DNA in histone gene promoters, as well as assembly and binding of transcriptional machinery at histone genes, could also be regulated by LPS (Liu et al., 2014).

It is possible that the cell prioritizes the transcription of pro-inflammatory genes, while stalling histones mRNA production. In tissues, macrophages are terminally differentiated and do not routinely divide; our primary macrophages were differentiated from precursor cells and therefore required cell division, but the relevance of the different transcriptional programs may have been maintained in our ex-vivo experiments (Liu et al., 2014). It has been shown that macrophages maintain proliferation in the presence of mitogens such as M-CSF that was used in our differentiation protocol, but when macrophages encounter invading microorganisms or microorganism-derived molecules such as LPS, proliferation is inhibited and gives rise instead to a rapid proinflammatory response (Liu *et al*., 2016). This change in fate is partly due to metabolic switches but may also involve chromatin remodeling. The downregulation of histone expression found in the cells stimulated with this inflammatory stimulus might be a cellular strategy to optimize the production of inflammatory mediators and other essential proteins needed for an immediate response to infection. It could be hypothesized that LPS-induced downregulation of histone expression in macrophages may be part of an integrated cellular response, the need to produce pro-inflammatory cytokines and chemokines outweighs the requirements for the synthesis of new histones for proliferation. Some histone variants have been implicated in cell proliferation and their downregulation could be required (Bannister et al., 2011). This aspect will be discussed below (in 5.2.3).

For future studies, because of their considerable regulation by LPS, the repression of the following histones should be further studied: Hist1h1a; Hist2h2be; Hist2h2ab; Hist4h4. The main regulator of histones in our experiments was infection by LPS, although other TLR agonists, such as *E. coli* (which includes LPS) and PGN, have also regulated some histones (Fig 4.13). Histone protein levels should also be determined by western blot, as this work already started but has not produced any results yet (Fig. 4.15) (Bannister et al., 2011).

As for Epi treatments, the increased concentration of Epi in treated cells from 0.5µM to 1µM and 2µM, have shown a trend towards a decrease in histone expression in both LPS-stimulated and unstimulated cells for histone mRNAs, such as Hist1h1a, Hist1h2ba and Hist2h2ab. Previous studies on anthracyclines mechanism of action showed that the ability of this drugs to intercalate within DNA causing genotoxic damage, including the binding to remodeling enzymes such as the topoisomerase II inhibition, and new studies suggesting that anthracycline can cause chromatin damage without DNA strand-breaks, caused by histone eviction from the DNA or the direct binding to regulatory factors such as transcription factors (NF-κB), (Chora *et al*., 2020), may inform about the regulation of histone mRNA.

The factors regulating histone transcription that are candidates for Epi interaction could be the transcriptional coactivator NPAT and the Tip60 histone acetyltransferase complex that are required for histone transcriptional activation (DeRan *et al*., 2007).

5.2.3 The role and expression of histone Hist2h2be

Hist2h2be is a histone variant that belongs to the histone H2B family, which, like other core histones, plays a crucial role in nucleosome formation and chromatin structure. Specific histone variants, despite sharing core structural features with canonical histones, can have distinct roles in chromatin dynamics, DNA repair, and gene regulation (He *et al*., 2021).

We are particularly interested in this histone because it showed a very clear trend across all three experiments that, there was a strong downregulation of Hist2h2be when the cells were stimulated with LPS. Given the observed downregulation of Hist2h2be by LPS stimulus, it is possible that this histone variant can have a specific role in immune regulation (He *et al*., 2021). This downregulation might be an adaptive strategy to alter chromatin accessibility, ensuring an optimal expression of genes crucial for the macrophages immune response. Macrophages are known for changing their physiology, including metabolism, in response to environmental signals (Liu *et al*., 2016). When macrophages are not activated and are instead resting cells in tissues, it is described that their metabolic profiles favor proliferation, which is required to maintain homeostasis of tissues and organs. On the other had when macrophages encounter proinflammatory stimuli, for example from microorganism invasion, cell proliferation programs are suppressed, and these activated cells acquire different metabolism such as an increase in glycolysis. Like this activated macrophage respond to the high energetic needs of mounting the inflammatory response. Hist2h2be has been described as a proliferation-associated protein that is upregulated in various cancer cells (He *et al*., 2021). To counteract proliferation, it is therefore possible that macrophages repress this histone variant when stimulated with LPS. In breast cancer cells, depletion of Hist2h2be led to impaired proliferation, colony formation, migration and invasion (He *et al*., 2021). The phenotype concerning inflammatory responses of Hist2h2be-depletion macrophages will be studied in the future as a follow-up project that is based on the work presented in this thesis. The preliminary results obtained in this thesis will be further explored and studied in the host laboratory to understand the role of histones regulation, with particular focus on Hist2h2be, in macrophage functions.

6 Conclusion and future perspectives

The main conclusions from this work are that Epirubicin is not a strong regulator of histones in macrophages, but histones may be regulated by inflammatory stimuli such as LPS, in particular the histone variant Hist2h2be. For future studies, there are some possible experimental approaches to continue this project such as:

- Test histone regulation by LPS at the protein level by Western Blot (This work has already started and in current undergoing);
- Thoroughly screen as many histones as possible to understand if regulation by LPS is a common feature;
- Test if other agonists also lead to histone up regulation, as suggested from the preliminary experiment in M4;
- Test if histone regulation is lost in macrophages from TLR4 KO animals, and from other innate receptor and transducer KOs, such as TLR2 and the TLR intracellular adaptor MyD88;
- Determine the phenotypes in macrophages of depletions of particular histones;
- Test in *in vivo* models of infection if histone regulation is observed in tissues and circulating immune cells; These experiments can for example be performed in an LPS or *E. coli* model of sepsis infection, and several organs, as well as blood, can be collected at distinct time points after infection for mRNA and protein analysis.

The work presented here will lead to more studies that will test the novel hypotheses and to explore the many factors with unknown contributions discussed throughout this thesis: factors like regulation by the cell cycle, other inflammatory stimuli, other types of histones, test other drugs from the anthracyclines groups (only Epirubicin was tested), and ultimately to attempt to test histone regulation by infection in primary human macrophages, either by *ex-vivo* pro-inflammatory stimulation or by comparing macrophages from patients with inflammatory diseases and infections with healthy donors.

7 References

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8 Supplementary data

Materials, Key resources and Solutions

Supplementary data 1 - Material used in the laboratory.

Laboratory materials	Source
Flasks	IGC washing room
Graduated pipettes (5ml, 10ml, 20ml)	IGC washing room
Glass serological pipettes	IGC washing room
Eppendorf tubes (1ml and 2ml)	IGC washing room
Cylinders	IGC washing room
Beakers	IGC washing room
Tips	IGC washing room
Falcon tubes (15ml and 50ml)	VWR
Filter tips	SARSTEDT
Culture flasks and plates	Nunc, Thermo Fisher Scientific
Cryovials 2ml	Nunc, Thermo Fisher Scientific
ELISA assay plates	PLATE IMMUNO F96 MAXISORP, Nunc, Thermo Fisher Scientific
qRT-PCR assay plates	384 well plates, Bioplastics
Plate seals	Polyester film SEALING TAPE, Nunc, Thermo Fisher Scientific
qRT-PCR Plate seals	MicroAmp Optical Adhesive Film, Applied Biosystems, ThermoFisher
Micropipettes, automatic pipettes and multichannel pipettes	Eppendorf, VWR, Costar
Neubauer counting chamber	VWR
Protein gel Hand-Casting Equipment	Mini-PROTEAN system and Mini Trans-Blot cell, Bio-Rad

Supplementary data 2 - Key resources.

Mediums, reagents and other resources	Source	Identifiers	Additional information
Strain, (male Mus musculus) C57BL/6J	Animal house at Instituto Gulbenkian de Ciência	JAX:000664	Male animals
Cell line (Homo sapiens) THP-1	ATCC	TIB-202	Human cells
DMEM Medium, high glucose, pyruvate	Gibco, ThermoFisher	41966-052	Component of cell culture media
RPMI 1640 Medium	Gibco, ThermoFisher	31870-025	Component of cell culture media
Fetal bovine serum (FBS)	Gibco, ThermoFisher	10500-064	Component of cell culture media
Penicillin–streptomycin	Gibco, ThermoFisher	15140-122	Component of cell culture media
Sodium pyruvate	Gibco, ThermoFisher	11360-039	Component of cell culture media
L-Glutamine	Gibco, ThermoFisher	25030-024	Component of cell culture media
Non-essential aminoacids solution	Gibco, ThermoFisher	11140-035	Component of cell culture media
HEPES buffer	Gibco, ThermoFisher	15630-056	Component of cell culture media
2-Mercaptoethanol	Gibco, ThermoFisher	31350-010	Component of cell culture media and Western Blot reagent
DMSO	Sigma-Aldrich	D2650	Component of freezing media
RNeasy Mini Kit	QIAGEN	50974106	Assay kit
RNase-Free DNase Set	QIAGEN	79254	Assay kit
cDNA Supermix kit Xpert	Grisp	GK86	Assay kit
Taq Universal SYBR Green Supermix	Bio-Rad	1725125	Assay kit
Human TNF-α ELISA MAX Standard	BioLegend	430201	Assay kit
Mouse TNF-α ELISA MAX Standard	BioLegend	430902	Assay kit
Quick Start Bradford reagent	Bio-Rad	5000205	Assay reagent
Epirubicin	Target Mol	282T0125	Drug
PBS buffer (Phosphate-buffered saline) 10x	IGC washing room	-	Buffer

BSA (Bovine Serum Albumin, Fraction V)	NZYtech	MB04602	Western blot reagent
SureCast Resolving Buffer pack (1.5M Tris HCl pH 8.8)	ThermoFisher	HC2212	Western blot reagent
SureCast Stacking Buffer pack (0.5 M Tris HCl pH 6.8)	ThermoFisher	HC2112	Western blot reagent
SDS (Sodium Dodecyl Sulfate)	Bio-Rad	1610301	Western blot reagent
30 % acrylamide/bis-acrylamide solution	Bio-Rad	1610158	Western blot reagent
APS Ammonium Persulfate)	Bio-Rad	1610700	Western blot reagent
TEMED	Bio-Rad	1610800	Western blot reagent
Rainbow Marker - Full range	Amersham, GE HEALTHCARE, LIFE SCIENCES	RPN800E	Western blot reagent
Antibody Anti-β-actin (mouse monoclonal)	Santa Cruz	sc-58673	1:2000, Western blot reagent
Goat F(ab) polyclonal Secondary Antibody to Mouse IgG H+L (HRP)	Abcam	ab6823	1:4000, Western blot reagent
Nitrocellulose Membrane roll, 0.45 micrometers	Bio-Rad	1620115	Western blot reagent
ECL prime WB detection	GE Healthcare	RPN2232	Western blot reagent

Supplementary data 3 – Mediums used in the cell culture.

Medium	Composition
DMEM medium	DMEM supplemented with 10% (v/v) FBS and 1% (v/v) Penicillin-Streptomycin
Macrophage RPMI medium	RPMI supplemented with 10% (v/v) FBS and 0.2 % (v/v) penicillin/streptomycin.
RPMI supplemented medium or C10	RPMI was supplemented as follows: 10% (v/v) FBS, 1% (v/v) Penicillin-Streptomycin, 1% (v/v) Sodium Pyruvate, 1% (v/v) L-Glutamine, 1% (v/v) non-essential amino acids, 1% (v/v) Hepes buffer and 0.05 M of 2-Mercaptoethanol
Freezing medium	FBS supplemented with 10% (v/v) DMSO

Materials, Key resources and Solutions

Supplementary data 4 - RIPA buffer (20 mL).

Reagents	Concentrations
NaCl [5 M stock]	150mM
Tris-HCl pH 7.4 [1 M stock]	50mM
EDTA [0.5 M stock]	1mM
Triton X-100	1 %
Deoxycholic acid [5% stock]	1 %
SDS [10 % stock]	0.1 %
PIC [25x stock] (Roche, ETDA-free cOmplete Protease Inhibitor Cocktail, #11873580001)	1 x
Distilled water	Fill up to desired volume

Supplementary data 5 – Standards used in the Bradford technique.

Standard 1	16µg/mL	1.6µL stock BSA	998µL H2O
Standard 2	8µg/mL	500µL standard 1	500µL H2O
Standard 3	4µg/mL	500µL standard 2	500µL H2O
Standard 4	2µg/mL	500µL standard 3	500µL H2O
Standard 5	1µg/mL	500µL standard 4	500µL H2O
Blank	0	-	500µL H2O

Supplementary data 6 - Separating gel solution.

Reagents	15% Separating gel (2 gels)
Distilled Water	0.75mL
1.5M Tris HCl pH 8.8 (RT)	2.5mL
10 % SDS (RT)	100µL
30 % AA/bisAA (4°C)	6.6mL
10 % APS (4°C)	100µL
TEMED (4°C)	10µL

Supplementary data 7 - Stacking gel solution.

Reagents	Stacking gel (2 gels)
Distilled Water	3.05mL
0.5M Tris HCl pH 6.8 (RT)	1.25mL
10 % SDS (RT)	50µL
30 % AA/bisAA (4°C)	650µL
10 % APS (4°C)	50µL
TEMED (4°C)	5µL

Supplementary data 8 - SDS Running buffer (1 L).

Reagents	Preparation
25 mM Tris	3.0g
190 mM Glycine	14.3g
0.1 % SDS [20% stock]	5mL
Distilled Water	Fill up to 1L

Supplementary data 9 - Wet Transfer buffer (1 L).

Reagents	Preparation
25 mM Tris	3.0g
190 mM Glycine	14.3g
20 % Ethanol	200mL
0.1 % SDS [20% stock]	5mL
Distilled Water	Fill up to 1L

Supplementary data 10- Solution 10x TBS (1 L).

Reagents	Preparation
Tris Base (formula weight 121.1 gr)	5.6g
Tris HCl (formula weight 157.6 gr)	24g
NaCl (formula weight 58.4)	87.7g
Distilled Water	Fill up to 1L

Supplementary data 11 – Solution 1x TBS-T (1 L).

Reagents	Preparation
10x TBS	100mL
0.1% Tween 20 [100%stock]	1mL

Distilled Water	Fill up to 1L

Supplementary data 12 - Blocking solution (100 mL).

Reagents	Preparation
BSA	5g
TBS-T	Fill up to 100mL

Supplementary data 13 - Pro-inflammatory agonists used in this study.

Notes	Agonists	Stock	Source	Concentration	TLR
Fixed Bacteria Gram-negative	*E. coli*	5.4milion/ µL	Previously fixed by the host lab (10.21203/rs.3.pex-1437/v1)	MOI 20	TLR4
Lipopolysaccharide (*E. coli* 055: B5)	LPS	3,9mg/mL	Santa Cruz sc-221855B	100ng/mL	TLR4
Peptidoglycan (*E. coli*)	PGN	1mg/mL	Invivogen, TLR-kit1mw	10 µg/mL	TLR2
Synthetic analog of double-stranded RNA (dsRNA)	PolylC HMW	1mg/mL	Invivogen, TLR-kit1mw	10 µg/mL	TLR3
Synthetic analog of double-stranded RNA (dsRNA)	PolyIC LMW	1mg/mL	Invivogen, TLR-kit1mw	10ug/mL	TLR3
FSL-1 (Pam2CGDPKHPKSF) is a synthetic lipoprotein derived from Mycoplasma salivarium	FSL-1	100ug/mL	Invivogen, TLR-kit1mw	100ng/mL	TLR2/6

Supplementary data 14 - Primers for human histones were obtained from Primer Bank (https://pga.mgh.harvard.edu/primerbank/); the selected primers originate amplicons between 100 to 150 bp in length.

Histone names	GeneCard symbol	Gene ID NCBI	Histone cluster and chromosomes	Primer sequences (5-3')
HIST1H1A	H1-1	3024	Cluster 1, chromosome 6	FW: AGGCAAAGAAACCTGCTAAGG RV: CGATCAGCTCTGACACGGAAG
HIST1H4A	H4C1	8359	Cluster 1, chromosome 6	FW: AAGGGTTTGGGTAAGGGGG RV: TAGATCAGACCAGAGATCCGC
HIST1H2BB	H2BC3	3018	Cluster 1, chromosome 6	FW: ATGCCTGAACCCTCTAAGTCT RV: CTGCGCTTACGCTTCTTACCA
HIST1H2AC	H2AC6	8334	Cluster 1, chromosome 6	FW: CTCGCGCCAAAGCGAAATC RV: CTGCGTAGTTGCCTTTACGGA
HIST1H3F	H3C7	8968	Cluster 1, chromosome 6	FW: TACTGTCGCCCTCCGTGAAA RV: CACCAGGTAAGCCTCGCAG
HIST2H2BF	H2BC18	440689	Cluster 2, chromosome 1	FW: GCCCCGAGGAAACAATGAC RV: GCAGGCGGATGGAAGAGAG
HIST3H2A	H2AC25	92815	Cluster 3, chromosome 1	FW: GGTCGTGGTAAGCAGGGTG RV: CGCTCCGAATAGTTGCCCTT
HIST4H4	H4C16	121504	Cluster 4, chromosome 12	FW: TGCTGCGGGACAATATCCAAG RV: AACCGCCGAAACCATAAAGGG
HIST2H2BE	H2BC21	8349	Cluster 2, chromosome 1	FW: ATGCCTGAACCGGCAAAATC RV: TGGATCTCGCGGGATGTGAT

Supplementary data 15 - Primers for mouse histones were obtained from Primer Bank (https://pga.mgh.harvard.edu/primerbank/), except for the primers for HIST2H3CA1, which were designed with primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) by the host laboratory; the selected primers originate amplicons between 100 to 150 bp in length.

Histone names	GeneCard symbol	Gene ID NCBI	Histone cluster and chromosomes	Primer sequences (5-3')
HIST1H1A	H1-1	3024	Cluster 1, chromosome 6	FW: ACTGCCACGGAGAAACCTG RV: AGAAACTGCCTGCACGATGAG
HIST1H4C	H4C3	8364	Cluster 1, chromosome 6	FW: ATGTCTGGACGTGGTAAGGGT RV: CCTGGATGTTATCACGGAGAAC
HIST1H3G	H3C8	8355	Cluster 1, chromosome 6	FW: GCTCGTACCAAGCAGACTG RV: TCACGGACTAGACGCTGGAAT
HIST1H2BA	H2BC1	255626	Cluster 1, chromosome 6	FW: GAGGTGGCGGTAAAGGGTG RV: CGCTCGAAGATGTCTGTCAC
HIST2H3CA1	-	-	Cluster 2, chromosome 1	FW: CAGCGCGGGAGTTTCAAGT RV: GAAGACACCCAAACGAGGAGC
HIST2H2BE	H2BC21	8349	Cluster 2, chromosome 1	FW: CTCCCAAGAAGGGTTCCAAGA RV: TTGCTATGCGCTCAAAGATGT
HIST2H2AB	H2AC21	317772	Cluster 2, chromosome 1	FW: CCATCTGCAACTAGCTGTGAG RV: TCCGTTTTCTTCGGAAGTAAGAC
HIST3H2A	H2AC25	92815	Cluster 3, chromosome 1	FW: AAGCTCGTGCAAAAGCGAAG RV: GCAGTCAGATATTCTAGCACAGC
HIST4H4	H4C16	121504	Cluster 4, chromosome 12	FW: GAAGCGCATCTCGGGTCTC RV: CATAGCCGTAACCGTCTTGC