UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE MEDICINA

TESIS DOCTORAL

Papel de TLR4 del neutrófilo en el ictus agudo experimental

Role of neutrophil TLR4 in experimental acute stroke

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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DOCTORAL THESIS

ROLE OF NEUTROPHIL TLR4 IN EXPERIMENTAL ACUTE STROKE

DOCTORAL THESIS PRESENTED TO EARN THE DOCTOR OF PHILOSOPHY DEGREE BY

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ABBREVIATIONS

APC: allophycocyanin

ATP: adenosine triphosphate

BBB: blood-brain barrier

BM: bone marrow

BSA: bovine serum albumin

CADASIL: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy

CARASIL: cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy

CCA: common carotid artery

CCL: chemokine (C-C motif) ligand

CCR: chemokine (C-C motif) receptor

CD: cluster of differentiation

cDNA: complementary DNA

CH: contralateral hemisphere

CNS: central nervous system

COX-2: cyclooxygenase-2

CXCL: chemokine (C-X-C motif) ligand

CXCR: chemokine receptor

DAMPs: damage-associated molecular patterns

DCs: dendritic cells

DHR123: dihydrorhodamine 123

DMEM: Dulbecco's modified eagle medium

DPBS: Dulbecco's Phosphate-Buffered Saline

dsDNA: double-stranded desoxiribonucleic acid

DsRed: red fluorescent protein

EDTA: ethylenediamine tetraacetic acid

FACS: fluorescence-activated cell sorter

FDR: false discovery rate

FITC: fluorescein isothiocyanate

fMLP: formyl-methionyl-leucyl-phenylalanine

FSC: forward scatter

GFP: green fluorescent protein

GSEA: gene set enrichment analysis

HBSS: Hank's balanced salt solution

HMGB-1: high-mobility group box 1

ICAMs: intracellular adhesion molecules

IFN: interferon

IGF-1: insulin-like growth factor 1

IL: interleukin

iNOS: inducible nitric oxide synthase

IRF3: interferon regulatory factor 3

IVM: intravital microscopy

IκB: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

KO: knock-out

LFA1: lymphocyte function-associated antigen 1

LPS: lipopolysaccharides

LyzM: lysozyme M promoter

MAC-1: macrophage-1 antigen

MD2: myeloid differentiation factor 2

MHC: major histocompatibility complex

MMP-9: matrix metalloproteinase 9

MPO: myeloperoxidase

MRI: magnetic resonance imaging

MyD88: myeloid differentiation primary response 88

NADPH: nicotinamide adenine dinucleotide phosphate

NE: neutrophil elastase

NET: neutrophil extracellular trap

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NLH: non-lesioned ipsilateral hemisphere

NMDA: N-methyl-D-aspartate

NO: nitric oxide

NSCs: neural stem/progenitor cells

PAMPs: pathogen-associated molecular patterns

PBS: phosphate buffer solution

PCA: principal component analysis

PCR: polymerase chain reaction

PE: phycoerythrin

Per1: period circadian protein homolog 1

PFA: paraformaldehyde

PMA: phorbol 12-myristate 13-acetate

pMCAO: permanent middle cerebral artery occlusion

PMN: polymorphonuclear

PPARγ: peroxisome proliferator-activated receptor gamma

PRR: pattern recognition receptor

PSGL-1: glycoprotein P-selectin glycoprotein ligand-1

RAGE: receptor for advance glycation end products

RBC: red blood cells

ROS: reactive oxygen species

rpm: revolutions per minute

RPMI: Roswell Park Memorial Institute

RT: room temperature

SEM: standard error of the mean

SSC: side scatter

ssRNA: single-stranded ribonucleic acid

STAT3: signal transducer and activator of transcription 3

TAN: tumor-associated neutrophil

TGF-β: transforming growth factor beta

TIA: transient ischemic attacks

TLR: toll like receptors

Tnfaip3: tumor necrosis factor alpha induced protein 3

TNF-α: tumor necrosis factor Alpha

tPA: tissue plasminogen activator

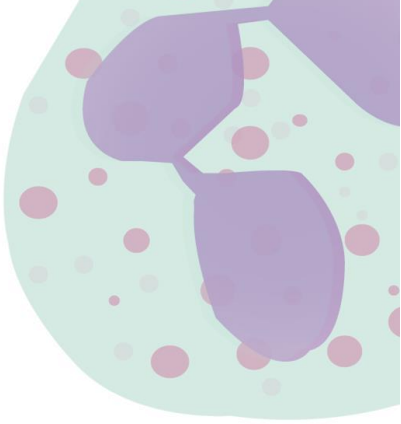
TRAM: TRIF-related adaptor molecule

TRIF: TIR-domain-containing adapter-inducing interferon-β

WBC: white blood cell

WT: wild type

ZT: *zeitgeber* time



RESUMEN

RESUMEN

El sistema inmune es capaz de provocar una respuesta contra un estímulo de tipo no patógeno, lo que se denomina inflamación estéril. Las moléculas endógenas que se liberan cuando un tejido se daña pueden iniciar una respuesta inflamatoria mediante la activación de receptores como el TLR4 (del inglés, *Toll-like receptor* 4). Este receptor se expresa en los neutrófilos, además de en otras células circulantes. Los neutrófilos constituyen la primera línea de defensa del sistema inmune innato ya que son las primeras células en responder cuando se produce un proceso de inflamación aguda. En la última década, los neutrófilos han sido reconsiderados como células complejas capaces de llevar a cabo un amplio conjunto de funciones especializadas, así como efectores de la respuesta inmune innata. La unión de DAMPs (patrones moleculares asociados a daño, del inglés, *damage-associated molecular patterns*) al TLR4 neutrofílico activa al neutrófilo. Una vez activados, los neutrófilos se adhieren al endotelio y comienzan el proceso de infiltración que culmina con la entrada en el tejido dañado, donde el neutrófilo es capaz de ejecutar distintas funciones como la fagocitosis, el estallido respiratorio, la degranulación o la NETosis.

En los últimos años, han surgido evidencias que sugieren la existencia de distintos subgrupos de neutrófilos que difieren en sus funciones, marcadores celulares de superficie, madurez o localización. El estudio de estas características llevó a la descripción de diferentes fenotipos de neutrófilos. Existen múltiples variables que han permitido discernir entre los distintos fenotipos de neutrófilos como pueden ser la edad, el estímulo de la microbiota o el comportamiento diferencial en una determinada patología (como

puede ser el cáncer). El estudio de los diferentes subgrupos de neutrófilos podría ser crucial para implementar terapias basadas en la intervención selectiva sobre un determinado fenotipo.

El ictus es una enfermedad muy común que representa la segunda causa de muerte en los países occidentales (la primera entre mujeres en España), la segunda causa de demencia y la causa principal de discapacidad en adultos. Puede ser clasificada en dos amplias categorías: el ictus isquémico y el ictus hemorrágico. El ictus isquémico se define como el infarto cerebral que resulta en muerte celular atribuible a la isquemia basado en pruebas neuropatológicas, de neuroimagen y/o evidencias clínicas de una lesión permanente (Sacco et al., 2013). Hasta la fecha, todas las terapias existentes se han centrado en restablecer el flujo sanguíneo al tejido isquémico ya que la rápida reperfusión mediante trombólisis venosa o mediante trombectomía endovascular permiten salvar el tejido lesionado. Sin embargo, en ambas opciones el tiempo transcurrido desde el inicio de los síntomas es un factor crítico y limitante. Debido a ello, hay una búsqueda activa de nuevas estrategias terapéuticas.

La respuesta inmune subsecuente a un ictus isquémico es un factor crucial en la fisiopatología y la resolución del ictus. Los neutrófilos se infiltran rápidamente en el tejido isquémico cerebral, respuesta que empieza de forma muy temprana, a las 6 h tras el ictus y llega al pico de infiltración 2 días después de la isquemia (Cai et al., 2019). Durante la fase aguda, los neutrófilos contribuyen a la ruptura de la barrera hematoencefálica, la extensión del infarto, la posible transformación hemorrágica y el empeoramiento de las secuelas neurológicas, *a priori*, todos efectos deletéreos. Sin embargo, varios estudios han mostrado un posible papel neuroprotector. Por ejemplo, la activación de los

receptores PPARγ induce la polarización de neutrófilos a un fenotipo N2 en el parénquima cerebral, lo que se asocia a una neuroprotección y a la mejor resolución de la inflamación (Cuartero et al., 2013).

Se conoce que TLR4 está implicado en el daño cerebral y la inflamación después del ictus (Caso et al., 2007). Este hecho ha sido demostrado también en pacientes, en los que la regulación al alza de TLR4 correlaciona con una mayor inflamación y peor desenlace de la patología (Brea et al., 2011). Además, se ha demostrado que la administración de un aptámero de DNA que se une a TLR4 induce un efecto protector frente al ictus isquémico en diversos modelos animales de la enfermedad (Fernández et al., 2018). Recientemente, nuestro laboratorio ha puesto de manifiesto que la ausencia de TLR4 induce la reprogramación neutrofílica hacia un fenotipo alternativo en isquemia cerebral. Además, encontramos una correlación negativa entre el número de neutrófilos y el tamaño del infarto en ratones que no expresan TLR4, que denominaremos TLR4KO (del inglés, TLR4 *knock-out*), lo que concuerda con un papel diferencial de los neutrófilos en ausencia de TLR4 (García-Culebras et al., 2019).

Con estos antecedentes, en la presente Tesis Doctoral, propusimos como hipótesis que el TLR4 neutrofílico es el responsable del daño inducido en el tejido cerebral después del ictus. Además, nos planteamos si la ausencia de TLR4 podría inducir la polarización del neutrófilo hacia el fenotipo N2, asociado con la neuroprotección. Finalmente, nos planteamos si la falta de TLR4 podría modificar las funciones del neutrófilo y, como consecuencia, alterar su contribución al proceso inflamatorio.

Para contrastar estas hipótesis utilizamos distintos ratones transgénicos, así como diferentes técnicas de laboratorio. Para el análisis transcriptómico se utilizaron ratones

de fenotipo salvaje (B6.C57BL/6J) así como ratones TLR4KO (B6.B10ScN-Tlr4$^{lps-del}$/JthJ). Para evaluar el papel del TLR4 neutrofílico en la neuroprotección tras ictus utilizamos ratones que no expresan TLR4 en las células mieloides (TLR4$^{loxP/Lyz-cre}$) así como sus respectivos controles (TLR4$^{loxP/loxP}$) en los que el volumen de infarto se determinó mediante imagen por resonancia magnética (IRM). El análisis estereológico se utilizó para determinar la infiltración de los neutrófilos y el fenotipo de los mismos. Además, se utilizó una combinación de citometría de flujo, microscopía confocal y microscopía intravital para evaluar las diferentes funciones neutrofílicas en ambos genotipos.

Los resultados obtenidos en esta Tesis Doctoral demuestran, mediante el análisis transcriptómico de los neutrófilos con y sin TLR4, que el TLR4 neutrofílico modula la contribución de los neutrófilos a la inflamación inducida por la isquemia, la migración al parénquima, así como su funcionalidad. Los neutrófilos que no expresan TLR4 fueron validados como una población de neutrófilos N2 ya que muestran una regulación al alza de genes involucrados en la caracterización fenotípica de los neutrófilos alternativos. Además, los neutrófilos que no tienen TLR4 son transcripcionalmente similares a los neutrófilos no envejecidos descritos por Frenette y colaboradores (2015), ya que ambas poblaciones muestran una alteración en la capacidad de participar en el proceso inflamatorio.

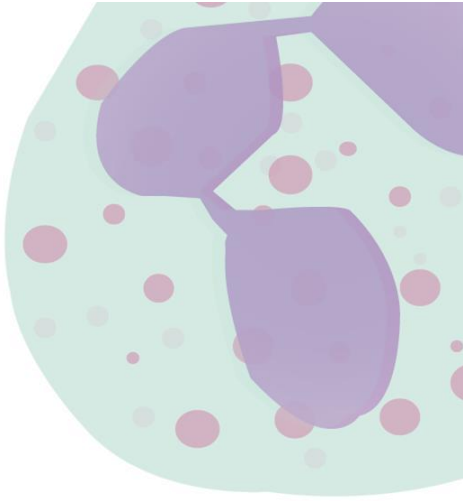
Hemos demostrado que la deficiencia específica de TLR4 en las células mieloides está involucrada en la neuroprotección después del ictus ya que el volumen de infarto de los ratones cuyos neutrófilos no expresan TLR4 es significativamente menor. Esta observación es concomitante con un aumento en la infiltración de neutrófilos sin TLR4

en el tejido isquémico. Además, la ausencia de TLR4 reprograma los neutrófilos hacia un fenotipo N2 que ha demostrado ser neuroprotector tras el ictus (Cuartero et al., 2013).

En homeostasis, la ausencia de TLR4 mantiene a los neutrófilos en un estado de "juventud" que se desregula, al menos en parte, tras un estímulo isquémico. Además, impide que los neutrófilos sigan su fluctuación circadiana normal y altera sus propiedades intrínsecas celulares como la complejidad, ya que los neutrófilos sin TLR4 son menos complejos que aquellos que lo expresan.

En relación a las funciones de los neutrófilos, aquellos que no expresan TLR4 muestran una actividad fagocítica mayor en el estado basal, son preferencialmente engullidos por la microglía tras el ictus, producen menos especies radicales de oxígeno en las primeras etapas del proceso inflamatorio y son más susceptibles de producir NETs *in vitro* pero no *in vivo*. No se encontró ninguna diferencia entre fenotipos en el proceso apoptótico. Finalmente, en modelos inflamatorios fuera del SNC (sistema nervioso central) los neutrófilos que no expresan TLR4 se adhieren a los vasos e infiltran con menor eficiencia en el tejido.

En conclusión, los resultados presentados en esta Tesis Doctoral indican que TLR4 representa un papel fundamental en la polarización del neutrófilo, ya que su ausencia reprograma a los neutrófilos hacia un fenotipo N2 y modula la inducción de diferentes rutas asociadas con el proceso inflamatorio, así como altera distintas funciones neutrofílicas. En conjunto, esta Tesis Doctoral contribuye a la idea de que TLR4, especialmente cuando es manipulado a nivel célula-específico, podría ser una diana para el desarrollo de nuevas estrategias neuroprotectoras.



ABSTRACT

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The immune system can elicit responses towards non-pathogen stimulus, which is called sterile inflammation. Endogenous molecules released when the tissue is damaged can initiate the inflammatory response by activating receptors such as TLR4. This receptor is expressed, among other circulating cells, in neutrophils. Neutrophils are the front-line defensive cells of the innate immune system being the first responders during acute inflammation. In the last decade, neutrophils have been reconsidered as complex cells capable of a significant array of specialized functions and as effectors of the innate immune response. The binding of DAMPs to the neutrophilic TLR4 activates the neutrophil. When activated neutrophils adhere to the endothelium and start the infiltration process which culminates in the infiltration into the damaged tissue where the neutrophil is able to execute functions such as phagocytosis, oxidative burst, degranulation or NETosis.

In the last few years evidence have come up suggesting the existence of distinct neutrophil subsets differing in functions, cell surface markers, maturity or localization. The study of those characteristics led to the description of different neutrophil phenotypes. There are multiple variables that have allowed to describe different neutrophil phenotypes, such as age, microbiome stimulation or differential behavior in a disease context (i.e. cancer). The study of different subsets of neutrophils could be vital to implement therapies based on selective intervention on a certain phenotype.

Stroke is a very common disease being the second cause of death in the western countries (first cause among women in Spain), the second cause of dementia and the leading cause of severe disability in adults. It can be classified into two wide categories:

ischemic stroke and hemorrhagic stroke. Ischemic stroke is defined as infarction of the brain resulting in cell death attributable to ischemia based on neuropathological, neuroimaging and/or clinical evidence of permanent injury (Sacco et al., 2013). Thus far, all therapies are focused on restoring the blood flow to the ischemic tissue. Quick reperfusion by intravenous thrombolysis and/or endovascular thrombectomy can salvage this tissue. However, both options are time-critical. Because of that reason there is an active search of new therapeutic approaches.

The immune response subsequent to an ischemic stroke is a crucial factor in its physiopathology and outcome. Neutrophils rapidly infiltrate into the ischemic brain tissue starting as early as 6 h after the stroke and peaking 2 days after ischemia (Cai et al., 2019). During this acute stage neutrophils will contribute to BBB disruption, infarct size, hemorrhagic transformation and worse neurological outcomes. However, several studies have reported a possible neuroprotective role. PPARγ activation induces the polarization of neutrophils toward an N2 phenotype in the brain parenchyma which is associated with neuroprotection and resolution of inflammation (Cuartero et al., 2013).

It is known that TLR4 is implicated in brain damage and inflammation after stroke (Caso et al., 2007). This fact has also been demonstrated in patients, in which the up-regulation of TLR4 correlated with higher inflammation and poor outcome (Brea et al., 2011). Moreover, it has been shown that the administration of a TLR4-binding DNA aptamer exerts a protective effect against acute stroke in different animal models (Fernández et al., 2018). Recently, our laboratory has proven that TLR4 absence induces neutrophil reprogramming toward an alternative phenotype in brain ischemia. We found a negative correlation between the number of neutrophils and the infarct volume in TLR4KO mice,

supporting a differential role of neutrophils in the absence of TLR4 (García-Culebras et al., 2019).

With this background, in this Doctoral Thesis we hypothesized that the neutrophilic TLR4 accounts for the harm induced to the brain tissue. Also, that TLR4 absence might induce neutrophil polarization towards an N2 phenotype, associated with neuroprotection. Finally, we asked if the lack of TLR4 could modify the neutrophil functions and therefore alter its contribution to the inflammatory process.

In order to prove this hypothesis, we used different transgenic mice and techniques. For the transcriptional analysis we used WT mice (B6.C57BL/6J) and TLR4KO mice (B6.B10ScN-Tlr4$^{lps\text{-}del}$/JthJ). In order to assess the role of the neutrophilic TLR4 in neuroprotection after stroke we used mice that do not express TLR4 in myeloid cells (TLR4$^{loxP/Lyz\text{-}cre}$) and its respective controls (TLR4$^{loxP/loxP}$), and infarct size was measured by MRI. A combination of flow cytometry and confocal microscopy and intravital microscopy were used to assess different neutrophil functions in both genotypes.

Our results show that, in accordance with the transcriptome analysis, neutrophilic TLR4 modulates the contribution of neutrophils to ischemia-induced inflammation, migration to the parenchyma and functional priming. Neutrophils without TLR4 were validated as an N2 population since they show an upregulation of genes involved in the phenotypic characterization of alternative neutrophils. Also, neutrophils lacking TLR4 are transcriptionally similar to non-aged neutrophils described by Frenette and cols. (2015) since both subsets show an alteration in the ability to participate in the inflammatory process.

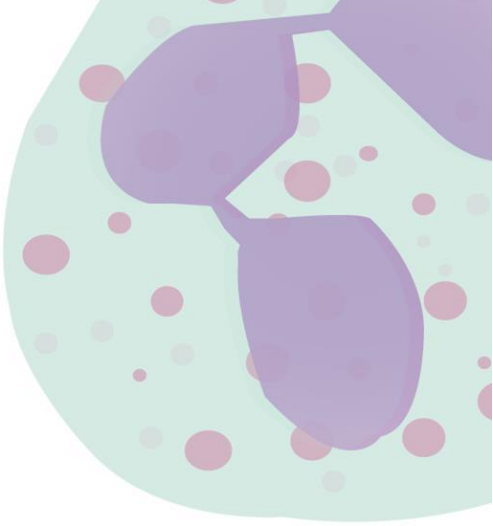
Abstract

We have proven that myeloid-specific TLR4 deficiency is involved in neuroprotection after stroke since the infarct volume in mice whose neutrophils do not express TLR4 is significantly smaller. That fact is concomitant with an increased infiltration of TLR4-lacking neutrophils into the ischemic tissue. Also, TLR4 absence skews neutrophils towards an N2 phenotype which is neuroprotective after stroke.

In homeostasis, the absence of TLR4 keeps neutrophils in a steady youth status that is dysregulated, at least in part, after an ischemic insult. It also prevents neutrophils from their normal circadian fluctuation and alters the intrinsic properties of the cell such as complexity, since neutrophils without TLR4 are less complex than those that express it.

Regarding neutrophil functions, TLR4-lacking neutrophils show a higher phagocytic activity in the basal state, they are preferentially engulfed by the microglia after stroke, produce less radical oxygen species (ROS) in the first stage of the inflammatory process and are more prone to undergo NETosis *in vitro* but not *in vivo*. We did not find any difference between genotypes in the apoptotic process. Finally, in an acute inflammation model outside the CNS, neutrophils lacking TLR4 seem to adhere and infiltrate less efficiently into the tissue.

In summary, TLR4 plays a crucial role in neutrophil polarization since its absence skews neutrophil polarization towards an N2 phenotype and modulates the induction of different pathways associated with the inflammatory process as well as different neutrophilic functions. Overall, this Doctoral Thesis contributes to the idea that TLR4, especially when targeted in specific cell types, could be a potential target to develop neuroprotective strategies.



INTRODUCTION

1. INTRODUCTION

1.1. INNATE IMMUNE RESPONSE TO ISCHEMIA: TOLL-LIKE RECEPTOR 4

1.1.1. Innate immunity

Traditionally, immunity has been considered as the ability of an organism to face infectious diseases. Throughout History, the possibility of infection has posed a significant hindrance to the growth, stability and progress of the humankind (Fitzgerald and Kagan, 2020). The immune response is the ensemble of physical barriers, cells and physiological processes that the immune system uses when facing an immune challenge, in order to eliminate it or to prevent its appearance in the future. This ensemble can be compartmentalized into two big blocks that work together to maintain the organism integrity. These blocks are the **innate immunity** (or non-specific) and the **adaptative immunity** (or specific or acquired). Innate immunity, which is the one relevant for this dissertation, is specialized in responding rapidly to a challenge. The main components of innate immunity are:

- Physical and chemical barriers: epithelia and antimicrobial chemicals produced by the organisms.
- Phagocytic cells: neutrophils, macrophages, dendritic cells, natural killer cells and other innate lymphoid cells.
- Circulating proteins: complement system and other inflammatory mediators.

The physiological function of the immune system is the defense against pathogens. In order to do so, the innate immune system recognizes molecular structures from microbial origin. These substances are called **pathogen-associated molecular patters**

(**PAMPs**). These structures include nucleic acids (ssRNA, dsRNA), proteins (N-formylmethionine, flagellin) or cell wall lipids (LPS, lipoteichoic acid). However, even noninfectious substances can elicit immune responses. There are pathogen infection-independent immune responses or, as it is also called, **sterile inflammation**. This process was firstly proposed by Polly Matzinger in 1994 when she named it the "danger" theory (Matzinger, 1994). This theory claims that danger signals released by distressed or damaged cells initiate immune responses and this response is not only driven by the "self" or "not-self" pattern that was believed until that moment. That theory led to the discovery of **damage-associated molecular patters** (**DAMPs**), several endogenous molecules released when the tissue is damaged (Land, 2003). DAMPs can initiate the inflammatory response by activating classical pattern recognition receptor (PRR) such as **Toll like receptors** (**TLR**) but not only. It also can be triggered by the activation by DAMPS of other receptors such as advanced glycation end products (RAGE) or ion channels. DAMPs can activate both non-immune cells, such as epithelial cells, endothelial cells and fibroblasts, and innate immune cells, such as neutrophils, monocytes and dendritic cells (DCs) (Figure 1) (Chen and Nuñez, 2010).

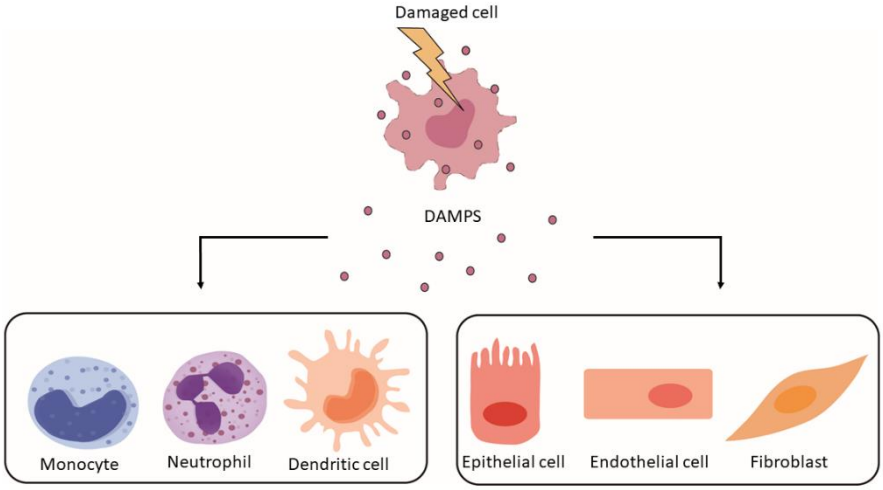


Figure 1. Selection of cells involved in DAMP-sensing and sterile inflammation. *DAMPs can activate innate immune cells that are going to release inflammatory mediators and therefore lead to the recruitment of inflammatory cells. Also, non-immune cells such as epithelial and endothelial cells and fibroblasts can be activated by DAMPs and facilitate the recruitment of immune cells into damaged tissue through pro-inflammatory cytokine production, adhesion molecule expression and altered vascular permeability.*

1.1.2. Toll-like receptors

TLRs are an evolutionarily conserved family of PRRs expressed on many cell types. They recognize molecules from microbes as well as molecules released and expressed by dying cells. TLRs were first described in *Drosophila melanogaster* where they are crucial to stablish the dorsoventral polarity during embryonic development (Hashimoto et al., 1988). TLRs are type transmembrane I receptors. They are homologous to the Drosophila Toll protein and the receptor for IL-1. A few years later, TLRs were found to play a role in innate immune responses to fungi and bacteria (Lemaitre et al., 1996). Until now, 10 TLRs have been identified in humans and 12 in mice (Barton and Medzhitov, 2003). They can be classified according to their location: cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10, whereas intracellular TLRs (localized in the endosome) are TLR3, TLR7, TLR8, TLR9, TLR11 and TLR13 (Kawai and Akira, 2010). The fist TLR described was **TLR4**. It

was identified as a receptor capable of driving responses in antigen-presenting cells that promote inflammation and adaptive immunity (Medzhitov et al., 1997). Shortly after that discovery, LPS was identified as the microbial stimulus that activates TLR, since a TLR4 mutation produced a diminished response to LPS in mice (Poltorak et al., 1998). Since then, mammalian TLRs have represented a main point in immunology research.

1.1.3. TLR4 pathway

When binding to its ligand, TLR4 dimerize (Figure 2). TLR4 forms a complex with MD2 and it starts the signaling pathway, which can elapse through the MyD88-dependent pathway or the TRIF-dependent pathway. The formation of the multimer, composed of two copies of TLR4 and two copies of MD2 and the ligand, initially transmits the signaling for the early-phase activation of NF-κB through MyD88 recruiting. Then the multimer complex is internalized and retained in the endosome where it is going to trigger the TRIF-dependent pathway. That pathway leads to the activation of IRF3 and the late-phase of NF-κB, resulting in the induction of type I IFN. Early and late-phase activation of NF-κB is required for the production of inflammatory cytokines (Kawai and Akira, 2010).

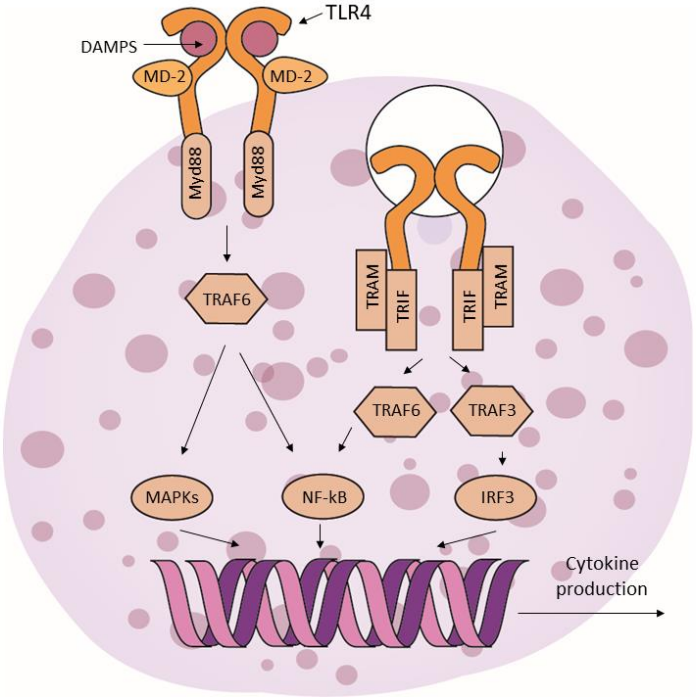


Figure 2. TLR4 pathway. *After DAMP stimulation, TLR4 dimer is formed. This dimer recruits MD-2 co-receptor. At this point two different routes can be followed. A) MyD88 dependent pathway leads to cytokines production through MAPK and NF-κB. B) MyD88 independent pathway is the alternative pathway which through TRIF and TRAM activation will again regulate the cytokine production through NF-κB and IRF3.*

1.1.4. TLR4 in the central nervous system (CNS)

The blood-brain-barrier (BBB) confers the CNS the status of isolation within the body. Even in this tightly controlled environment, TLR ligands have been reported and cells conforming the CNS have the ability to orchestrate immune responses through TLR activation. Microglia are the resident immune cells of the CNS. They are the major cells in the CNS that express TLR4 (Lehnardt et al., 2003). Neurons also express TLR4, especially when in pathological environments (Tang et al., 2008) and so do astrocytes (Jou et al., 2006) and oligodendrocytes (Church et al., 2016). Not only mature cells express TLR4 in the CNS, TLR4 can also be found on neural stem cells and progenitors (Rolls et al., 2007) showing that this receptor has an active role in adult neurogenesis (Palma-Tortosa et al.,

2019). Other members of the neurovascular unit such as the vascular endothelium also expresses TLR4 (Nagyoszi et al., 2010). Regarding brain structures, TLR4 is expressed in meninges, ventricular ependyma and circumventricular organs (Chakravarty and Herkenham, 2005).

1.1.5. TLR4 in circulating cells

In mice as in humans, circulating myeloid cells such as monocytes, macrophages, myeloid DCs and granulocytes (neutrophils and eosinophils) exhibit high levels of TLR4 expression. TLR4 is also expressed in lymphocytes (B and T) (Vaure and Liu, 2014). Exposure to LPS or DAMPS decreases TLR4 expression in macrophages and neutrophils and does not affect the expression in monocytes. Also, pre-exposure to LPS reduces the sensitivity to a following LPS exposure. This phenomenon is called LPS tolerance and it is due to the loss of TLR4 surface expression (Nomura et al., 2000).

1.2. NEUTROPHILS

1.2.1. Neutrophils in health

Neutrophils have always been considered as simple front-line defensive cells of the innate immune system equipped with limited proinflammatory duties. They are the first responders during acute inflammation. In the last decade, neutrophils have been reconsidered as complex cells capable of a significant array of specialized functions, and as an effector of the innate immune response.

Neutrophils are white blood cells that are continuously generated in the bone marrow from myeloid precursors. Up to $2x10^{11}$ cells are produced every day. During the maturation process the neutrophil goes through different stages: myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell and polymorphonuclear cell. Humans and mice differ in their quantities of circulating neutrophils. While in humans neutrophils represent 50-70% of total white blood cells, in mice, only 10-25% of leukocytes are neutrophils (Mestas and Hughes, 2004). The main characteristics of neutrophils is that their nucleus is segmented, and their cytoplasm is filled with granules and secretory vesicles (Figure 3). Granules are stores of proteins that are released by neutrophils with the aim to kill microbes and digest tissues.

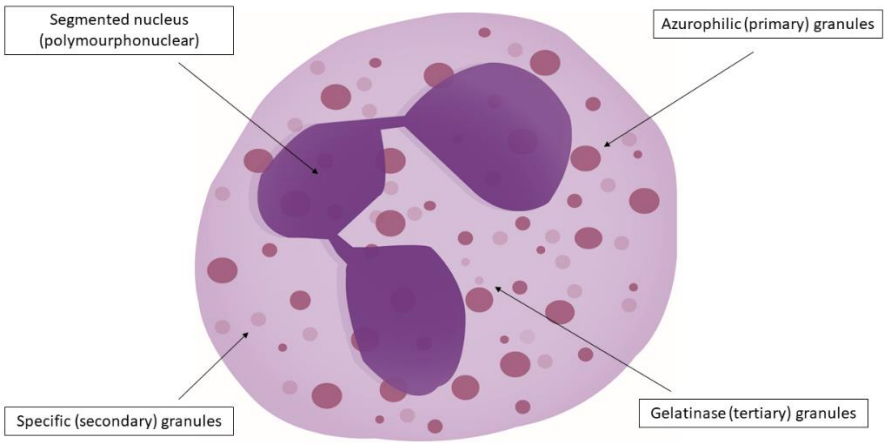


Figure 3. Schematic neutrophil representation. *The main characteristic of neutrophils is their polymorphonuclear shape. Neutrophils present three different types of granules (azurophiric, specific and gelatinase) which differ in their content.*

There are three types of granules which contain different types of pro-inflammatory molecules, such as (Borregaard, 2010):

- Azurophilic (also called primary) granules: they contain myeloperoxidase (MPO, which is a member of the heme peroxidases family), neutrophil elastase (NE), phospholipase A2, acid hydrolases, serine proteases and lysozyme, among others.
- Specific (also called secondary) granules: which contain, among others, lactoferrin, alkaline phosphatase, lysozyme and NADPH oxidase.
- Gelatinase (or tertiary) granules: which contain phosphatases and matrix metalloproteinase 9 (MMP9) also known as gelatinase B.

The half-life in circulation of a neutrophil is 1.5 h in mice and 8 h in humans. For that reason they have been traditionally considered short-lived cells (Galli et al., 2011). However, this time is expanded when an inflammatory process occurs and neutrophils become activated. In this case, their life span increases by several fold which allows the presence of activated neutrophils at the site of inflammation (Colotta et al., 1992). Moreover, by studying photo-activated neutrophils entering the injured tissue in mice, it was shown that a neutrophil can have a lifespan as long as 48 h (Wang et al., 2017). This ability of expanding their life span is crucial for neutrophils to be able to contribute to the resolution of inflammation.

1.2.2. Neutrophil functions

Neutrophils, while executing their functions, must follow a carefully programmed plan since their antimicrobial arsenal is deadly and unable to discriminate between the host and the threat, so they could potentially damage the host. The deployment of those

weapons must be precise, contained and effective in order to preserve the integrity of the organism. The process through which a neutrophil transforms from an inactive cell into a destructive weapon is called *activation*. During this process, the cell is going to mobilize secretory vesicles and granules, identify chemotactic gradients, move towards them while reorganizing its actin skeleton, infiltrate into tissues, begin transcription of inflammatory molecules and ultimately unleash a wide variety of molecules (Amulic et al., 2012).

1.2.2.1. Neutrophil activation

At sites where an inflammatory process is starting, inflammatory signals are abundant and stimulate the endothelial cells. These molecules, such as TNF-α, IL-1β or IL-17, promote the expression of adhesion molecules (P-selectins, E-selectins, ICAMs) on endothelial cells (Borregaard, 2010). Neutrophils are constantly probing the endothelial wall, particularly in the postcapillary venules, seeking for stimulated endothelial cells (Figure 4) (Lawrence et al., 1997). Neutrophils constitutively express the glycoprotein P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin which are going to bind to P and E-selectins from the endothelial wall, leading to tethering of the neutrophil to the endothelium (McEver and Cummings, 1997). Right after this attachment, the "rolling" process begins, where the neutrophil rolls over the endothelial wall until they reach the "firm adhesion" state when the neutrophil stops and prepares for migration. The neutrophil needs to change its cytoskeleton in order to infiltrate into the tissue. It forms a leading-edge lamellipodium which concentrates chemokine and phagocytic receptors (Xu et al., 2003). After infiltration, the neutrophil faces the inflammatory milieu. Many receptors, including PRRs such as Toll-like receptor TLR4 (which will be further discussed

later on) activate and lead the neutrophil to a fully activate state. At this point, the neutrophil is able to execute functions such as phagocytosis, oxidative burst, degranulation and NETosis.

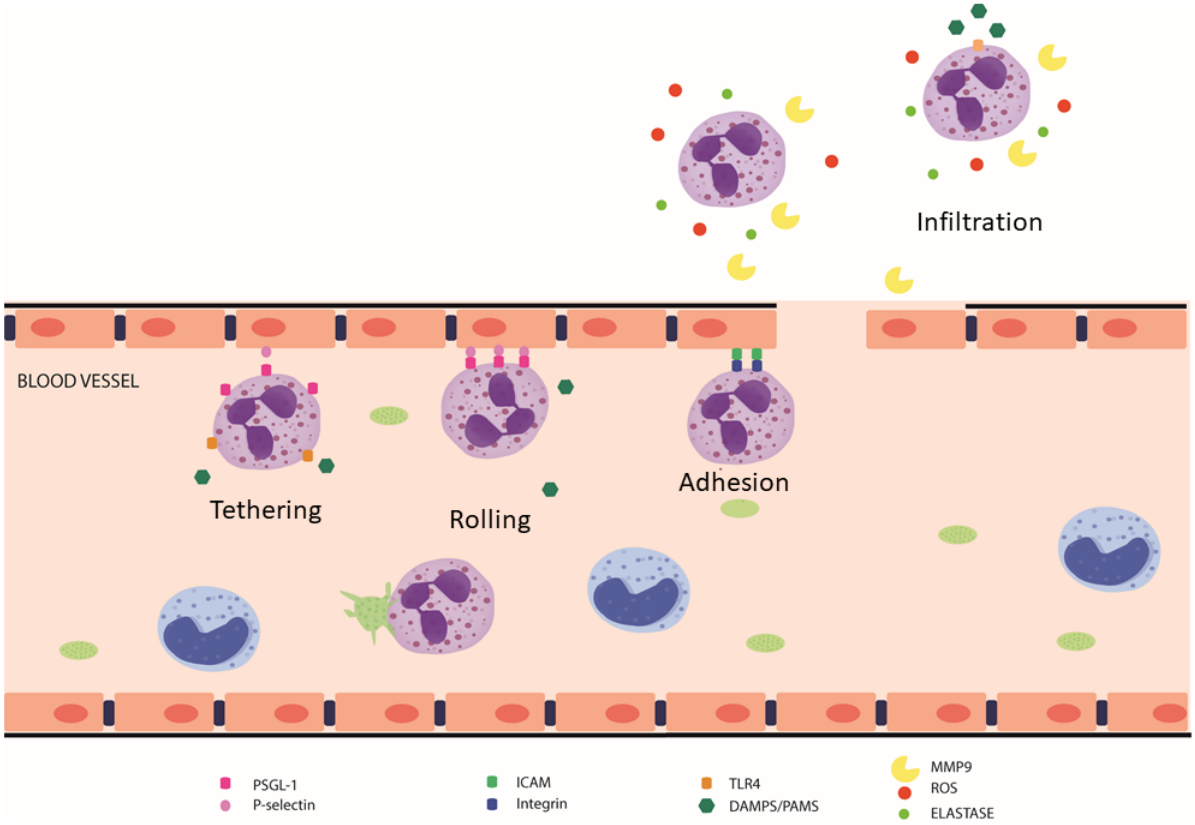


Figure 4. Neutrophil recruitment cascade. *The recruitment process starts with the initial capture of the neutrophil on the endothelial wall mediated by endothelial selectins and neutrophil selectin ligands. These molecules bind and unbind producing the "rolling" movement which decelerates as the integrins activate leading to the adhesion state. Finally, the neutrophil can infiltrate into the damaged tissue.*

1.2.2.2. Oxidative burst

When activated, neutrophils produce reactive oxygen species (ROS) in a process called "respiratory burst" or "oxidative burst". Reactive oxygen species are an array of different molecules that differ in their stability, reactivity and permeability; however they

all can modify and damage other molecules (Hampton et al., 1998). In essence the oxidative burst process consists of a rapid increase in oxygen consumption upon neutrophil activation due to production of ROS by the NADPH oxidase (Amulic et al., 2012). NADPH oxidase in an enzyme able to reduce molecular oxygen to superoxide which dismutates forming hydrogen peroxide (Figure 5). Both superoxide and hydrogen peroxide react with different molecules producing a cascade of more oxidant molecules such as peroxynitrite (when superoxide reacts with nitric oxide) or hypoalus acids (when hydrogen peroxide reacts with MPO). Clearly this mechanism is useful when combating an infection but ROS can also modify and damage host tissue (Bogdan et al., 2000).

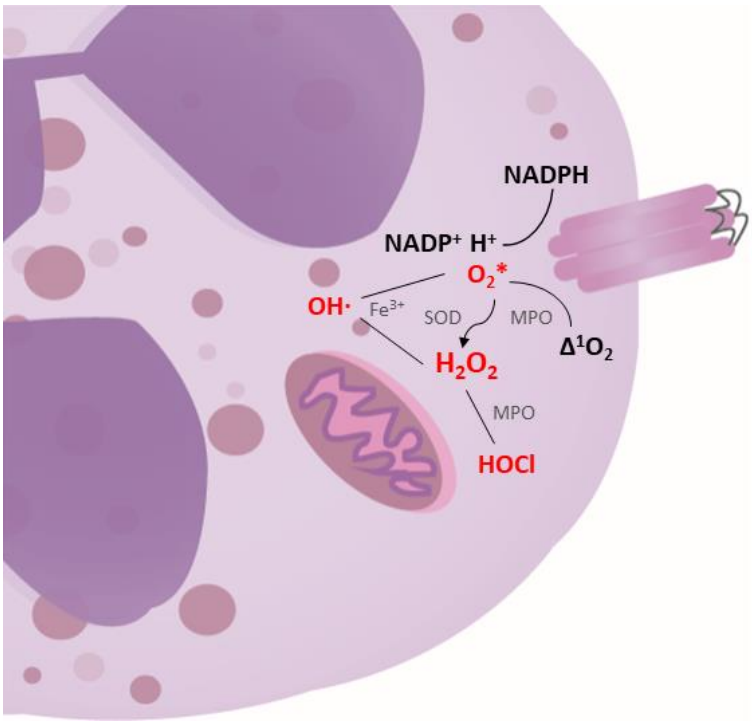


Figure 5. Oxidative burst metabolism in neutrophils. *Activated NADPH oxidase catalyzes the transfer of electrons from NADPH to molecular oxygen generating $O_2^{\cdot-}$. To contain the damage the superoxide dismutase (SOD) dismutates $O_2^{\cdot-}$ to hydrogen peroxide. MPO can convert H_2O_2 to HOCl which is bactericide. MPO can also convert O_2^- into singlet oxygen (Δ^1O_2). Finally, ferric iron can convert O_2^- and H_2O_2 into hydroxyl radical.*

1.2.2.3. Phagocytosis

Phagocytosis is a process by which certain cells ingest or engulf other cells, pathogens or cell debris. It is an active process mediated by a receptor. When the receptor is activated the particle is internalized by the cell membrane which is going to form a vacuole called phagosome. Subsequently, in neutrophils, the phagosome fuses with primary azurophilic granules, which contain preformed microbicidal molecules, including MPO and NE. The NADPH oxidase complex will assembly on the phagosomal membrane where electrons are transferred to molecular oxygen, with massive production of ROS (Amulic et al., 2012). Neutrophils eagerly phagocytose apoptotic cells and are the most important scavengers of cell debris in biological fluids (specially blood) (Manfredi et al., 2018). The depletion of phagocytes before sterile acute tissue injuries cause the accumulation of cell debris which influence the outcome (Capobianco et al., 2017). This fact suggests that neutrophils, and more specifically, its phagocytic function, is a key player in the maintenance of tissue homeostasis and the resolution of the inflammatory process.

1.2.2.4. Neutrophil Extracellular Traps (NETs)

Neutrophils can undergo three types of death: apoptosis, necrosis and *NETosis*. NETs are large, extracellular, web-like structures composed of cytosolic and granule proteins that are assembled on a scaffold of decondensed chromatin (Papayannopoulos, 2018). Following activation, neutrophils depolarize, the nuclear envelope disassembles and the chromatin decondenses. After that, the plasma membrane permeabilizes and NET expand into the extracellular space (Fuchs et al., 2007). There are different pathways that lead to NET generation. They differ in terms of dependence of oxygen species, kinetics of

the process and also the fate of the involved neutrophils (which can eventually die or survive after NET formation) (Manfredi et al., 2018), In order to start the NETosis process, two enzymes of the ROS pathway are critical. NAPH oxidase stimulate MPO to trigger the activation and translocation of NE from azurophilic granules to the nucleus where MPO and NE act in conjunction decondensing chromatin (Figure 6) (Papayannopoulos et al., 2010). This MPO-NE pathway can be activated during the host defense process against pathogens or in a sterile inflammation context. TLR4 ligands, such as HMGB1 (released by activated platelets, activated leukocytes and necrotic cells), triggers platelet activation which promotes the association of platelets with neutrophils and stimulates the formation of NETs (Clark et al., 2007). NETs are able to damage the surrounding tissue and regulate inflammatory cytokines directly or indirectly by modulating other immune cells (Papayannopoulos, 2018).

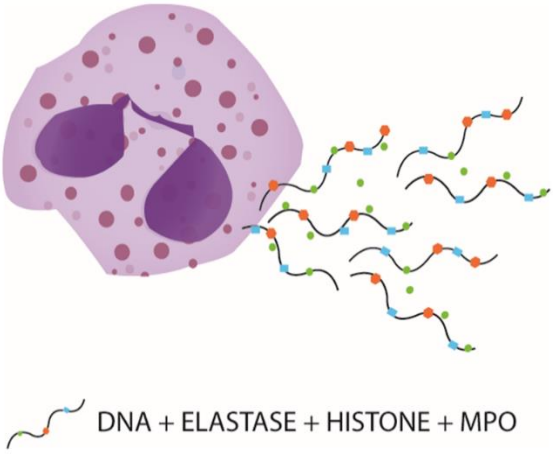


Figure 6. NET release. *The enzyme complex NADPH oxidase generates ROS which can disintegrate granules resulting in the release of NE. NE induces chromatin decondensation and plasma membrane rupture which leads to the release of NETs composed mainly by DNA, elastase, histone and MPO.*

1.2.2.5. Neutrophil apoptosis and clearance

Neutrophils must be removed from the injured tissue to prevent further damage and to contribute to the resolution of the inflammatory process. Once neutrophils have executed their functions, they die *via* apoptosis which removes cells from the injury and also sends a negative feedback signal for leukocyte recruitment. For example, in the brain, neutrophils are going to be engulfed and phagocytosed by activated microglia (Neumann et al., 2015).

1.2.3. Neutrophil plasticity

In the last few years evidence have come up suggesting the presence of distinct neutrophil subsets in different pathologies such as infections, inflammation and cancer (Cuartero et al., 2013; Fridlender et al., 2009; García-Culebras et al., 2019; Tsuda et al., 2004). Different neutrophil populations have been proposed based on numerous parameters such as function, cell surface markers, maturity or localization. Those distinct populations characterized by a specific molecular signature and/or function can be defined as *neutrophil phenotype* (Deniset and Kubes, 2018). It is extremely difficult to discriminate between differentiation and activation and where this process occurs.

The main characteristic that allowed scientists to differentiate between different neutrophils subtypes was the age. The typical nuclear segmentation of neutrophils varies with age: banded neutrophils are younger than segmented (Casanova-Acebes et al., 2013). Aged neutrophil phenotype is defined by a decrease in CD62L expression and the presence of CXCR4 (Casanova-Acebes et al., 2013). The regulation of this population is believed to follow a circadian pattern (Adrover et al., 2019). Not only time affects

phenotype. It has been described that the microbiome, by TLR4 stimulation, is able to drive the aged phenotype (Figure 7). Depletion of microbiota and therefore the lack of TLR4 activation reduces the number of circulating aged neutrophils and improves the inflammatory process (Zhang et al., 2015).

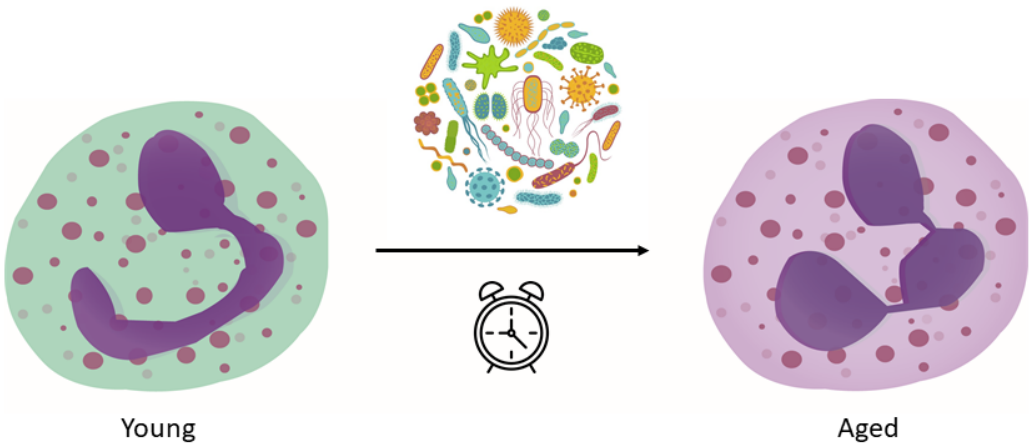


Figure 7. Differential features of young and aged neutrophils. *TLR4 agonists derived from the microbiota and time through their internal clock drive the aging process. These signals trigger aging associated changes such as nuclear morphology.*

All previous examples were neutrophil subsets that exist in homeostatic situations but there are other phenotypes when in an inflammatory setting. M1/M2 dichotomy has been traditionally used to divide macrophages in two groups, pro-inflammatory or anti-inflammatory. The first confirmation of this dichotomy also existing in neutrophils came when studies in cancer identified N1/N2 tumor-associated neutrophils (TAN) in mice (Fridlender et al., 2009) which, in this case, were antitumorigenic or protumorigenic, respectively. In this study, TGF-β blockade resulted in an influx of TANs that were hypersegmented, more cytotoxic and with higher levels of proinflammatory cytokines which resulted in an antitumor phenotype (N1), whereas TGF-β favored the N2 phenotype and therefore enhance tumor growth (Fridlender et al., 2009). Transcriptomic

analysis of both populations confirmed that they were two different phenotypes (Shaul et al., 2016). This N1/N2 dichotomy has also been studied in cardiovascular diseases. In the stroke context, the expression of a M2 marker has been described in neutrophils: $Ym1^{+}$ neutrophils were detected both in blood and bone marrow after the treatment with a PPARγ agonist. This treatment produced neuroprotection and this correlated with an increased number of N2 neutrophils in the injury site (Cuartero et al., 2013). In addition, during the restorative stage after a myocardial infarct neutrophils express progressively the mannose receptor, which traditionally has been considered as a M2 marker (Ma et al., 2016).

The study of different subsets of neutrophils could be vital to implement therapies based on selective intervention on a certain phenotype. These "specialized neutrophils" or subsets are for the most part not found in blood, suggesting this transition happens specifically in tissue. If neutrophils express the same surface markers in blood and they polarize in the tissues, it is necessary to establish a local approach but, if they express different markers since their generation in the bone marrow, an intravenous therapy could be useful. What has proven wrong is depleting all neutrophils since they are indispensable to fight infections, which are very common in some pathologies such as cancer or stroke.

1.3. ISCHEMIC STROKE

Stroke is a very common disease. In the western countries, stroke is the second cause of death (the first cause among women in Spain), the second cause of dementia and the leading cause of severe disability in adults. Stroke can be classified into two wide

categories: ischemic stroke and hemorrhagic stroke. Ischemic stroke is defined as infarction of the brain, spinal cord or retina resulting in cell death attributable to ischemia based on neuropathological, neuroimaging and/or clinical evidence of permanent injury (Sacco et al., 2013).

Transient ischemic attacks (TIA) can also occur and they are defined as a temporal interruption of blood flow that resolves before causing permanent injury. Although ischemic stroke and TIA are two different categories of stroke, the pathogenesis is the same and the research for the underlying cause is identical as well as the possible prevention strategies. Stroke research started with the identification of a hypoperfused, hibernating, electrically non-functional part of the brain that was named ischemic *penumbra* (Astrup et al., 1981). This region of the brain would end up irreversibly injured over time unless reperfused. Therefore, all therapies are focused on restoring the blood flow to this area. Quick reperfusion by intravenous thrombolysis and/or endovascular thrombectomy can salvage this tissue and allow to recover normal function (Trialists, 1997). However, both therapeutic options are time-critical (Emberson et al., 2014).

1.3.1. Epidemiology of ischemic stroke

Stroke is the leading cause of death and disability world-wide. Ischemic stroke represents approximately 71% of all strokes globally (Roth et al., 2018). It affects 13.7 million people globally per year and causes 5.5 million deaths which means that an estimated of 1 in 4 adults will experience a stroke in their lifetime (World Stroke Organization, 2019). The incidence and prevalence have evolved over time. The global incidence, mortality and disability adjusted life years for ischemic stroke decreased over the last three decades but not equally when separated by the level of a country's income:

all these values decreased in high-income countries, but no differences were found in low or middle-income countries (Krishnamurthi et al., 2013).

1.3.2. Risk factors

Risk factors can be modifiable or non-modifiable depending on whether they can be changed willingly over the course of the subject lifetime. Non-modifiable risk factors for ischemic stroke include age, sex and genetic factors. The influence of age differs depending on the development of the country but in general is higher in aged individuals (O'Donnell et al., 2010). The incidence of ischemic stroke is also higher in men than in women (Feigin et al., 2017). Although most stroke cases are sporadic some monogenic causes of stroke have been identified such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) syndromes.

There are several modifiable risks factors for ischemic stroke. Intervention on those factors is the reason of the decrease in stroke incidence in high-income countries. According to the INTERSTROKE study, 10 factors were the cause of 91.5% risk of stroke in all groups of age, regions and sex. These factors were a clinical history of hypertension, sedentarism, a high apolipoprotein B to apolipoprotein A ration, poor diet, high waist-to-hip ratio, psychosocial stress and depression, smoking, cardiac issues, high alcohol consumption and diabetes mellitus, being hypertension the one that carried the strongest risk of all (O'Donnell et al., 2010).

1.3.3. Pathophysiology

To know the cause of an ischemic stroke is very important since it can guide therapeutic strategies for the prevention of recurrent strokes. In this context, most ischemic strokes have a thromboembolic origin (Figure 8).

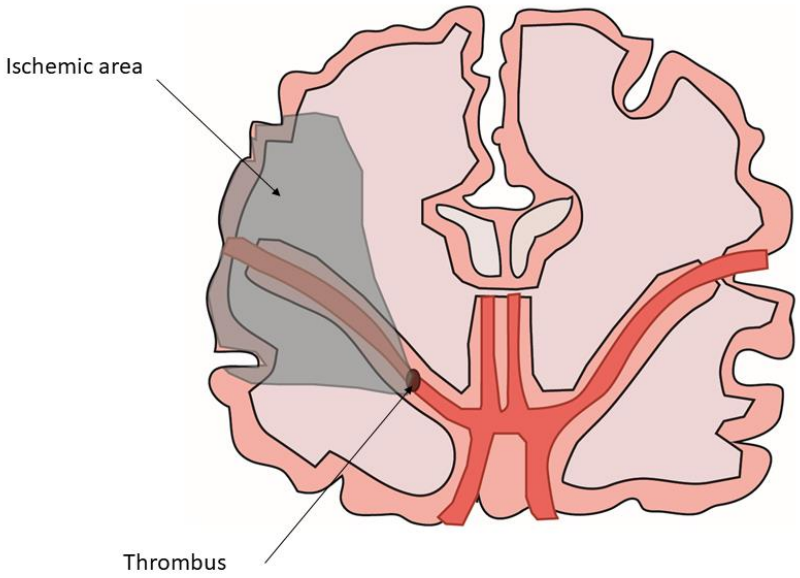


Figure 8. Schematic representation of an ischemic stroke in a human brain. *The thrombus produces a blockage of blood flow in the brain which leads to a hypoxic situation in which an area of the brain does not receive the appropriate supply of oxygen and glucose.*

The embolism can be atherothrombotic o cardioembolic. The causes can be categorized as follows:

- Arterial causes of stroke:
    - Atherosclerosis: an embolus originated from a stenotic atherosclerotic plaque. These thrombi mostly occlude large vessels distally.
    - Small vessel disease: generic term that refers to intracranial vascular disease based on various processes such as loss of smooth muscle

cells, degeneration of elastic lamina and proliferation of fibroblasts resulting in the reduction of blood flow to the brain parenchyma. This situation can lead to lacunar stroke, leukoaraiosis, cerebral microbleeds and intracerebral hemorrhage.

    - Arterial dissection: this is particularly important in younger patients. A dissection in the intimal layer of an artery, most commonly the extracranial carotid and vertebral arteries, can occlude the artery or cause thrombus formation.

- Cardiac causes of stroke:
    - Atrial fibrillation: blood can be retained in the left atrial appendage and allow thrombosis and subsequent embolism to the cerebral circulation.
    - Patent foramen ovale: Around 25% of individuals have a degree of residual patency in the heart that creates a potential mechanism for embolism (Hagen et al., 1984).
    - Infective endocarditis: bacterial endocarditis can cause septic emboli.

Independently of the cause of the stroke, the viability of the penumbra area is going to depend vastly on the collateral blood flow of each individual. When a vessel is occluded, collateral circulation, as alternative blood flow pathways are called, can sustain those areas for a limited time. This collateral circulation varies not only between individuals but also can vary over time in the same individual (Campbell et al., 2013).

1.3.4. Ischemic cascade

The blockage of blood flow in the brain leads to a hypoxic situation which means that there is a lack of oxygen and glucose supply (Figure 9). This causes a mismatch between energy requirements and availability of resources. When there is no oxygen and glucose, neurons are not able to maintain the transmembrane gradient. This results in an excessive release of neurotransmitters such as glutamate (Obrenovitch et al., 1993). Since the clearing of excitatory neurotransmitters requires energy, they are going to accumulate in the synaptic space activating NMDA receptors and thereby causing excitotoxicity. NMDA activation leads to an excess of intracellular calcium that is going to start harmful pathways in all type of brain cells. In neurons, it is going to stimulate ROS production leading to mitochondrial injury and subsequent activation of the apoptotic cascade (cytochrome c release, caspase activation, DNA fragmentation) (Love, 2006). Microglia are one of the first responders after the ischemia. Upon activation they release pro-inflammatory cytokines that are going to contribute to neuronal damage and to the recruitment of macrophages and neutrophils to the brain parenchyma. When activated, microglia and macrophages adopt a phagocytic phenotype and clear cellular debris (Wimmer et al., 2018). In astrocytes, the excess of calcium is going to prevent its homeostatic function and they will start releasing trophic factors that contribute to the formation of the glial scar (Liddelow et al., 2017). Also, oligodendrocytes are jeopardized since excitotoxicity produces cell dysfunction which leads to demyelination and white matter loss (Dewar et al., 2003).

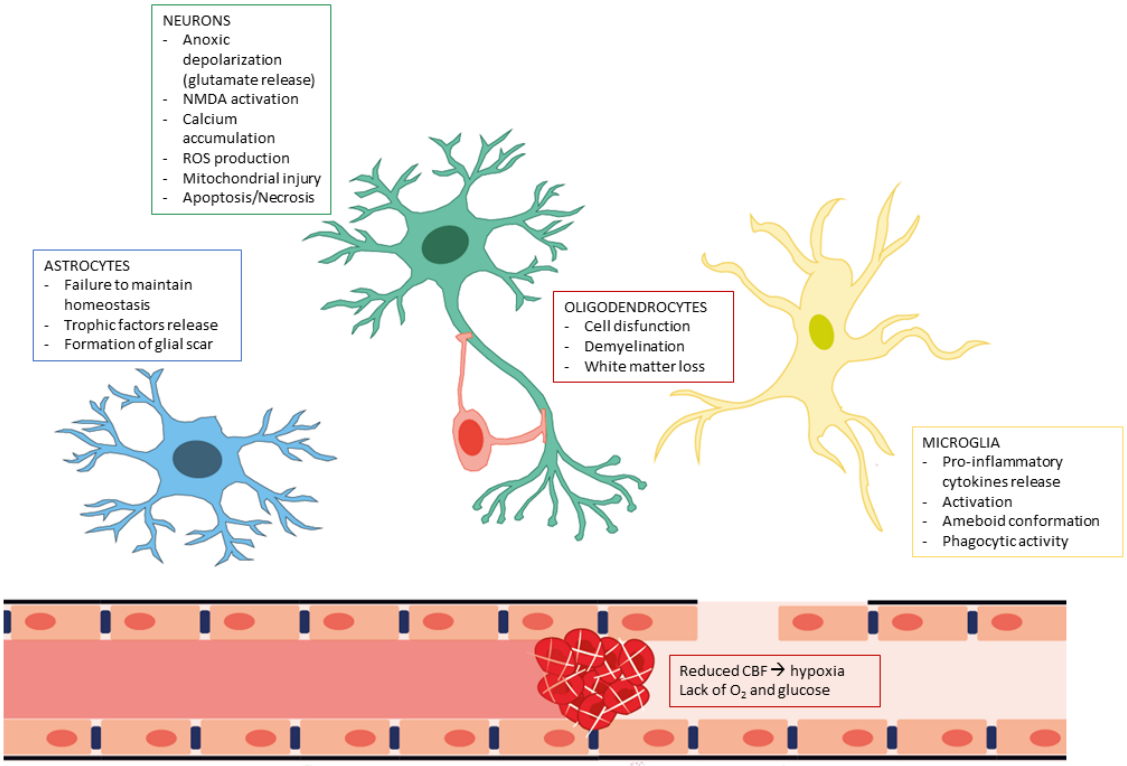


Figure 9. Schematic representation of the ischemic cascade*. The lack of oxygen and glucose results in a cascade of events that ultimate leads to cell death.*

1.3.5. Immune response in stroke

The immune response subsequent to an ischemic stroke is a crucial factor in stroke physiopathology and outcome. This response starts locally, in the ischemic brain tissue, but rapidly inflammatory mediators generated *in situ* propagate through the whole organism creating a systemic response followed by immunosuppression. Traditionally, inflammation considered as the activation of intravascular leukocytes and the release of proinflammatory mediators is potentially damaging for the tissue. While initially inflammation is aimed at restoring the initial situation prior to the lesion it usually comprises collateral damage. Since inflammation in stroke occurs in the acute phase and is a non-dependent pathogen inflammation innate immunity is going to play a key role.

1.3.5.1. Intravascular inflammation

The inflammatory cascade is activated within seconds after vessel occlusion and therefore the inflammatory process starts in the intravascular compartment (Iadecola and Anrather, 2011) (Figure 10). The clot blocking the blood vessel alters the environment of the vascular endothelium and activates platelets. This change of the stasis results in the placement of P-selectin expression on the surface of endothelial cells (stored in Weibel-Palade bodies) and platelets (stored in α-granules) (De Meyer et al., 2016). Selectins are single-chain transmembrane glycoproteins that mediate weak and transient interactions between leukocytes and the endothelial wall (Futosi et al., 2013). Platelets' P-selectin can also bind to leukocytes and contributes to stabilize leukocytes clusters causing more intravascular clogging and therefore more damage (De Meyer et al., 2016). Endothelial cells and leukocytes produce ROS (mainly O_2^- by NADPH oxidase) that lead to a loss of NO promoting vasoconstriction and therefore enhancing leukocyte and platelet aggregation (Atochin et al., 2007). Also, the aberrant vascular reactivity contributes to aggravate the ischemic insult by reducing blood flow to the ischemic area (Atochin et al., 2007). ROS, by a direct mechanism, can also affect the vascular function by stimulating the constriction of pericytes, producing microvascular occlusions (Yemisci et al., 2009). Proteases expressed by the vascular cells and released by leukocytes contribute to the BBB damage and leukocyte infiltration (Konsman et al., 2007).

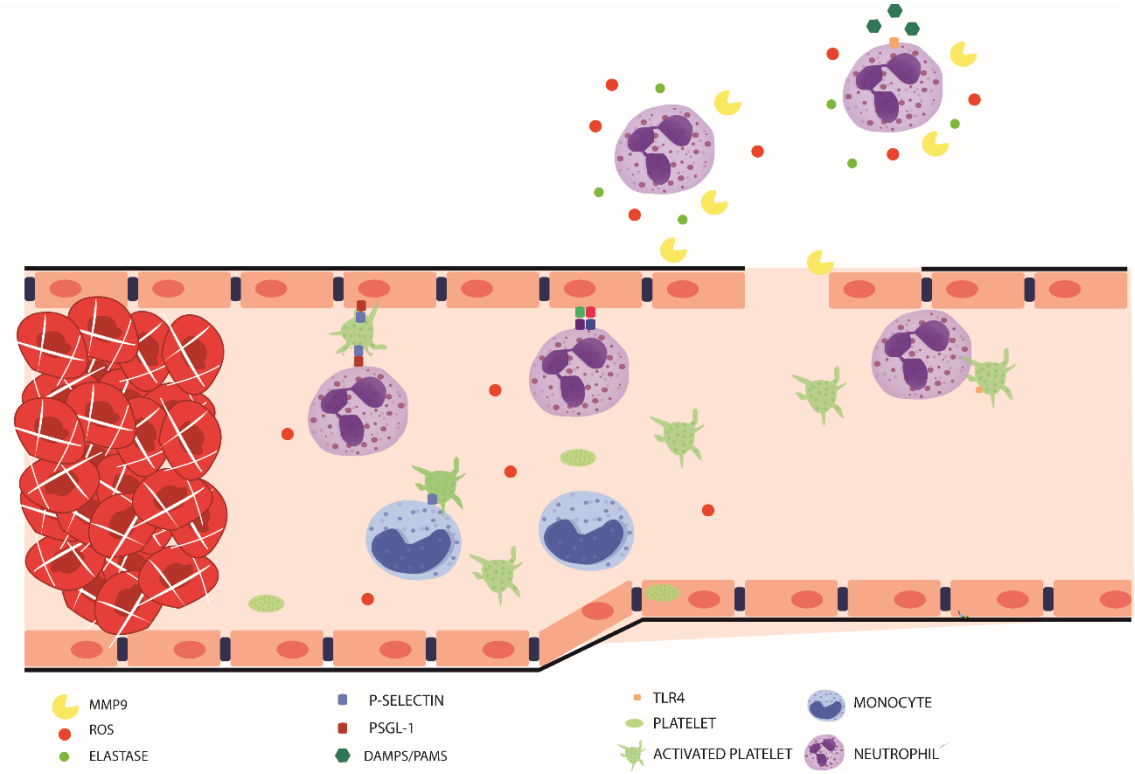


Figure 10. Intravascular inflammation. *The clot alters the environment of the vascular endothelium and activates platelets. Translocation of P-selectin on the surface of platelets and endothelial cells leads to platelet-leukocyte aggregation. Loss of NO promotes vasoconstriction and enhances leukocyte and platelet aggregation. MMP activation could lead to BBB breakdown facilitating leukocyte extravasation.*

1.3.5.2. Microglial activation

Following the intravascular inflammatory process, the perivascular area and the brain parenchyma are going to be exposed to the proinflammatory milieu. As a result of neuronal and glial depolarization and consequent cell death, extracellular ATP increases (Melani et al., 2005) (Figure 11). ATP activates P2x7 receptors in microglia activating this type of cells. In the early stage of inflammation, microglia produce TNF, IL-1β, ROS and other pro-inflammatory mediators. This activation also leads to a conformational change, from the ramified (Nimmerjahn et al., 2005) to an ameboid morphology (Schiefer et al., 1999). Microglia activation happens very early but continues for several weeks after the

insult (Lalancette-Hébert et al., 2007). Activated microglia have the ability to phagocytose contributing to the restoration of tissue homeostasis by clearing cell debris (Neumann et al., 2009). Current studies suggest that microglia engulf and phagocytose cells, for example, neutrophils, to protect the surrounding tissue conferring a neuroprotective role to these glial cells (Neumann et al., 2009). Microglia not only activate but also proliferate after stroke. The population of microglia increases in the ipsilateral hemisphere but remains at basal levels in the contralateral (Gelderblom et al., 2009).

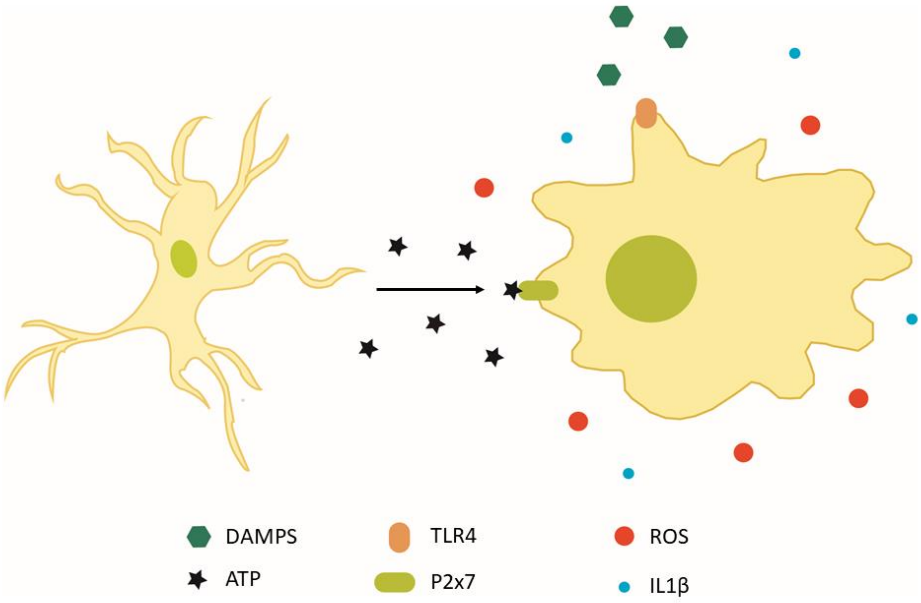


Figure 11. Microglia activation. *Cell death leads to nucleotide release (ATP) which activate purinergic receptors (P2x7) in microglia, leading to the production of proinflammatory cytokines. Ischemic cell death leads to the formation of DAMPs, which activate TLR4. As a consequence, microglia produce IL-1β, ROS and other pro-inflammatory mediators which also lead to a conformational change, from the ramified to an ameboid morphology.*

After an ischemic insult microglia can swift phenotypes (M1/M2) depending on the type of stimulation. M1 microglia are considered pro-inflammatory since they produce TNF-α, IL-1β and IFN-γ and they tend to induce neuronal death (Hu et al., 2012). On the

other hand, M2 microglia are considered anti-inflammatory since they contribute to recovery after damage and they secrete mediators such as IL-10, TGF-β, IL-4, IL-13, IGF-1 and other neurotrophic factors (Taylor and Sansing, 2013). After ischemia, local microglia exhibits a M2 phenotype but gradually transforms into M1 in peri-infarct regions (Hu et al., 2012). We can distinguish both types of microglia by detecting specific markers such as MHC class for M1 and Ym1 and CD206 for M2 (Perego et al., 2011). In summary, microglia respond differently depending on the time point, with M2 at the early phase followed by a switch to M1.

1.3.5.3. Monocyte infiltration

The intravascular inflammation and parenchymal cell activation lead to diminished blood brain-barrier function, increased expression of endothelial cell adhesion molecules, recruitment of inflammatory cells and leukocyte capillary plugging. However, the inflammatory response is not locally restricted. Stroke induces a pronounced inflammatory response in the periphery, leading to increased leukocyte counts and pro-inflammatory chemokines levels within the circulation (Smith et al., 2004). Circulating monocytes infiltrate into the brain parenchyma early and massively (Figure 12). After ischemia, pro-inflammatory monocytes ($Ly6C^{high}$) and anti-inflammatory monocytes ($Ly6C^{low}$) infiltrate into the parenchyma. Recruitment and infiltration of $Ly6c^{high}$ monocytes decrease over time while the recruitment and infiltration of $Ly6c^{lo}$ monocytes increase (Kim et al., 2014). Moreover, the selective depletion of $CCR2^{+}$ monocytes induced hemorrhagic transformation supporting the role of monocytes in maintaining the vascular integrity after ischemia (Gliem et al., 2012). Monocyte heterogeneity is also being discussed. The exact contribution of each subset is not clear. For example, it has

been proved that $CCR2^{+}$ monocytes infiltrated into the ischemic brain play a protecting role reducing the infarct volume due to their differentiation into an anti-inflammatory subset (M2) and by promoting the polarization of microglia towards a M2 phenotype (Chu et al., 2015). The role of anti-inflammatory or $Ly6C^{low}$ monocytes has been poorly addressed. Taken all together, these results emphasize the presence of a dynamic shift in the recruitment and infiltration of monocytes subsets and therefore it is possible that the functions of each subset could be different depending on the time point after the ischemia. Pro-inflammatory monocytes could be implicated in limiting the damage in the early stages of the inflammatory process by eliminating cell debris and maintaining the microvasculature stability, and anti-inflammatory monocytes could be implicated in tissue remodeling in the sub-acute phase.

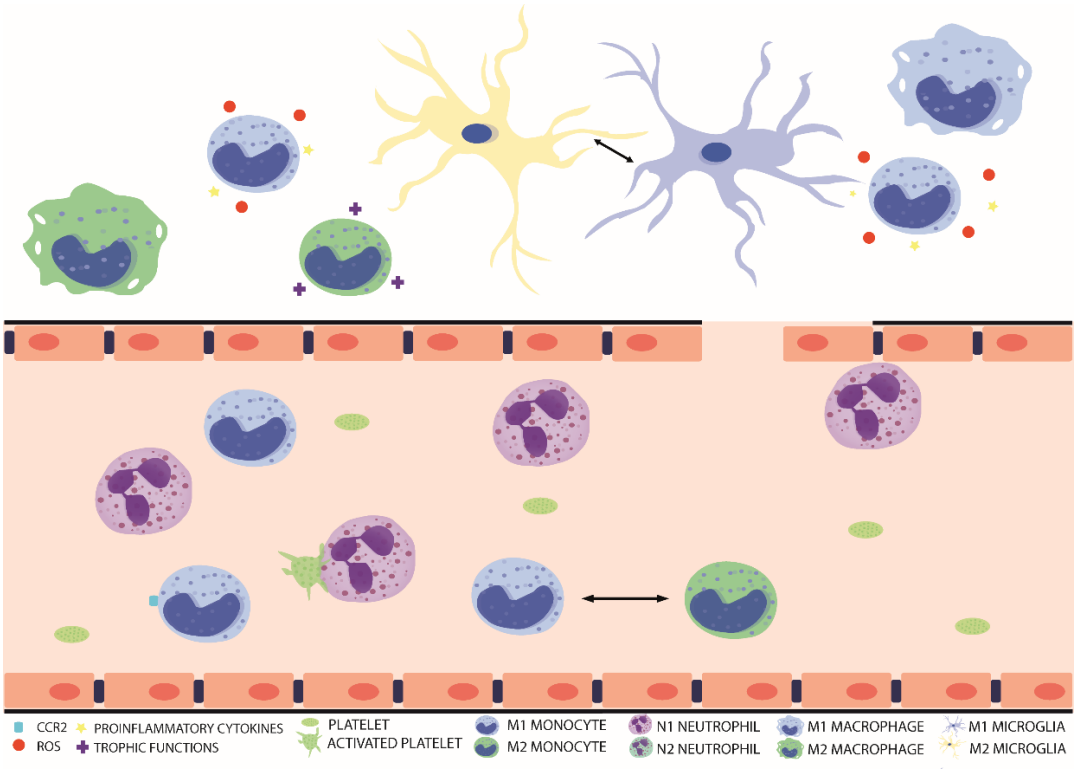


Figure 12. Schematic representation of monocyte infiltration. *Circulating monocytes infiltrate into the brain parenchyma early and massively. Monocytes also play a role in the polarization of microglia towards a M2 phenotype.*

1.3.5.4. Neutrophil infiltration

Neutrophils rapidly infiltrate into the ischemic brain tissue. Neutrophils contribute to the sterile inflammation situation caused by the ischemia (Figure 13). Firstly, as quick as after 15 minutes after ischemia, neutrophils start expressing several endothelial adhesion molecules such as PSGL-1, intracellular cell adhesion molecule-1 (ICAM-1), integrins (such as CD11a, b and c) and macrophage-1 antigen (MAC-1). These molecules allow the neutrophils to roll and adhere to the brain vessels (Kataoka et al., 2004). Adhesion molecules facilitate diapedesis through the endothelial cells. After adhesion, neutrophils follow a chemokine gradient and start to infiltrate into the injured tissue. At this point, neutrophil accumulation in the vessels can contribute to the “no-reflow” phenomenon

obstructing the vessels and hindering the possible reperfusion (Ames et al., 1968). Also, rupture of the BBB after the ischemic insult facilitates the neutrophil entry into the brain.

Chemokine receptors are expressed in the neutrophil plasma membrane and their activation can lead neutrophils to sustain and amplify the inflammatory process. Neutrophil infiltration starts between 6 and 12 h after stroke, peaks 1-2 days after ischemia and after 7 days few neutrophils remain in the injury site (Cai et al., 2019). During this acute stage neutrophils will contribute to BBB disruption (Konsman et al., 2007), infarct size (Buck et al., 2008), hemorrhagic transformation (Zhang et al., 2019) and worse neurological outcomes (Kumar et al., 2013). Whereas the detrimental effects dominate the literature, it is being discussed whether they can also contribute to the tissue repair or even have a neuroprotective role. When neutrophils are activated, they produce ROS which are going to damage the surrounding tissue. Moreover, they also release other molecules from different granules such as MMP-9, cathepsin G, collagenase, gelatinase, heparinase, which contribute to the extracellular matrix breakdown and vascular damage (Ruhnau et al., 2017).

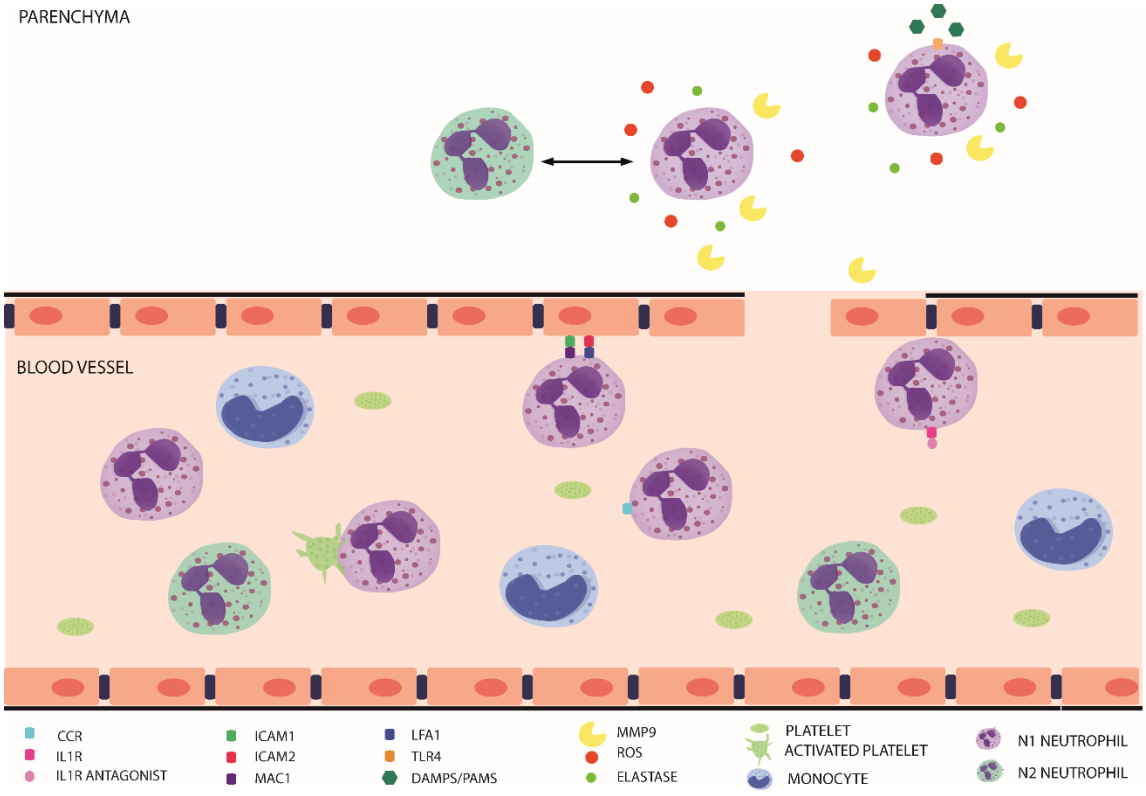


Figure 13. Schematic representation of neutrophil infiltration. *Neutrophils, under inflammatory conditions, activate and contribute to the inflammatory process. They are able to skew to different phenotypes (N1/N2), produce and release different cytokines, form platelet-neutrophil complexes, adhere to the endothelium and infiltrate into damaged tissues.*

Neuronal ischemia promotes NET formation which will further increase neuronal death (Cai et al., 2019). When forming NETs, neutrophils also release NE, an enzyme that contributes to vascular permeability (Stowe et al., 2009). Neutrophils accumulate both in the perivascular spaces and in the brain parenchyma (Perez-de-Puig et al., 2015). Despite every fact described above, neutrophils are not only harmful when it comes to stroke pathology. Several studies have reported a possible neuroprotective role. Previous studies in our laboratory showed that PPARγ activation induces the polarization of neutrophils toward an N2 phenotype in the brain parenchyma characterized by the expression of Ym1 marker (a marker traditionally used for M2 macrophages) (Cuartero

et al., 2013). This shift is associated with neuroprotection and resolution of inflammation after stroke since these neutrophils are more easily engulfed by microglia and therefore more rapidly cleared from the injured tissue (Cuartero et al., 2013). PPARγ activation not only shifted neutrophils towards the N2 phenotype but also increased the number of infiltrated neutrophils which crashed frontally with the previous literature since the accepted theory claimed that more neutrophils implied more damage. In the same study, when depleting neutrophils in mice treated with a PPARγ agonist, the neuroprotection that the depletion should have provided was not evident indicating that N2 neutrophils do not play a deleterious role after stroke (Cuartero et al., 2013).

1.3.5.5. Lymphocyte infiltration

In later stages, around three days after the ischemia, lymphocytes (T and B) are also recruited (Gelderblom et al., 2009). Similar to other immune cells, such as neutrophils and monocytes, lymphocytes release inflammatory cytokines and cytotoxic substances, such as ROS. Lymphocyte infiltration has also been linked to the severity of the injury, as that of neutrophils, since the prevention of their entry confers neuroprotection after ischemia (Becker et al., 2001). Specifically, whereas T lymphocytes are believed to contribute to amplifying inflammation after ischemic stroke, B lymphocytes seem to be implicated in the cognitive impairment following stroke (Doyle et al., 2015). The general consensus establish that T lymphocytes respond at sterile lesions as they would do to an infection, therefore releasing cytokines and cytotoxins that could contribute to the lesion (Kim et al., 2016). However, as well as in monocytes and neutrophils, there are lymphocytes subpopulations which could play different roles in ischemia. When Treg lymphocytes are depleted, infarct volume increases (Liesz et al., 2009).

1.3.6. Clinical management of stroke

Stroke is a medical emergency. Ideally, when patients suffer a stroke, they will be taken to a stroke unit. These units present unified treatment protocols and a rigorous control of the admitted patient allowing a tighter control. Also, it is especially important that the emergency response team acts in a coordinated manner with the hospital network in order to treat the patients in the fastest and most efficient way, keeping them monitored until they reach the stroke unit (Ringelstein et al., 2013). Nowadays, two possible reperfusion therapies are usually administered together to increase the success rate, intravenous thrombolysis and endovascular thrombectomy. The choice of which reperfusion therapy is applied will depend on the location of vessel occlusion, the presence of viable brain tissue and the time elapsed since stroke onset.

1.3.6.1. Intravenous thrombolysis

Intravenous thrombolysis aim is the recanalization of the occluded artery by tissue plasminogen activator (tPA) intravenous administration. tPA cleaves plasminogen to plasmin, which degrades fibrin and dissolves the thrombus. Traditionally, tPA was administered up to 4.5 h after stroke onset since the efficacy reduced and the secondary effects increased after that time (Lees KR et al., 2010). Recent studies have extended the time window for this therapy; for example, the EXTEND trial demonstrated the tPA effectivity when administered up to 9 h after onset (Ma et al., 2012). The main drawback of this therapy is its main adverse effect, hemorrhagic transformation. Aside of the time, eligibility for this therapy also depends on other exclusion factors such us past history of intracerebral hemorrhage, recent surgery, use of anticoagulants or uncontrolled hypertension (Campbell et al., 2019).

1.3.6.2. Endovascular thrombectomy

Endovascular thrombectomy consists in the removal of the thrombus under image guidance leading to rapid recanalization of the blood flow. At the beginning the results were not very promising but with the irruption of new improved removal devices the positive results became evident. Most recent trials demonstrated a major benefit of reperfusion up to 24 h after onset (Nogueira et al., 2018). Endovascular thrombectomy has fewer contraindications than intravenous thrombolysis. Patients not eligible for treatment with tPA can be treated with endovascular thrombectomy. Despite its advantages, intravenous thrombolysis remains the standard therapy for all eligible patients. After tPA is administered, thrombectomy is also performed to increase the odds of reperfusion. Ongoing trials are studying whether direct thrombectomy without intravenous thrombolysis is non-inferior to both treatments. The main issue with this way of treatments lays on the availability of an immediate thrombectomy procedure since most patients are first treated in a primary stroke center, where they receive intravenous thrombolysis and then are transferred to a comprehensive stroke center capable of endovascular thrombectomy (Campbell et al., 2019).

1.3.7. Role of TLR4 in the acute damage after ischemia

Our group was pioneer in describing the implication of TLR4 in brain damage and inflammation after experimental stroke (Caso et al., 2007). After that, several groups have proven that the lack of TLR4 entitles improved neurological and behavioral outcomes in different animal models of experimental stroke (Cao et al., 2007; Hua et al., 2007). The

absence of TLR4 produces a significant decrease of IκB phosphorylation, NFκB activity, pro-inflammatory mediators (such as TNF-α and IL-6) and enzymes such as iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) (Caso et al., 2007). This fact has also been demonstrated in patients, in which the up-regulation of TLR4 correlates with higher inflammation and poor outcome (Brea et al., 2011). After ischemia, HMGB-1 (high-mobility group box 1) has been described to activate TLR4 and to induce MMP-9 upregulation in neurons and astrocytes, which contribute to the damage of the BBB as part as the previously mentioned ischemic cascade (Qiu et al., 2010). Recently, it has been shown that the administration of a TLR4-binding DNA aptamer exerts a protective effect against acute stroke in animal models (Fernández et al., 2018).

TLR4 is not only implicated in inflammation after stroke, but also in neurogenesis. TLR4 deficiency promotes subventricular zone cell proliferation but its presence promotes neuroblast migration and increases the number of new cortical neurons after stroke proving to have a modulating role in neurogenesis (Moraga et al., 2014). Recently, it has been demonstrated that TLR4 activation is necessary to keep an increased proliferation of neural stem/progenitor cells (NSCs) as well as to facilitate the neuroblast differentiation and migration (Palma-Tortosa et al., 2019).

Surprisingly, TLR4 stimulation prior to the ischemic insult leads to suppression of inflammation and contributes to neuroprotection. This phenomenon, called ischemic preconditioning, occurs when minor ischemic events lead to a decrease in the neuronal damage of a posterior stroke (Pradillo et al., 2009).

The fact that TLR4 is expressed in CNS as well as in circulating cells makes it harder to understand the implication of the receptor in a systemic way. The peripheric contribution

of TLR4 to the ischemic process has not been fully studied. Chimeric mice with bone marrow lacking TLR4 showed a reduced infarct volume and edema after experimental stroke indicating that peripheral cells have an important role in stroke pathophysiology (Yang et al., 2008).

Following this idea, our laboratory kept exploring the role of TLR4 in neutrophil reprogramming toward an alternative phenotype in brain ischemia. In this study we showed that TLR4 absence increases neutrophil infiltration (as previously seen with PPARγ activation) which was again concomitant to neuroprotection after permanent middle cerebral artery occlusion (pMCAO) (García-Culebras et al., 2019). Interestingly, the absolute count of infiltrated neutrophils into the infarcted area was not significantly affected by the reduced infarct volume but, when normalized by the infarct volume, an increased number of neutrophils (neutrophils/mm^3) was found.

As previously stated, literature indicates that neutrophil infiltration is associated with brain damage. We found indeed a positive correlation between the number of neutrophils and infarct volume in WT mice but the opposite in TLR4-KO mice, supporting a differential role of neutrophils in the absence of TLR4. Blood neutrophilia is attenuated in TLR4-KO mice despite their increased infiltration to the ischemic tissue. All these aforementioned results suggest a differential role of neutrophils depending on the presence or absence of TLR4. To further confirm this statement, we eliminated neutrophils *in vivo* after pMCAO. WT mice treated with antiLy6G leukocyte antibody presented a significantly smaller infarct volume than those treated with the IgG isotype. However, neutrophil-depleted TLR4-KO animals had a significantly higher infarct volume.

Altogether, these data support the notion that neutrophil infiltration into the ischemic tissue has functional and opposite consequences in the presence or absence of TLR4.

As we did in previous studies, we selected Ym1 to explore whether TLR4 presence affects the relative proportions of classical (N1) versus alternative (N2) neutrophil populations in the ischemic brain, and we found that N2 neutrophils were more abundant in the infarcted area of TLR4-KO mice.

Finally, to determine whether TLR4 deficiency was relevant in specific cell subsets, we obtained mice with TLR-deficient myeloid lineage by using the $TLR4^{loxP/LyzMcre}$ construction. Those mice showed dramatically decreased lesion volumes confirming our hypothesis, that specific ablation of TLR4 in neutrophils conferred neuroprotection after pMCAO.

1.3.8. Targeting neutrophils in ischemic stroke

All the aforementioned data evidence that neutrophils have key roles in sterile inflammation and, in what concerns to us in this particular dissertation, in ischemic brain injury. For such reason, neutrophils are of great interest as targets to treat and prevent ischemic stroke.

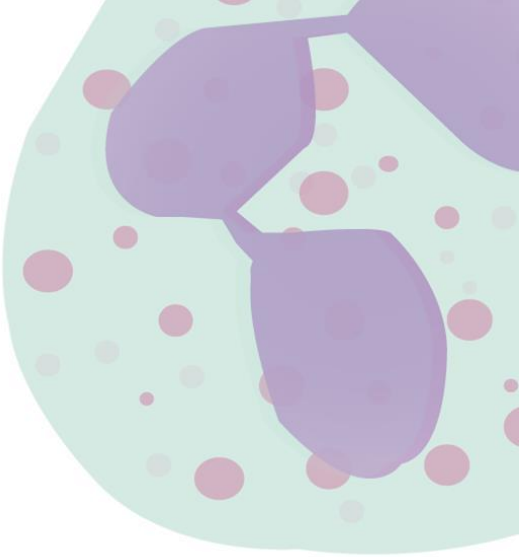
The inhibition of neutrophil recruitment to the ischemic tissue is a strategy that could be theoretically approached by targeting different signaling pathways. Neutrophils react to chemokines and cytokines released after stroke to initiate activation and infiltration. Inhibition of such molecules has shown variable results in ischemic stroke. Inhibition of CXC chemokines resulted in impaired neutrophil activation but it did not show any effect on stroke outcome (Copin et al., 2013). However, inhibition of both CXCR1 and CXCR2,

but not of CXCR2 alone, reduced the infarct volume and improved the outcome in a stroke animal model (Brait et al., 2011). Antagonizing IL-1R has shown a decrease in neutrophil infiltration, infarct size and cerebral edema and an improvement in behavioral outcomes (Rothwell, 2003). When it comes to the recruitment phase, a possible target could be the inhibition of the P-selectin-PSGL-1 interaction which mediates platelet-neutrophil adhesion whose blockage has shown to be neuroprotective (Sreeramkumar et al., 2014).

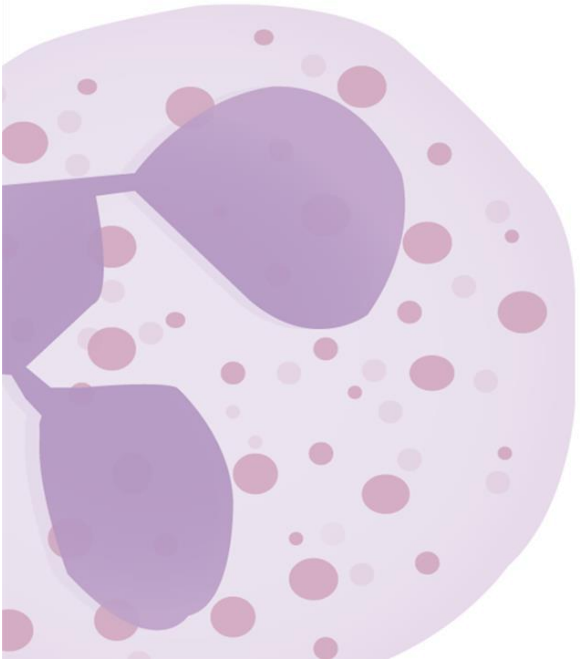
Another potential approach is to impede neutrophil infiltration by manipulating integrins and selectins. The blockade of ICAM-1 showed some promising results in animal models but failed to be effective in patients (Enlimomab Acute Stroke Trial Investigators, 2001; Zhang et al., 1995). The effect of blocking LFA1 (CD11a/CD18) and Mac1(CD11b/CD18), which mediate neutrophil adhesion through ICAM1 and ICAM2 in endothelial cells, has also been explored (Arumugam et al., 2004; Soriano et al., 1999; Zhang et al., 2003). Again, the results have been different depending on the timing and the model used.

Neutrophil-mediated detrimental effects are also possible targets. Neutrophils contribute to the disruption of the BBB through the release of proteases (such as MMP-9 and elastase) and ROS. Pharmacological and genetic approaches to block these molecules have shown promising results in animal models (Gidday et al., 2005; Stowe et al., 2009). However, this potential therapy remains to be proved beneficial in patients, especially considering the potential reparative actions of MMP-9, also known to promote angiogenesis/neurogenesis in the chronic phase (Hao et al., 2011; Lee et al., 2006).

Finally, as it has been extensively described previously, the modulation of neutrophil phenotype towards phenotypes with pro-resolving properties in stroke is an interesting novel therapeutic possibility, a possibility that pharmacologically may be achieved by aiming at specific targets such as, for instance, PPARγ and TLR receptors. In summary, current knowledge underlines the importance of the identification of the myeloid cell subsets directly associated to neuroprotection after stroke. In addition, it is also essential to characterize specific states of activation in myeloid cells associated to brain ischemia and to explore the signaling pathways by which these states determine stroke progression and outcome, as well as the mechanisms and consequences of platelet–leukocyte interactions. The comprehension of these processes may pave the way to novel therapeutic avenues to stop the cytotoxic phase of the inflammatory response and to promote neuroprotection and tissue repair after ischemic stroke. Furthermore, a better characterization of the myeloid cell subsets that orchestrate the response to brain ischemia and their dynamics may help us to design specific treatments within specific therapeutic windows with the aim to increase the benefits of the modulation of cell– cell interactions in the immune response after stroke



HYPOTHESIS AND OJBECTIVES



2. HYPOTHESIS AND OBJECTIVES

2.1. HYPOTHESIS

TLR4 plays a key role in the inflammatory process and tissue damage that occur after a stroke. It has been described that in TLR4KO mice there is a negative correlation between infiltrated neutrophils and infarct volume. Moreover, neutrophil-depleted TLR4KO mice exhibit a significantly bigger infarct volume. However, the specific mechanisms by which TLR4 participates of those processes are not fully understood.

We hypothesize that the neutrophilic TLR4 is the responsible for the harm induced to the brain tissue. Also, that TLR4 might induce neutrophil polarization towards an N2 phenotype, associated with neuroprotection. Finally, that the lack of TLR4 could modify the neutrophil functions and therefore alter its contribution to the inflammatory process.

2.2. OBJECTIVES

The objectives stablished in order to prove this hypothesis were the following:

I. Study of the $TLR4^{+/+}$ and $TLR4^{-/-}$ transcriptome analysis

II. Study of the effect of TLR4 specific myeloid ablation on stroke

III. Characterization of $TLR4^{-/-}$ neutrophils dynamics

IV. Study of the effect of specific myeloid TLR4 ablation in neutrophil functions in homeostasis and after stroke

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. *IN VIVO* TECHNIQUES

3.1.1. Mice

Adult mice from 8 to 10 weeks were used to perform the experiments. Different genotypes of mice were used. WT mice, B6.C57BL/6J were obtained from Harlan. B6.B10ScN-Tlr4$^{lps\text{-}del}$/JthJ (TLR4KO mice) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice resulting from the breeding of both strains were used to obtain F2 mice, TLR4KO and their littermate controls. To elucidate the specific role of TLR4 in different cells transgenic mice expressing the Cre recombinase enzyme under specific drivers were used (B6.129P2-Lyz2tm1(cre)Ifo/J, Jackson Laboratory). Specifically, we used LyzM^{Cre} mice, where this enzyme, under the lysozyme M promoter (LyzM), is expressed mainly in myeloid cells, combined with TLR4$^{loxP/loxP}$ mice, kindly donated by Prof. Timothy Billiar (University of Pittsburgh, USA) to obtain mice lacking TLR4 specifically in myeloid cells. LyzM^{cre} mice were also crossed with GFP mice (B6.Cg-Gt(ROSA)26Sol-m6CCAG-ZsGreen11Hze¡J, Jackson Laboratory) to create LyzM^{cre}/GFP mouse model where myeloid express the green fluorescent protein.

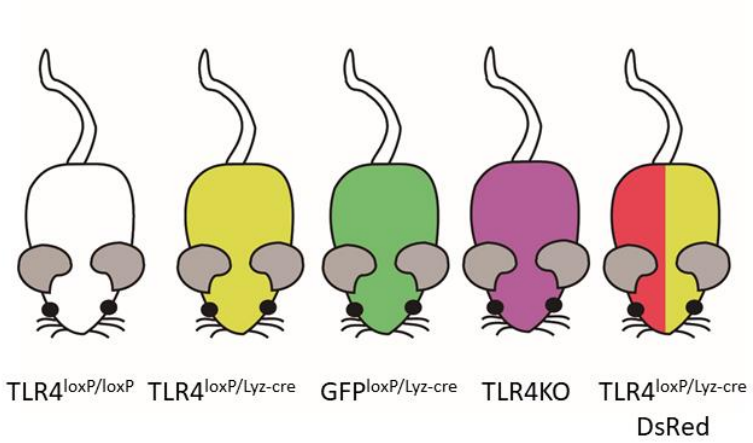


***Figure 14. Transgenic mice**. TLR4$^{loxP/loxP}$, control mice; TLR4$^{loxP/LyzM\text{-}cre}$ mice lacking TLR4 in myeloid cells; GFP$^{loxP/LyzM\text{-}cre}$ mice whose myeloid cells express the GFP; TLR4KO mice lacking TLR4; TLR4$^{loxP/LyzM\text{-}cre}$ + DsRed chimeric mice.*

Genotyping of these mice was made by Polymerase Chain Reaction (PCR) using DNA obtained from tail biopsy. DNA extraction was performed by the "Hot-Shot" technique. Tail biopsy is mixed with 70 µl of NaOH 1M in a 250 µl Eppendorf tube. The tube is placed in a PCR machine (25 min 90°C) and, when finished, 70 µl of neutralization buffer is added and mixed. Then 2 µl of the mix is used as DNA sample for the PCR. Primers used for genotyping can be found on table 1.

To obtain reporter mice for intravital microscopy (IVM) studies we used mice expressing DsRed under the control of the b-actin promoter (B6.Cg-Tg(CAG-DsRed*MST)1Nagy, kindly donated by Dr Hidalgo).

Gene	Primer
TLR4	F:5´-GCA AGT TTC TAT ATG CAT TCT C -3´ R: 5´- CCT CCA TTT CCA ATA GGT AG -3´
$TLR4^{loxP}$	F:5´- AGGGAGATGTGTGTGAAGAAGCCT -3´ R: 5´- TGACTTTCTCAGTTTGGTCCTGGG -3´
Lyz^{cre}	F:5´- TTACTCACCCTACACCAAATTTCCCTCC -3´ R: 5´- CCT CCA TTT CCA ATA GGT AG -3´
Internal control	F:5´-GCA AGT TTC TAT ATG CAT TCT C -3´ R: 5´- CCT CCA TTT CCA ATA GGT AG -3´

Table 1. *Primers used for transgenic mice genotyping.*

All experimental protocols were performed in accordance with the European Parliament and of the Council Directive 2010/63/EU and Spanish legislation (Real Decreto 53/2013) and were approved by the Ethics Committee on Animal Welfare of University Complutense (PROEX 016/18). Mice were housed under standard temperature and humidity conditions in a 12 h light/dark cycle room. Mice had access to rodent chow and water *ad libitum*. All groups were performed and quantified in a randomized fashion by investigators blinded to each specific condition.

3.1.2. Induction of focal ischemia

Surgery was conducted under anesthesia with isoflurane in a mix of O_2 and synthetic air (0.2/0.8 L/min). Through the procedure body temperature was maintained at 37.0°C using a servo-controlled rectal probe-heating pad. Surgical procedure is a variant of the one described by Chen and collaborators (Chen et al., 1986). Firstly, the ipsilateral

common carotid artery was permanently occluded (Figure 15A). Then, an incision is made through the middle line between the left eye and the auditive conduct (Figure 15B). Temporal muscle is then exposed and retracted. A small craniotomy is made over the trunk of the left MCAO (Figure 15C). pMCAO was performed by ligature of the trunk just before its bifurcation between the frontal and parietal branches with a 9-0 suture (proximal occlusion) (Figure 15D). Flow disruption was confirmed visually under an operating microscope. These experimental conditions led to moderately sized cortical infarcts (Figure 15E). Mortality is non-existent after MCAO in this model and it is unaffected by the different experimental groups.

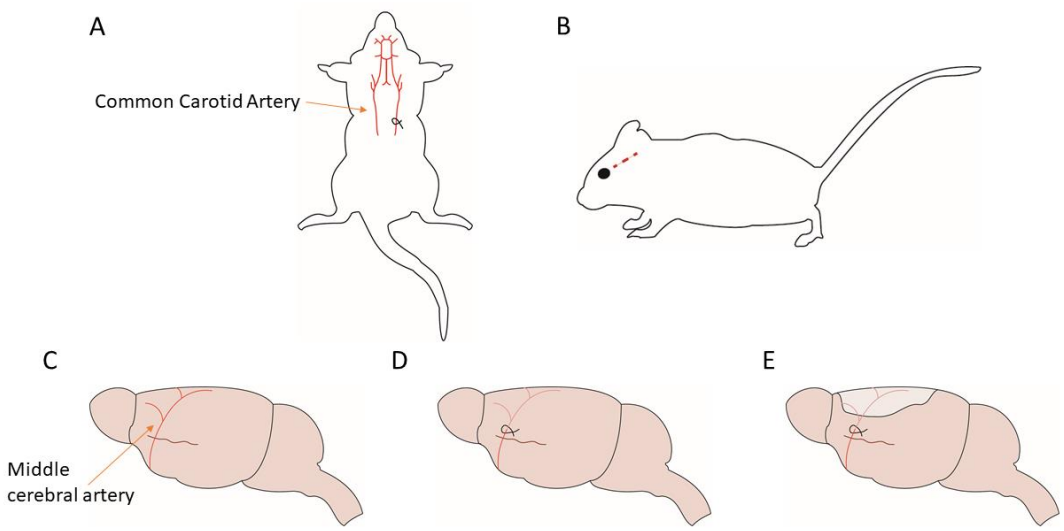


Figure 15. Induction of focal ischemia*. A: ligature of the CCA; B: incision through the middle line between the left eye and the auditive conduct; C: trunk of the left MCAO; D: pMCAO performed by ligature of the trunk just before its bifurcation between the frontal and parietal branches; D: moderately sized cortical infarcts.*

3.1.3. Brain infarct determination

The infarct extension was determined by magnetic resonance imaging (MRI). MRI was performed 24 h after pMCAO using a BIOSPEC BMT 47/40 (Bruker, Ettlingen, Germany). T2-weighted images were acquired, and infarct volume was calculated using the MRI analysis calculator application from Image J software (NIH, USA). To calculate the infarct volume as the percentage of the hemisphere that is infarcted, we estimated the volume of the contralateral hemisphere (CH) and that of the non-lesioned ipsilateral hemisphere (NLH) in 16 coronal sections between -1.78 and -3.64mm posterior to bregma (450 µm apart). The % of the infarcted hemisphere was then calculated using the formula = (CH-NLH/CH) x 100. The value resulting from this formula is then normalized by the edema index which is the ratio between the volume of the contralateral and ipsilateral hemisphere.

3.1.4. Neutrophil depletion

To deplete neutrophils, mice were injected either with mouse anti-neutrophil antibody (anti-mouse Ly6G 1A8 clone BioXCel, which is specific to neutrophils within myeloid cells) 0.5 mg per day i.p. or control isotype (rat IgG2A BioXCel) daily for 3 days, starting 24 h before surgery (Figure 18).

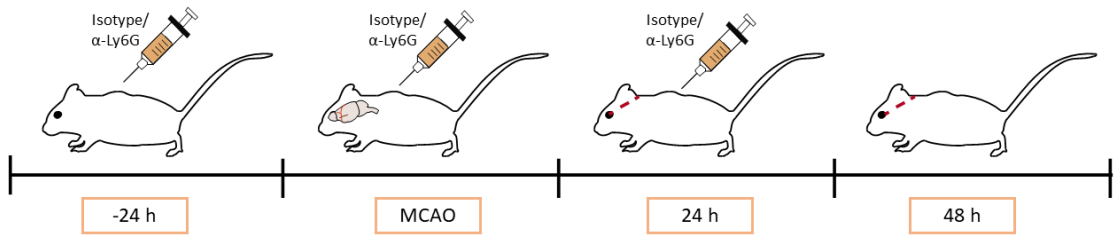


Figure 18. Representative outline of the neutrophil depletion protocol. *Mice were injected either with mouse anti-neutrophil antibody 0.5 mg per day i.p. or control isotype daily for 3 days, starting 24 h before surgery.*

3.1.5. Bone marrow transplant for chimeric mice

To compare the neutrophils ability to migrate from the vessel into the infarcted tissue we created mice with a mixed bone marrow (BM) constituted by myeloid precursors with and without TLR. Recipient mice from $TLR4^{loxP/loxP}$ were irradiated (13 Gy in two doses) and 24 h after, BM was reconstituted. Donor BM was harvested from $GFP^{loxP/LyzM\text{-}cre}$ and $TLR4^{loxP/LyzM\text{-}cre}$ by flushing both tibiae and femurs with FACS (fluorescence-activated cell sorter) buffer into a 50 ml Falcon tube. Tissue was homogenized by flushing it through a mixing needle. Cell suspension was centrifuged (400xg, 5 min) and supernatant aspired. Cell pellet was then mixed with 1 ml red blood cells (RBC) lysis buffer for 1 minute. Finally, 9 ml of FACS buffer was added and centrifuged for 5 min at 400xg and cells are resuspended in FACS buffer being ready to be injected into the bloodstream.

3.1.6. Intravital microscopy of the cremaster muscle

Intravital microscopy of the cremaster muscle after TNF-α stimulation (R&D Systems, 0.5 mg intrascrotal injection) was performed as previously reported (Hidalgo et al., 2009) (Figure 16) using the VIVO system indicated above. For confocal IVM, we used laser stacks for 488, 561 and 640 nm beams coupled with a confocal scanner (Yokogawa CSUX-A1; Yokogawa, Japan) and images were acquired with 0.5 mm Z-intervals. The SlideBook software was used for acquisition and analysis. Ten to twenty venules segments per mouse were analyzed 150 to 210 min after TNF-α treatment in multiple fluorescence channels (Cy3/561 for PE, FITC/488 for FITC and Cy5/640 for APC) and bright-field images with 1x1 or 232 binning with 3 second interval for 2 min on each field of view. For double staining with PE- and FITC-conjugated antibodies, acquisition was facilitated in single (FITC) and quadrant (PE) filters in order to avoid bleed-through of fluorescent signals between channels. For the visualization of leukocytes, anti-Ly6C-FITC antibody was injected intravenously at 1 mg/mouse. For analysis of rolling and adhered cells to the inflamed endothelium we used the SlideBook software. Counts of rolling or adhered cells in 2-minute captures (captured at 3 second intervals) were normalized using the width of the vessel to allow comparison among all vessels. For adhesion or rolling efficiency indices, these data were compared with the frequency of free-flowing WT and experimental cells in the blood for each mouse, which was obtained from cytometric analysis of blood neutrophils for BM chimeric mouse. Kinetic parameters for crawling neutrophils were calculated using ImageJ, with the help of the Manual Tracking plugin (Fabrice Cordelieres, Institut Curie, France) and the Chemotaxis and Migration Tool (Gerhard Trapp and Elias Horn, ibidi GmbH, Germany).

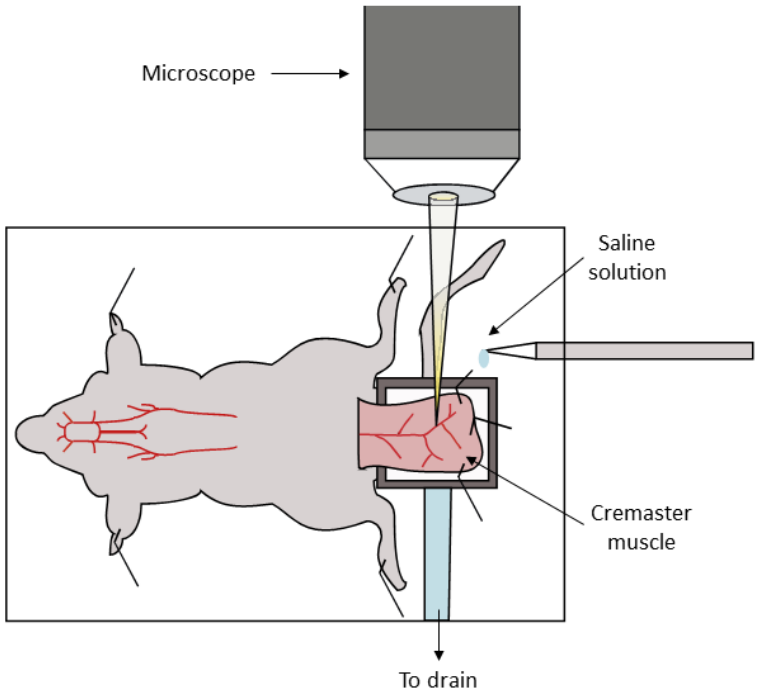


Figure 16. Representative image of the intravital microscopy of the cremaster muscle. *After TNF-α intrascrotal injection, the cremaster muscle is exposed and fixed into a methacrylate base bathed in saline solution that drains through one side of the base.*

3.1.7. Zymosan-Induced peritonitis

Transplantation chimeras were treated with zymosan (1mg, intraperitoneal injection, Sigma). 2 h later, blood samples were collected, and the peritoneal lavage was obtained for cytometric analyses and cell count. The ratios of neutrophils from each donor in the peritoneum and blood were compared to estimate the migration efficiencies of mutant cells (ratio in peritoneum / ratio in blood).

3.2. *IN VITRO* TECHNIQUES

3.2.1. Blood processing

Blood samples were obtained either from the tail vein or directly from the right ventricle before perfusion. Blood cells were lysed in RT lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L CaCl2, 0.02% NaN3, 1% Triton X-100).

3.2.2. Microglia isolation

Microglia was isolated as previously described (Grabert and McColl, 2018). Firstly, mice were perfused transcardially with saline. The ipsilateral hemisphere to MCAO was minced by scalpel blade in ice-cold HBSS, then centrifuged (400xg, 5 min, 4ºC), resuspended and incubated for 1 h at 37°C in enzymatic cocktail (50 U/ml collagenase, 8.5 U/ml dispase, 100 µg/ml Na-tosyl-L-lysine chloromethyl ketone hydrochloride, 5 U/ml DNaseI in HBSS). Tissue was then dissociated by using a Dounce homogeniser and the enzymatic reaction was terminated by addition of an equal volume of 10% fetal bovine serum HBSS. Homogenates were centrifuged and pellets resuspended in 35% Percoll overlaid with HBSS and then centrifuged (800xg, 45 min, 4°C). Cells were resuspended in MACS buffer and CD11b microbeads (Miltenyi Biotec) were added followed by an incubation (15 min, 4°C). After incubation, 100 µl of buffer was added to the cells and they were flushed through a LS magnetic column placed in the magnet. The flow-through collected is the negative selection fraction (Figure 17). To obtain the positive selection fraction the column is removed from the magnet and placed into a 15 ml tube. 5 ml of buffer was added, and bead-bound microglial cells were flushed out by firmly pushing the plunger.

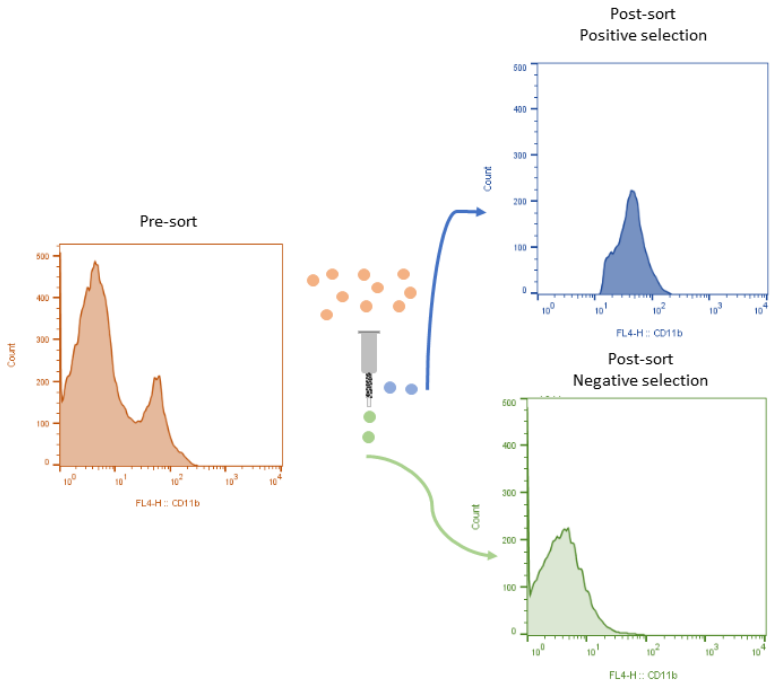


Figure 17. Microglia isolation. *FACS plots showing CD11b signal before and after the sorting process.*

3.2.3. Immunofluorescence

Animals were euthanized 48 h after pMCAO by isoflurane overdose followed by transcardiac perfusion with 0.1M phosphate buffer followed by 4% p-formaldehyde (PFA) in 0.1M phosphate buffer (pH 7.4). Brains were removed, placed in 4% PFA overnight and then transferred into a 50 ml Falcon tube filled with 30% sucrose solution for 48h. Coronal series sections (40 µm) were sliced on a freezing microtome (Leica SM2000R, Leica Microsystems GmbH, Wetzlar, Germany) and stored in a cryoprotective solution. Double-label immunofluorescence was performed on free-floating sections and incubated overnight at 4°C with the following primary antibodies: rabbit anti-mouse Ym1 (Stem Cell Tech. Inc.), Biosciences), rat anti-mouse NIMP-R14 (Abcam) and anti-laminin biotin-conjugated (Novus Biological). Secondary antibodies used were goat anti-rabbit biotin or

goat anti-mouse biotin (Vector laboratories) in combination with Alexa488 streptavidin (Molecular Probes), donkey Cy3 anti-rat (Jackson Immunoresearch) and donkey Cy3 anti-mouse (Vector Laboratories). Controls performed in parallel without primary antibodies showed very low levels of nonspecific staining. Image acquisition was performed with a laser-scanning confocal imaging system (Zeiss LSM710) and image analysis was performed with the ZEN 2009 software (Zeiss). All co-localization images shown were confirmed by orthogonal projection of z-stack files.

3.2.4. Stereological analysis

The stereology system consists of a Nikon Eclipse TE300 microscope fitted with a XYZ motorized computer stage and controller (Ludl Electronics Products, Hawthorne, NY, USA) and with the StereoInvestigator software (Microbrightfield version 7.003 software). Quantification of positive cells for the different markers was performed with the 40X objective in the outlined area of the infarct, using the optical fractionator method [23] with the following sampling parameters: (1) a counting frame area of 1600 μm^2, (2) a dissector height of 14 μm^2, (3) a guard zone of 2 μm^2. The estimated total positive cell number (N) was calculated using the equation: $N= \Sigma Qx\ 1/ssf\ x\ 1/asf\ x\ 1/tsf$, where ΣQ- is the total number of cells counted with the fractionator, ssf is the section sampling fraction, asf is the sampling fraction area, and tsf is the sampling fraction thickness. The reliability of the sampling scheme was confirmed by the calculation of the Schmitz-Hof coefficient of error, which was <0.1.

3.2.5. Cell sorting by flow cytometry

Blood from a pool of 6 WT animals and 6 TLR4KO mice was harvested 48 after ischemia, RBC lysed, and leukocytes stained with Ly6G-Pacific Blue (1A8, Biolegend). Neutrophils were sorted on a FACS Aria III (BD Biosciences) resulting in > 95% purity and immediately processed for RNA extraction.

3.2.6. RNA isolation

Total RNA from each replicate of the two groups was isolated using NucleoZOL (MACHEREY-NAGEL, 740404.200) according to the manufacturer's protocol. Absorbance was measured at 260/280 nanometers for mRNA purity and a ratio above 1.9 was achieved for all samples used.

3.2.7. RNA array

Neutrophils were isolated from the blood and total RNA was extracted using Nucleozol® reagent (Invitrogen, USA). cDNA was synthesized and amplified using Transcriptor Universal cDNA Master kit (Roche) according to the manufacturer´s protocol and processed with GeneChip® WT PLUS Reagent Kit (Affymetrix®), hybridized with GeneChip® Mouse Gene 2.0 ST Array (Affymetrix®) and scanned with a GeneChip® Scanner 3000 7G (Affymetrix®). Raw data were processed with RMA algorithm included in Affymetrix® Expression Console™ for normalization and gene level analysis. For each experimental condition, three microarray experiments corresponding to three independent RNA replicates were processed and analysed. Fold changes between experimental conditions were calculated as a ratio between the mean of the gene expression signals. Statistical analysis was performed with the LIMMA package. For each

experimental condition, three microarray experiments corresponding to three independent RNA replicates from pools of 6 mice, were processed and analysed. Raw data were processed for background removal, quantile normalization and media-polished gene-level summarization with the RMA methods of oligo R package (Carvalho and Irizarry, 2010), (Yu et al., 2012). Differentially expressed genes were determined with the LIMMA package, using linear models and empirical Bayes moderation (Ritchie et al., 2015). Functional enrichment for GO terms (Ashburner et al., 2000) was performed using hypergeometric test and multiple testing correction and FDR control, as implemented in the clusterProfiler R package (Yu et al., 2012). Gene Set Enrichment Analysis (Subramanian et al., 2005) was also used as implemented in the clusterProfiler R package, using 25000 permutations for the Enrichment Score significance level estimation, multiple testing correction and FDR control. Gene Sets for Gene set enrichment analysis was performed with the sets from MsigDB v6.1 (Liberzon et al., 2011) as provided by the msigdbr package (https://github.com/igordot/msigdbr). Mouse orthologs assignments were performed using those provided by the HUGO Gene Nomenclature Committee at the European Bioinformatics Institute.

3.2.8. Flow cytometry

All cell pellets were resuspended in FACS buffer (DPBS containing 0.1% low endotoxin BSA). Cell suspensions were transferred to a 96-well plate and low affinity Fc receptors blocked by cell incubation with anti-CD16/32 antibody for 30 min. Plates were centrifuged (400xg, 5 min), supernatants discarded, and cell pellets disrupted by gentle agitation of plates. For flow cytometric analysis after MCAO or naïve animals, brain, blood and bone marrow cell suspensions were added to a 96-well plate for cell surface labelling

and stained with the following fluorochrome-conjugated monoclonal antibodies for 30 min: anti-mouse CD62L (clone 17A2, 1 µg/ml, BioLegend); anti-mouse/human CD11b-APC-Cy7 (clone M1/70, 1 µg/ml, BioLegend); anti-mouse CXCR4 (clone AFS98), 1 µg/ml, BioLegend); anti-mouse Ly6G (clone HK1.4, 1 µg/ml, BioLegend); anti-mouse Gr1 (clone 1A8, 1 µg/ml, BioLegend. Plates were centrifuged (400 g, 5min), supernatants discarded, and cells resuspended in FACS buffer. For Ym1 intracellular labelling, anti-Ym1 antibody (Stem Cell) was added over night after fix/perm treatment for 20 min (Fixation Buffer, BioLegend). Finally, cells were washed again and then incubated with goat-anti-mouse antibody conjugated to FITC (Poly 4053, Biolegend, 1 mg/ml) for 30 min. Data were acquired using a FACS Calibur (BD Biosciences) and analyzed using FlowJo software. Compensation was performed using single-labeled samples as references and positive regions of staining were defined based on unstained and/or isotype-stained controls. In order to assess cell complexity, mean fluorescent intensity detected in gated neutrophils was measured in SSC and FSC.

3.2.9. Phagocytic activity of myeloid cells

Blood was collected transcardially into a 15 ml Falcon tube previously filled with 10% citrate solution. RBC were lysed with 5 ml RBC lysis buffer (7 min) and blood centrifuged for 5 min at 1800 rpm. Supernatant was discarded and pellet resuspended in 5 RPMI after PBS 1x washing. Beads (latex beads carboxylate-modified polystyrene 1 um) were added to a final dilution of 1:100 and suspension was incubated for 1 h at 37°C. After incubation, cells were washed 3 times with PBS 1x, resuspended in FACS buffer and stained for surface markers (Figure 19).

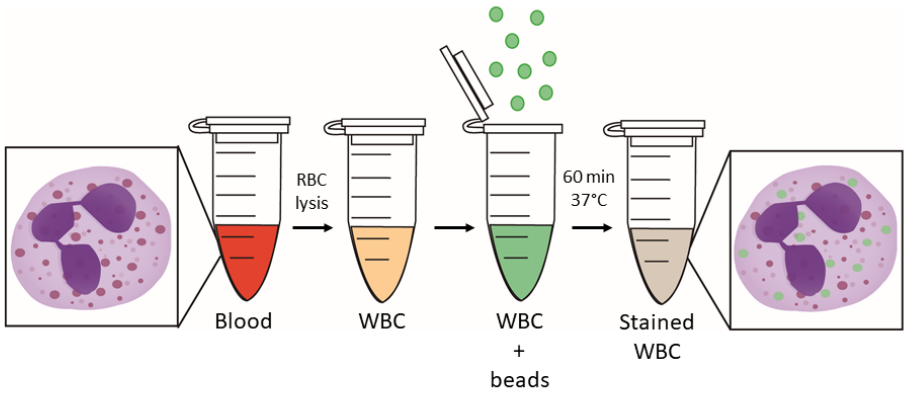


Figure 19. Experimental design of phagocytic activity of myeloid cells assay. *RBC are removed and beads are added to white blood cell (WBC) suspension. After 60 minutes at 37°C incubation cells are stained.*

3.2.10. Apoptosis assay

In order to study the apoptotic process in circulating neutrophils, after blood processing, cells were resuspended at a density of 10^6 cells/ml in FACS buffer. 0.2 mL cell suspension was pipetted into a flow cytometry test tube. 5 µl of 0.2 mM NucView 488 substrate stock solution was added to tube and mixed well to obtain a final concentration of 5 µM. Then, cells were incubated at RT for 15 minutes, protected from light. Finally, after washing cells, 200 µL FACS was added to each tube for cell staining.

3.2.11. Microglia engulfment

Neutrophil engulfment by microglia was performed as previously described (Cuartero et al., 2013). To assess neutrophil engulfment, merged staining of Iba1 and NIMP-R14 or Iba1, NIMP-R14 and Ym1 was analyzed using a laser-scanning confocal imaging system (Zeiss LSM710). All ischemic core was photographed using the 20x magnification objective. Quantification of double or triple labelled cells in the orthogonal projection of

z-stack files was performed using the cell counter tool of the software Image J (NIH). The parameters analyzed were: 1) % of microglia/macrophages engulfing neutrophils (% of Iba1+ cells containing NIMP-R14+ particles) was calculated as the ratio between the number of Iba1+ cells engulfing NIMP-R14+ particles divided by the total number of Iba1+ cells found in the field; 2) % of cleared neutrophils (% of neutrophils engulfed by microglia/macrophages) was calculated as the ratio between the number of NIMPR14+ cells engulfed by Iba1+ cells divided by the total number of NIMP-R14+ cells found in the field; 3) % of cleared Ym1+ neutrophils (% of Ym1+ neutrophils engulfed by microglia/macrophages) was calculated as the ratio between the number of Ym1+ NIMPR14+ cells engulfed by Iba1+ cells divided by the total number of Ym1+ NIMP-R14+ cells found in the field; 4) % of cleared Ym1- neutrophils (% of Ym1- neutrophils engulfed by microglia/macrophages) was calculated as the ratio between the number of Ym1- NIMPR14+ cells engulfed by Iba1+ cells divided by the total number of Ym1- NIMP-R14+ cells found in the field.

3.2.12. Neutrophil oxidative burst assay

Blood was collected transcardially and placed in a 15 ml Falcon tube previously filled with 10% citrate solution. After removing RBC, WBC were washed with 300 ul of DMEM (+ glucose – phenol red) and divided in 100 ul aliquots. 1 µM of DHR 123 and 1 µM of W-peptide (when required) were added and cells were incubated for 20 min at 37ºC. During that time DHR123 is transformed into rhodamine which is a fluorescent compound and therefore detectable with flow cytometry (Figure 20). After incubation, cells were placed in an ice bath for 10 minutes to stop the reaction. Cells were centrifuged at 1800 rpm,

4ºC for 5 min and cell pellet resuspended in 300 µl of ice-cold HBSS. Cells were then stained for flow cytometry analysis.

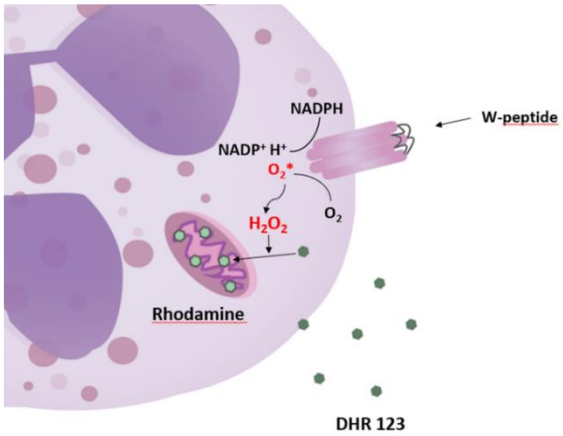


Figure 20. Representative scheme of the oxidative burst assay in the neutrophil. *1 µM of DHR 123 and 1 µM of W-peptide were added and cells were incubated for 20 min at 37ºC. During that time DHR123 is transformed into rhodamine which is a fluorescent compound and therefore detectable with flow cytometry.*

3.2.13. Neutrophil isolation with magnetic beads

Neutrophil isolation was achieved by using a commercial kit by Miltenyi Biotec. Briefly, after removing RBC, cells were resuspended in 200 µl of neutrophil isolation buffer (PBS 1X, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA). 50 µl of Neutrophil Biotin-Antibody Cocktail was added and cells were well mixed and incubated for 10 min in the refrigerator. 10 ml of buffer was added to wash cells and suspension was centrifuged at 300xg for 10 minutes. Pellet was resuspended in 400 µl of buffer and 100 µl of Anti-Biotin MicroBeads were added. Cell suspension was well mixed, and cells were incubated for 15 minutes in the refrigerator. After incubation, cells were washed with 10 ml of buffer and centrifuged at 300xg for 10 minutes. Cell pellet was resuspended in 500 µl of buffer. The column was placed in the magnetic field (MACS Separator) and prepared by rinsing with

500 µl of buffer. Cell suspension was applied onto the column. Flow-through was collected, representing the enriched neutrophils. Then, column was washed three times and flow-through combined with the one obtained in the previous step.

3.2.14. NET formation assay

After neutrophil isolation, $1x10^6$ cells were resuspended in RPMI. Neutrophils were then added to an Ibidi chamber (µ-Slide 8 Well Glass Bottom). Neutrophils were incubated to 15 min at 37°C so that cells adhere to the bottom of the chamber. PMA (20 nmol/L in RPMI) was added and cells are incubated at 37°C for 3h. After incubation, supernatant was removed and 4% PFA is added in order to fix the cells (10 min RT). After fixation, cells were washed and stained for NIMP-R14, elastase and histone H3 markers (rat anti-NIMP 1:200, rabbit anti-elastase 1:300, rabbit anti-histone H3 1:400) (Figure 21). Confocal images were taken in order to quantify the percentage of formed NETs.

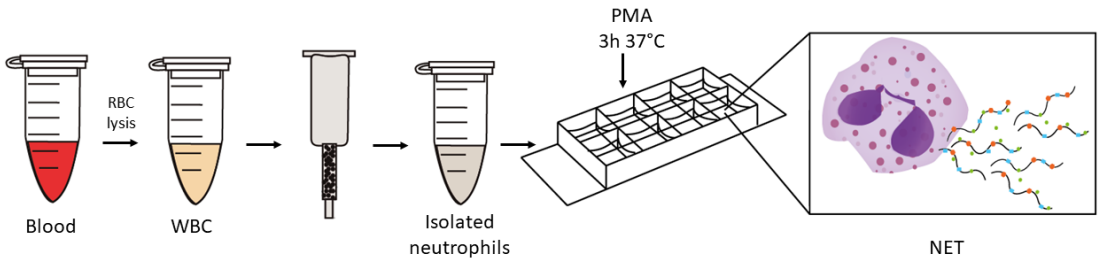


Figure 21. NET formation assay. *Isolated neutrophils are seeded into an Ibidi chamber and stimulated with PMA (20 nmol/L) for 3h. After incubation time cells are fixated and staining for specific NET markers.*

3.2.15. Neutrophil migration transwell assay

After blood processing, WBC were resuspended in RPMI media containing 10% FBS. Transwell inserts (6.5 mm, 5.0 mm pore size (3421; Corning Costar Corporation) were pretreated with 50 µl of RPMI for 20 min prior to adding cell suspension. Lower compartments were filled with 600 µl of DMEM containing 0.05 ng per ul of CXCL1 (Figure 22). Inserts were placed into the compartments and cells seeded (100 µl, $1x10^5$ cells). Plates were incubated for 1.5 h at 37°C. After incubation, liquid at lower compartment was collected and cells were stained for flow cytometry analysis.

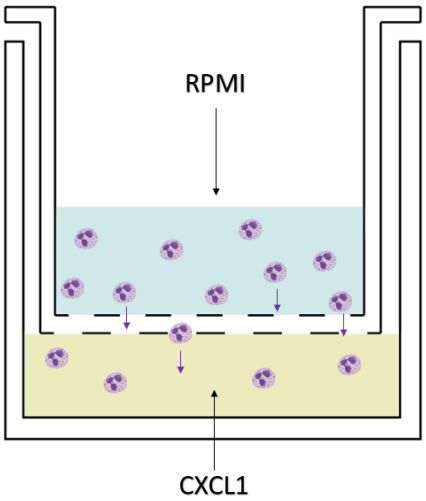
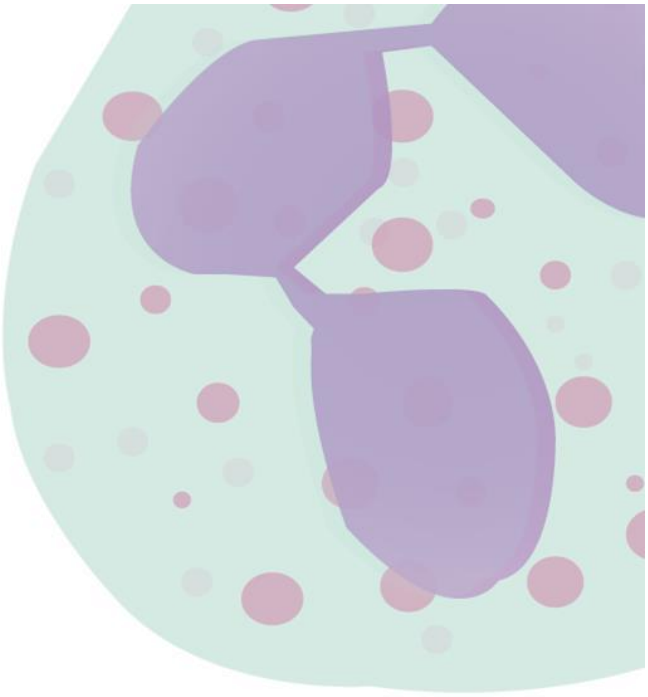


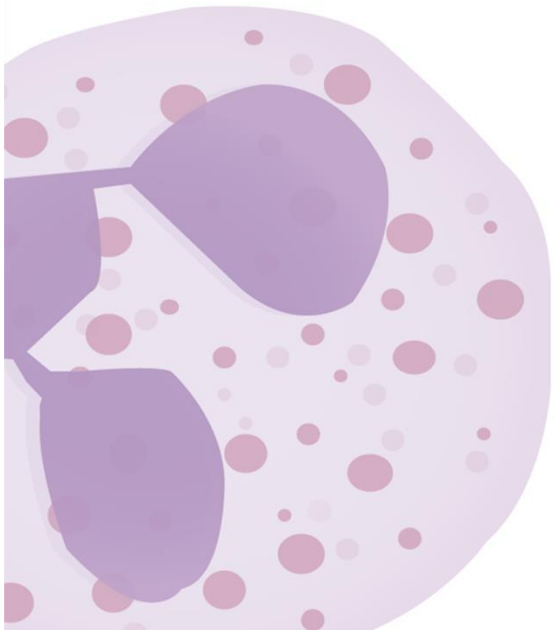
Figure 22. Neutrophil migration transwell assay. *Neutrophils are seeded in the top compartment. The lower compartment is filled with DMEM + CXCL1 which is going to trigger the neutrophil migration.*

3.2.16. Statistical analysis

Results are expressed as mean±SEM for the indicated number of experiments. Statistical significance was determined by t test, or 1- or 2-way ANOVA was used to compare >2 groups or parameters with the Tukey and Bonferroni post hoc tests, respectively. Values of $P<0.05$ were considered statistically significant.



RESULTS



4. RESULTS

4.1. TLR4$^{+/+}$ AND TLR4$^{-/-}$ NEUTROPHIL TRANSCRIPTOME ANALYSIS

4.1.1. mRNA microarray assessment of global gene expression in WT and TLR4-KO blood neutrophils after stroke

As previously stated, since we had seen that TLR4$^{-/-}$ mice showed a smaller infarct volume which could be due to the protective role of TLR4-lacking neutrophils, the starting point of this Doctoral Thesis was to elucidate the molecular mechanisms underlying TLR4-mediated neutrophil phenotype. Our first approach to the issue was to perform a genetic characterization of neutrophils without TLR4. With this aim we performed a cDNA microarray analysis of neutrophils isolated from adult WT and TLR4-KO mice blood 48 hours after ischemia, using Affymetrix platform and posterior Gene Set Enrichment Analysis (MIT Broad Institute). As shown in Fig. 23, the heat map of normalized expression for over 1500 selected genes ($P<0.05$) shows a clearly different signature between WT and TLR4-KO cells.

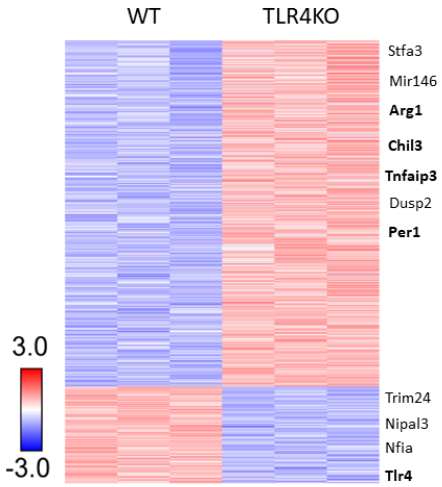


Figure 23. mRNA microarray assessment of global gene expression. *Heat map of normalized expression for 1499 selected genes (P<0.05) between WT and TLR4-KO neutrophils highlighting, among others, some selected genes validated. The color scale of the heat map indicates fold change of expression for each gene (red, upregulation; blue, downregulation).*

To link genes to specific pathways, we evaluated key pathways and gene groups using bioinformatic approaches. Gene Set Enrichment Analysis of the pathways enriched for the 500 genes more differentially expressed in TLR4-KO neutrophils showed 3 major groups of upregulated pathways, related to inflammation, circadian rhythms, and cell death. Interestingly, it also revealed an alteration in pathways related to neutrophil migration such as the IL (interleukin)-6–related pathways, to neutrophil trafficking and positive regulation of locomotion and to cell surface interactions at the vascular wall (Figure 24). These results suggest that neutrophil TLR4 modulates the contribution of these cells to ischemia-induced inflammation, their migration into the parenchyma, and their functional priming.

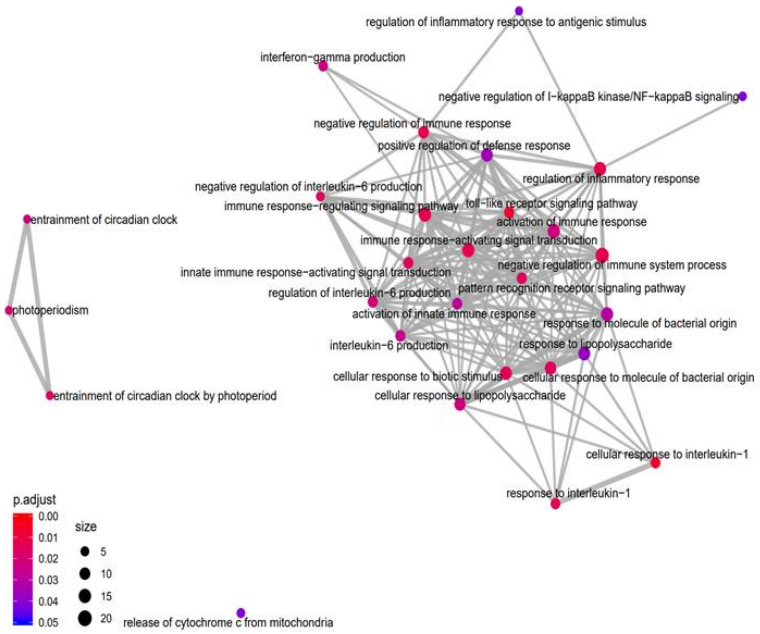


Figure 24. Gene Set Enrichment Analysis map representation. *Pathways enriched for the 500 genes that are more differentially expressed in TLR4-KO than in WT neutrophils.*

4.1.2. mRNA microarray validation of key genes related to N2 subset features

To further explore our hypothesis, we conducted a quantitative polymerase chain reaction validation of some of the gene expression changes in WT versus TLR4-KO. The results were highly concordant between the quantitative polymerase chain reaction and the microarray data, especially regarding the change in expression. Given the analogy found between neutrophils without TLR4 and N2 neutrophils previously described in out laboratory we decided to study its similarity with N2 neutrophils. We specifically explored genes involved in N2 neutrophil characterization such as Chil3, Arg1, Tnfaip3 and Per1. Our results show higher levels of Chil3 and Arg1 mRNA in TLR4-KO than in WT neutrophils. Expression of NF-κB (nuclear factor κB)–related genes, such as Tnfaip3 (tumor necrosis factor alpha induced protein 3; negative regulation of IκB kinase and NF-κB signaling), were also increased in TLR4-KO vs WT. We also studied one gene of the molecular clock,

Per1 (period circadian protein homolog 1), observing that its expression was again increased in TLR4-KO vs WT (Figure 25).

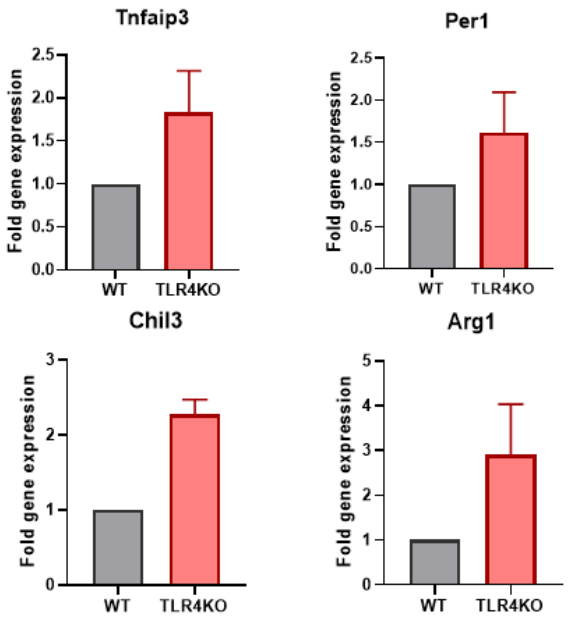


Figure 25. Quantitative polymerase chain reaction validation. *qPCR for Chil3, Arg1, Tnfaip3, and Per1 confirmed significant upregulation of expression in TLR4-KO mice 2 d after middle cerebral artery occlusion in comparison to WT-operated animals. Data are mean±SEM.*

4.1.3. Transcriptional changes similarities between TLR4-/- neutrophils and "non-aged" neutrophils

As described in the Introduction section, a recent study proposed that neutrophil aging is driven by external signals induced by microbiome produced TLR4 agonists (Zhang et al., 2015). In order to study whether TLR4 KO neutrophils were similar to the phenotypes they described (non-aged/aged), we compared the 500 most overexpressed genes in TLR4-KO neutrophils versus WT ones with the transcription changes observed in aged and non-aged neutrophils as reported by Frenette and collaborators (Zhang et al., 2015). The analysis showed that genes overexpressed in TLR4$^{-/-}$ neutrophils were also

significantly enriched among those overexpressed in the non-aged group (Normalized Enrichment Score, −1.38; false discovery rate q-value, 0.00229; Figure 26).

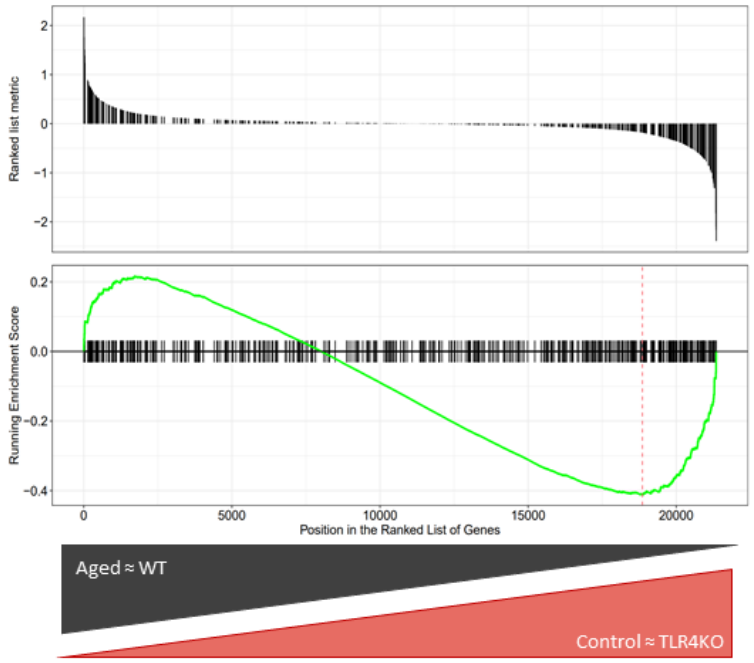


Figure 26. Gene Set Enrichment Analysis comparing both groups. *GSEA analysis showed that TLR4-KO neutrophils are similar to the control group by Frenette and cols (Zhang et al., 2015).*

Interestingly, when performing an enrichment assay only for the genes at the right side of the leading edge (dotted red line) in the Gene Set Enrichment Analysis, 3 upregulated pathways were found, corresponding to regulation of inflammatory response, negative regulation of immune effector process, and negative regulation of IL-6 production (Figure 27). These data demonstrate an alteration of both TLR4-KO and non-aged neutrophils in the ability to participate in the inflammatory process, strongly supporting the important role of TLR4 in neutrophil function.

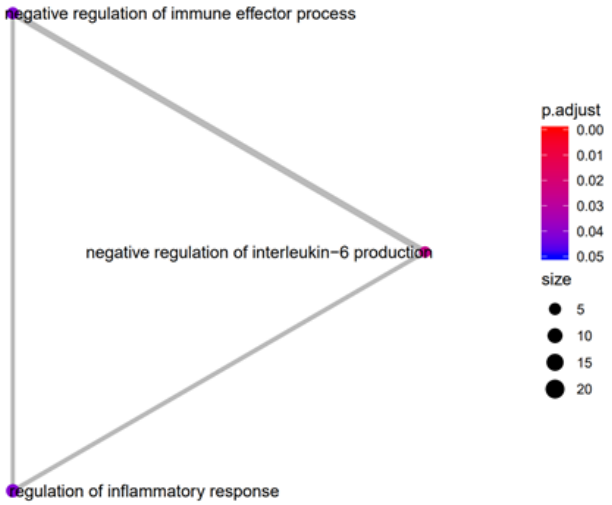


Figure 27. Gene Set Enrichment Analysis map representation*. Enrichment assay (right) only for the genes at the right side of the leading edge in the GSEA analysis showing the 3 altered pathways shared by control and TLR4-KO neutrophils.*

Since neutrophil heterogeneity is an uprising area of research, not only Frenette and cols have described transcriptional changes in different neutrophil subsets. We performed clustering and PCA analysis comparing out transcriptional information with the transcriptional signatures of “fresh” and “old” neutrophils taken at zeitgeber time 5 (ZT5, i.e., 5 hours after lights on) or at zeitgeber time 13, described by Hidalgo and cols. (Adrover et al., 2019). However, we did not find a clear similarity between both groups of subsets. PCA analysis did not show a clear clustering pattern between the different subsets (Figure 28).

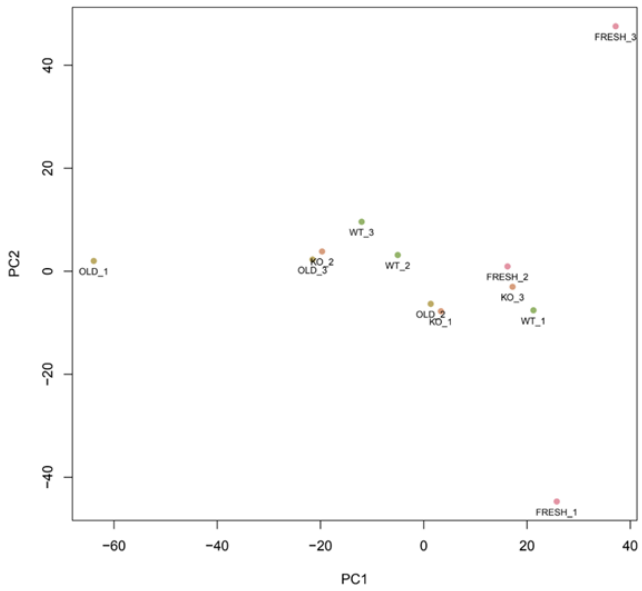


Figure 28. Principal component analysis. *PCA analysis of differentially expressed genes in circulating neutrophils from WT neutrophils at ZT5 ("old") and ZT13 ("fresh") and from WT and TLR4KO at ZT5.*

4.2. EFFECT OF TLR4 SPECIFIC MYELOID ABLATION ON STROKE

Given our previous observations showing that the lack of TLR4 induces neuroprotection after stroke and that neutrophil infiltration into the ischemic tissue has functional and opposite consequences in the presence or absence of TLR4 (García-Culebras et al., 2019), we asked whether TLR4 deficiency was specific for hematopoietic myeloid subsets in specific cell subsets, such as myeloid cells.

4.2.1. Validation of specific TLR4-deficient myeloid lineage genetic construction

To determine whether TLR4 deficiency was specific for hematopoietic myeloid cell subsets, we obtained mice with TLR4-deficient myeloid lineage by using the $TLR4^{loxP/LyzM\text{-}cre}$ genetic construction. Because of their mesodermal origin, microglia share many features with other myeloid cell types in the body. Therefore, the suitability of this

genetical approach to obtain a TLR4-lacking neutrophil model could be jeopardized by the presence of TLR4-lacking microglia. To discard this possibility, we isolated the microglia by magnetic columns sorting, checked the purity of the sorted population by FACS (Figure 29A) and then assessed the TLR4 expression by PCR. Our data confirmed that microglia in TLR4$^{\Delta lyzM}$ mice expresses TLR4 thus discarding any bias arising from the absence of TLR4 in these brain resident cells (Figure 29B). Also, since this genetical approach is based on eliminating TLR4 from myeloid cells, monocytes should be taken into account. The possibility that the lack of TLR4 in monocytes could be a contributing factor to our studies will be explored further ahead.

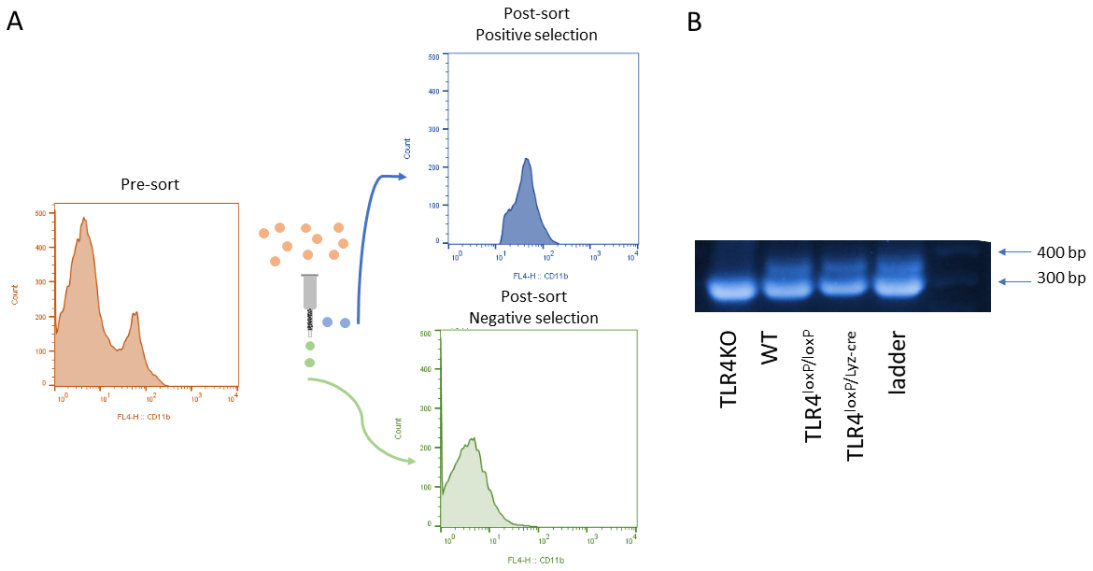


Figure 29. Purity of adult microglia isolated from naïve mouse whole brain. *A. Flow cytometric comparison of positive selected microglia (upper right) with pre-sorted (left) and non-selected samples (lower right) demonstrates highly pure CD11b+ positive selected microglia. B. Agarose gel electrophoresis of TLR4 PCR product; 1–4: TLR4 PCR product (390 bp) and control gene (324 bp) in isolated microglia, 5: ladder (1Kb ladder marker).*

4.2.2. Specific myeloid ablation of TLR4 induces neuroprotection after stroke: role of neutrophils

In accordance with previous results from our laboratory, specific myeloid ablation of TLR4 induces neuroprotection (*P<0.05) after MCAO measured 24 h after the surgery by MRI (Figure 30).

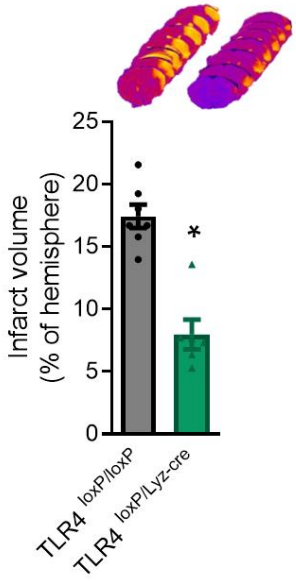


Figure 30. Effect of specific myeloid TLR4 ablation on infarct volume. *$TLR4^{loxP/loxP}$ and $TLR4^{loxP/LyzM\text{-}cre}$ mice were subjected to MCAO; infarct volumes were determined by magnetic resonance imaging (MRI) 24 h after ischemic insult in both groups. Infarct volumes in $TLR4^{loxP/loxP}$ and $TLR4^{loxP/LyzM\text{-}cre}$ mice (n=6–7; *P<0.05 vs $TLR4^{loxP/loxP}$). Data are mean±SEM.*

As previously mentioned, the increase in infarct volume found after depletion of neutrophils in TLR4KO mice led us to the hypothesis that neutrophils accounted for neuroprotective effect in this genotype (García-Culebras et al., 2019). LyzM is expressed in myelomonocytic cells, including monocytes, macrophages and granulocytes in mice. However, studies crossing LyzM-cre mice with GFP mice have shown that in the bone marrow the vast majority of cells expressing GFP were neutrophils and a very small percentage were monocytes (Faust et al., 2000). In order to confirm our hypothesis, and

to discard the possibility that the lack of TLR4 in other myeloid subsets (such as monocytes) had an effect in our model, we performed the same experiment in $TLR4^{loxP/LyzM\text{-}cre}$ mice. Specifically, $TLR4^{loxP/loxP}$ mice and $TLR4^{loxP/LyzM\text{-}cre}$ mice were injected with either mouse anti-neutrophil antibody or control isotype (Figure 31A). Specific depletion of neutrophils in $TLR4^{loxP/LyzM\text{-}cre}$ animals increased the infarct volume as opposed to the reduction found in control $TLR4^{loxP/loxP}$ mice vs the non-depleted animals. A significant interaction was found for infarct volume ($F[1,22]=40,11$; $P<0.0001$; n=6-7; Bonferroni post hoc: *$P<0.05$ vs MCAO $TLR4^{loxP/loxP}$ isotype; #$P<0.05$ vs MCAO $TLR4^{loxP/LyzM\text{-}cre}$ isotype) (Figure 31B). These data support that myeloid-specific TLR4 deficiency is involved in neuroprotection in these mice after MCAO.

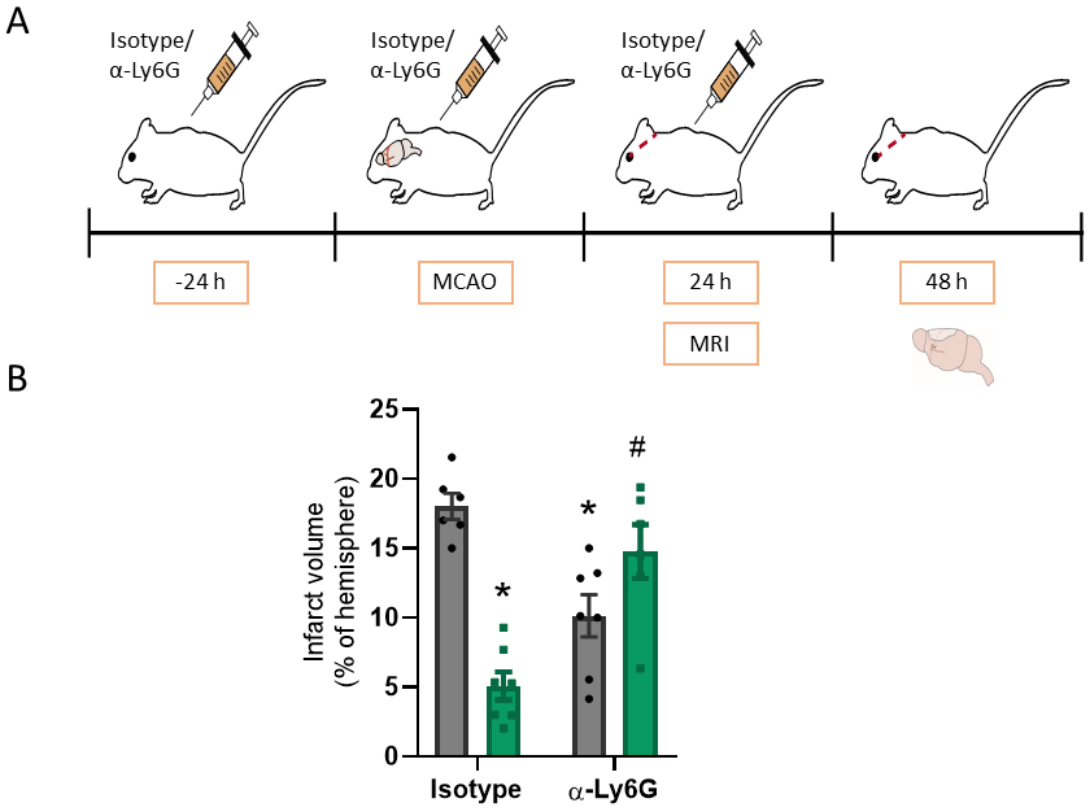


***Figure 31. Effect of neutrophil depletion on infarct outcome**. A. Experimental design. Mice were injected with either mouse antibody or control isotype daily for 4 days starting 24 h before MCAO; infarct volumes were determined by MRI 24 h after MCAO. B. Infarct volumes from the different genotypes with their respective treatments. A significant interaction between treatment and genotype was found for infarct volume ($F[1,22]=40,11$; $P<0.0001$; n=6–7; Bonferroni post hoc: *$P<0.05$ vs MCAO $TLR4^{loxP/loxP}$ isotype; #$P<0.05$ vs MCAO $TLR4^{loxP/LyzM\text{-}cre}$ isotype). Data are mean±SEM.*

4.2.3. The absence of myeloid TLR4 increases infiltrated N2 neutrophils concomitant to neuroprotection after middle cerebral artery occlusion (MCAO)

Since we had previously found a negative correlation between the density of neutrophils and the infarct volume in TLR4-KO mice, defying the traditional dogma that states that more infiltrated neutrophils mean more damage, we decided to examine the role of TLR4 in neutrophil infiltration. In order to do so we generated chimeric mice by transplanting bone marrow from $GFP^{loxP/LyzM\text{-}cre}$ mice (that express GFP in myeloid cells)

and TLR4$^{loxP/LyzM\text{-}cre}$ (that do not express TLR4 in the myeloid lineage) into TLR4$^{loxP/loxP}$ recipients. Two months after bone marrow reconstitution, chimeric mice were exposed to MCAO and brains were obtained 48 h after for immunostaining (Figure 32).

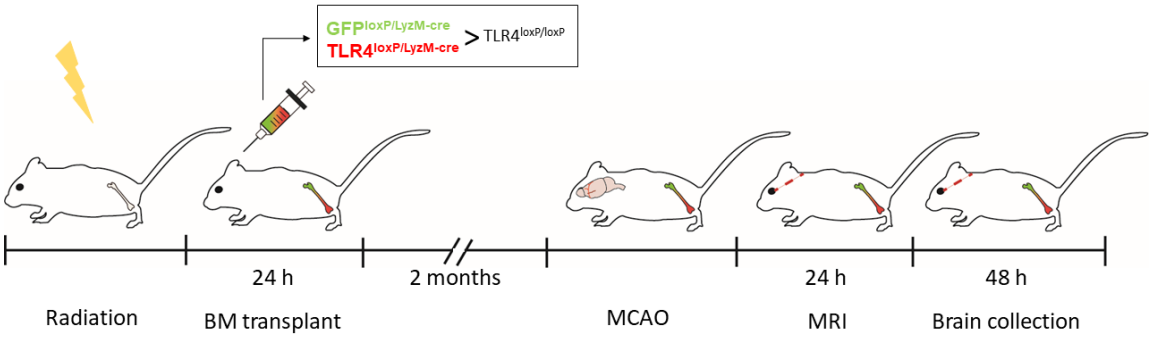


Figure 32. Differential myeloid cell infiltration in chimeric mice after stroke. *Experimental design: recipient mice from TLR4$^{loxP/loxP}$ (n=3) were lethally irradiated and 24 h after they were reconstituted with donor BM. Donor BM was harvested from GFP$^{loxP/LyzM\text{-}cre}$ and TLR4$^{loxP/loxP}$, and equal number of cells from each were mixed and intravenously injected into irradiated mice. Two months later, mice were subjected to MCAO and their brains collected for immunofluorescence studies 48 h later.*

Our data show that the percentage of TLR4-expressing myeloid cells (GFP^{+}/NIMP-R14^{+}) into brain parenchyma of TLR4$^{loxP/loxP}$ mice was significantly lower than that of TLR4-lacking myeloid cells (GFP^{-}/NIMP-R14^{+}) (Figure 33A). There were no differences between the percentage of both groups at the luminal level. These results strongly support that TLR4-deficient neutrophils have a higher ability to infiltrate into the ischemic brain tissue.

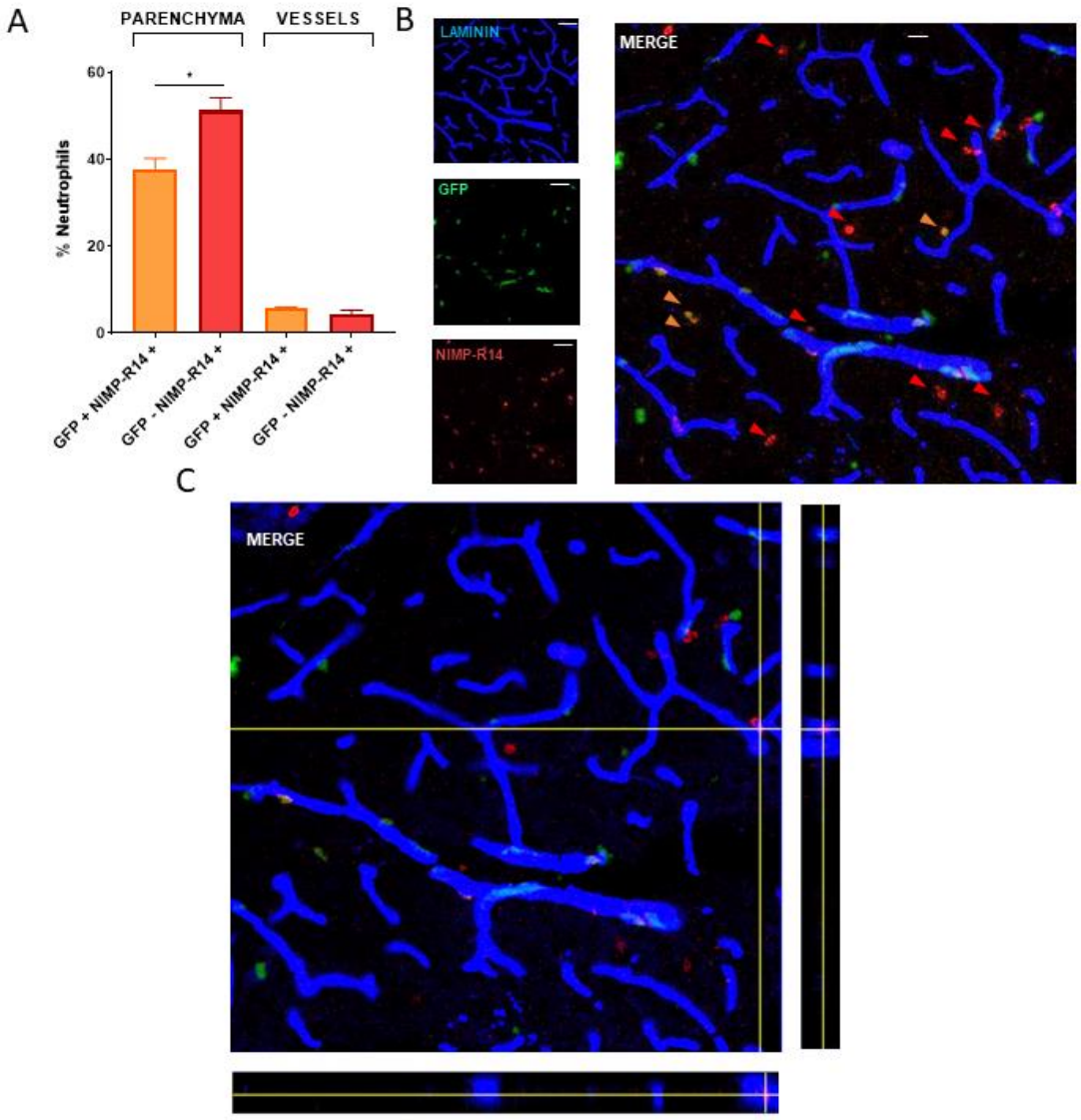


Figure 33. Differential myeloid cell infiltration in chimeric mice after stroke. *A. Percentage of myeloid cells expressing (GFP^+/$NIMP\text{-}R14^+$) or not expressing TLR4 (GFP^-/$NIMP\text{-}R14^+$) into the parenchyma and inside the vessels within the ischemic core (n=3; *P<0.05). B. Representative photomicrographs showing GFP (green), neutrophils (red), and laminin (blue) immunostaining. Arrowheads indicate colocalization of GFP^+/$NIMP\text{-}R14^+$ cells (orange) and GFP^-/$NIMP\text{-}R14^+$ cells (red) (scale bar=50 µm). Data are mean±SEM.*

4.2.4. The absence of myeloid TLR4 induces an N2 phenotype in infiltrated neutrophils after stroke

Previous results from our laboratory indicated that the absence of TLR4 on neutrophils, using TLR4-KO mice, drove neutrophil polarization towards an N2 phenotype which displayed beneficial properties after experimental stroke (Cuartero et al., 2013; García-Culebras et al., 2019). In order to confirm these results and to explain the reduction in infarct volume in TLR4$^{\Delta lyzM}$ mice despite an increased parenchymal infiltration, we studied by stereological analysis the number of N2 infiltrated neutrophils (Ym1^{+}/NIMP-R14^{+} neutrophils) normalized by the infarct volume, as well as the percentage of N1 (Ym1^{-}/NIMP-R14^{+}) and N2 (Ym1^{+}/NIMP-R14^{+}) infiltrated into the infarct core. This analysis was performed by sampling 7 coronal sections with the optical fractionator technique (StereoInvestigator software). First, we observed a marked increase in the density of N2 neutrophils (number of neutrophils normalized by infarct volume; *P<0.05, n=6; Figure 34A) vs. the control group (TLR4$^{loxP/loxP}$). When we quantified the percentage of N1/N2 neutrophils infiltrated in the parenchyma, we found a completely inverted ratio in TLR4$^{loxP/LyzM\text{-}cre}$ mice after stereological analysis 48 h after MCAO (*P<0.05, n=6) (Figure 34B), supporting that TLR4 absence skews neutrophils towards an N2 phenotype which is neuroprotective after MCAO.

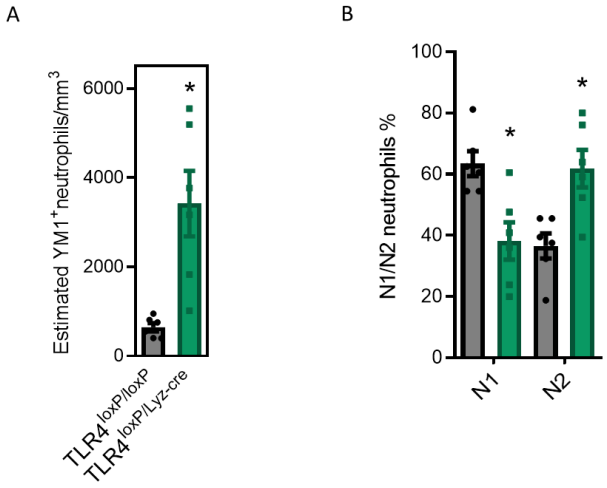


Figure 34. Effect of the absence of TLR4 on neutrophil polarization after MCAO. *A. Quantification of Ym1⁺ neutrophil infiltration, performed by sampling Ym1⁺/NIMP-R14⁺ cells and expressed normalized by infarct volume in mm³ (*P<0.05; n=6). B. Percentage of neutrophils N1 (Ym1⁻/NIMP-R14⁺) vs N2 (Ym1⁺/NIMP-R14⁺) in the ischemic core of both groups (*P<0.05 vs TLR4$^{loxP/loxP}$, n=6). Data are mean±SEM.*

4.3. CHARACTERIZATION OF TLR4$^{-/-}$ NEUTROPHIL DYNAMICS

4.3.1. Effect of myeloid TLR4 absence on "aging" surface markers in neutrophils from TLR4$^{\Delta lyzM}$ mice

According to our neutrophil transcriptome analysis, N2 neutrophils are similar to non-aged neutrophils as described by Frenette and cols (Zhang et al., 2015). With this hypothesis in mind we wanted to check whether our subset of neutrophils was similar to the one described as "young" neutrophils, consistent with the lack of activation by TLR4 (Zhang et al., 2015). According to this report, neutrophils freshly released from the bone marrow are CD62L^{hi} CXCR4^{lo} CD11b^{lo} and transition to a CD62L^{lo} CXCR4^{hi} CD11b^{hi} phenotype during daytime in mice. These alterations in the surface markers occur alongside functional changes. For example, aged neutrophils are more prone to initiate an inflammatory response or produce ROS (Uhl et al., 2016) which could influence the

progression and outcome of the ischemic stroke and, therefore, we thought they were worth it exploring. In order check if TLR4$^{-/-}$ neutrophils have the same aging pattern as those described by other groups, we analyzed three neutrophil aging markers (CD62L, CXCR4 and CD11b) in blood neutrophils at a basal state and 24 and 48 h post stroke. Our previous results, obtained by the transcriptome analysis, showed that neutrophils lacking TLR4 had a similar profile to the "young" or control group described by Frenette and cols. The analysis of the neutrophils aging markers by flow cytometry showed that, in the basal state (Figure 35A), neutrophils without TLR4 have a higher expression of CD62L and we can observe a trend to a decrease of CD11b and CXCR4 expression. Those results are in line with the ones obtained by the transcriptome analysis since that profile matches with the "non-aged" neutrophils. Interestingly, 24 h after the ischemia (Figure 35B), we cannot longer see the difference in CD62L expression. Moreover, the observed trend towards a decrease in CD11b and CXCR4 expression also disappeared. Again, 48 h after the stroke (Figure 35C), there were no differences in the expression of all three markers between genotypes. These results suggest that, in homeostasis, the absence of TLR4 keeps the neutrophils in a steady youth status. However, after a strong inflammatory insult, such as stroke, this regulation is lost, and the aging process is, at least in part, altered.

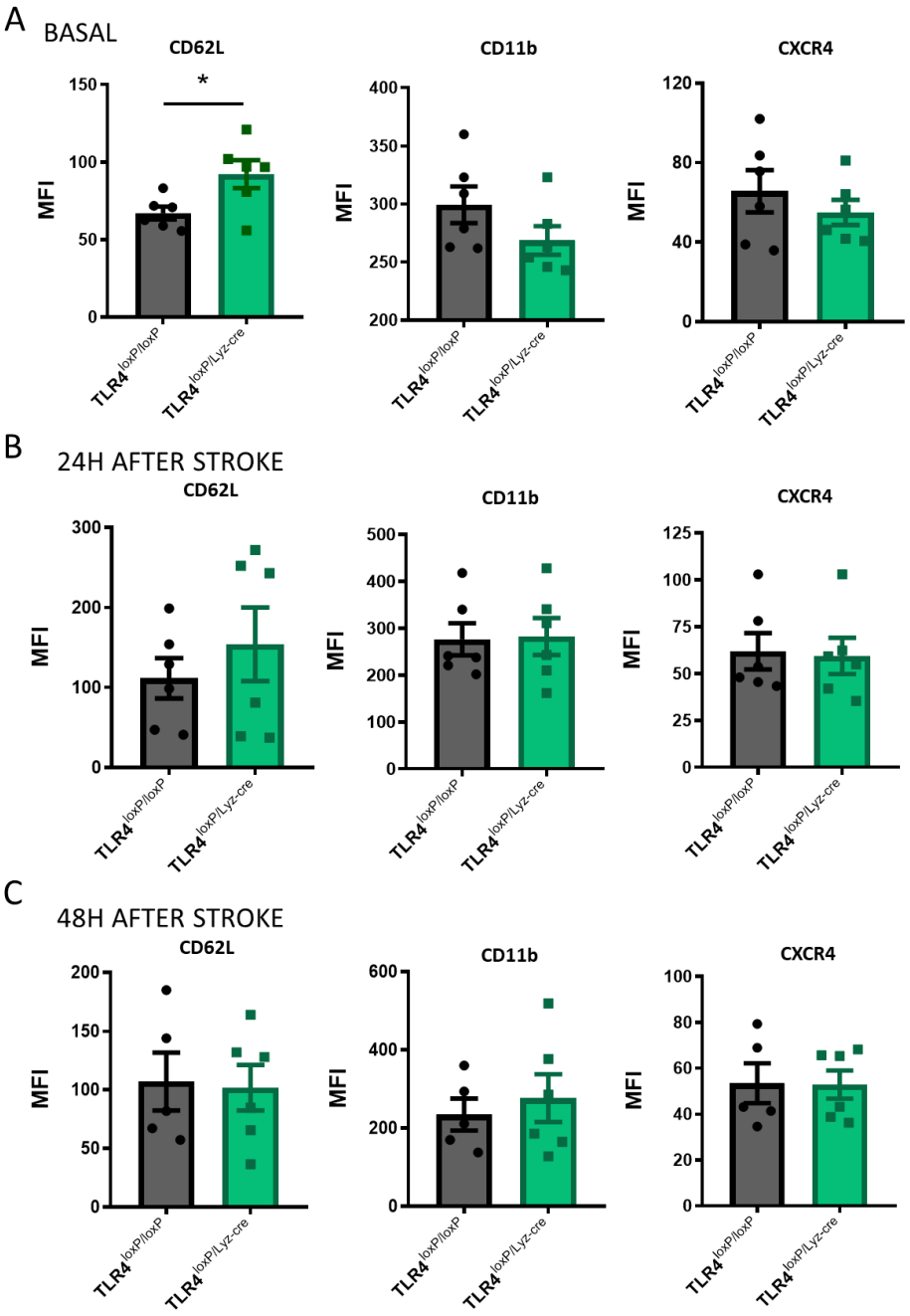


Figure 35. Neutrophil phenotype according to the neutrophil aging markers. *A. Flow cytometry analysis of the mean fluorescence intensity of CD62L, CD11b and CXCR4 in neutrophils in the basal state at ZT1 (n=6; *P<0.05). B. Flow cytometry analysis of the mean fluorescence intensity of CD62L, CD11b and CXCR4 in neutrophils 24 h after stroke. C. Flow cytometry analysis of the mean fluorescence intensity of CD62L, CD11b and CXCR4 in neutrophils 48 h after stroke. Data are mean±SEM.*

4.3.2. Study of the neutrophil circadian pattern in the absence of TLR4

As described in the introduction, neutrophils follow a circadian pattern that influence its function. Recently it has been described that fresh neutrophils display a higher infiltration efficiency towards inflamed tissue; also, the presence of aged neutrophils in the circulation correlates with a poor outcome after vascular ischemia and reperfusion damage (Adrover et al., 2019). Absolute neutrophil counts in blood follow circadian fluctuations, alternating between periods of active release between ZT17 and ZT5 and a period of clearance from the blood from ZT5 to ZT13 (Casanova-Acebes et al., 2013). Interestingly, markers of TLR4-dependent aging described by Frenette and cols coincide with those displayed in circadian aging (Adrover et al., 2019). Therefore, we checked whether circadian pattern of neutrophils is affected by TLR4 absence. The quantification of absolute number of neutrophils in blood by flow cytometry (Figure 36) showed that while $TLR4^{loxP/loxP}$ mice exhibit a circadian pattern (in accordance with the current literature), this pattern was altered in $TLR4^{loxP/LyzM\text{-}cre}$ mice. Two-way ANOVA analysis showed a significative difference in the variable time between genotypes ($P<0.05$, $F[2, 8]=5,369$). Bonferroni *post-hoc* analysis showed a lower number of neutrophils at ZT5 in blood of $TLR4^{loxP/LyzM\text{-}cre}$ mice than in that of $TLR4^{loxP/loxP}$ mice (*$P<0.05$ vs $TLR4^{loxP/loxP}$). This result suggests that the absence of TLR4 prevents neutrophils from their normal circadian fluctuation.

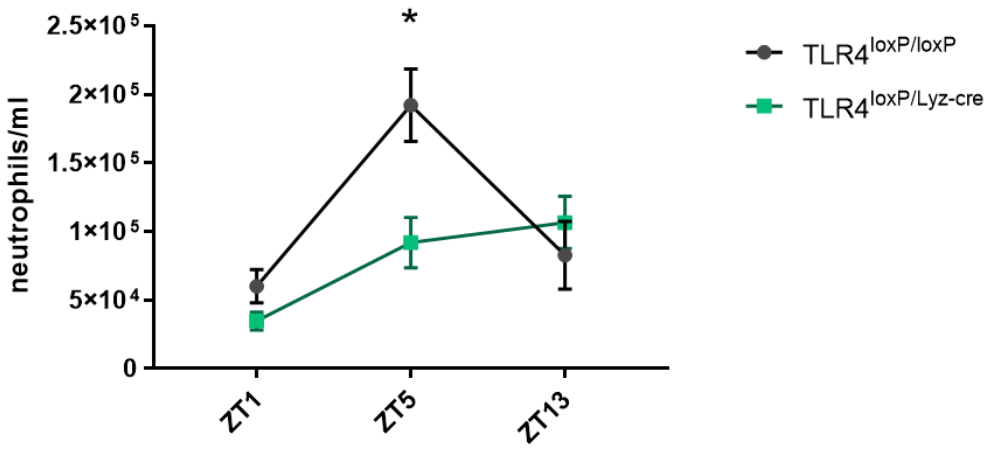


Figure 36. Kinetics of neutrophils in blood. *Total number of neutrophils in blood determined by flow cytometry at ZT1, ZT5 and ZT13 in TLR4$^{loxP/loxP}$ mice and TLR4$^{loxP/LyzM\text{-}cre}$ mice. Two-way ANOVA analysis showed a significative difference in the variable time between genotypes (P<0.05, F[2, 8]=5,369, n=6). Bonferroni post-hoc analysis showed that at ZT5 there were less neutrophils in blood in TLR4$^{loxP/LyzM\text{-}cre}$ mice than in TLR4$^{loxP/loxP}$ mice (*P<0.05 vs TLR4$^{loxP/loxP}$). Data are mean±SEM.*

Regarding the circadian expression of aging markers, whereas virtually all neutrophils have been reported to be CD62L^{hi} at ZT13, at ZT5 there is a mixture of CD62L^{hi} and CD62L^{lo} neutrophils. Moreover, the number of CD62L^{lo} neutrophils fluctuate over time whereas CD62L^{hi} remain stable (Casanova-Acebes et al., 2013). In the light of these data, since our data show a difference in CD62L neutrophil expression in basal conditions, we asked whether the loss of the circadian pattern that we had observed in neutrophils lacking TLR4 also affected the proportion of CD62L$^{hi/lo}$ neutrophils. Indeed, CD62^{lo} expression was found to match with the typical circadian pattern in both genotypes (Figure 37A). However, while TLR4$^{loxP/loxP}$ CD62L^{hi} neutrophils did not fluctuate as expected, neutrophils lacking TLR4 showed a different pattern. Two-way ANOVA analysis showed a trend towards a difference (P=0.07, F[1, 10]=4,016) between the two genotypes and the Bonferroni *post-hoc* test showed a significative higher expression of

CD62L at ZT1 (*P<0.05 vs $TLR4^{loxP/loxP}$; Figure 37B). This result suggests that, at this time point, there are more "young" neutrophils in blood in $TLR4^{loxP/LyzM\text{-}cre}$ mice than in $TLR4^{loxP/loxP}$ mice, consistent with a phenotype associated to neuroprotection, observed in our previous publications and in agreement with the transcriptional profiling results described in the beginning of the Results section when neutrophils lacking TLR4 where compared with the non-aged neutrophils described by Frenette and cols.

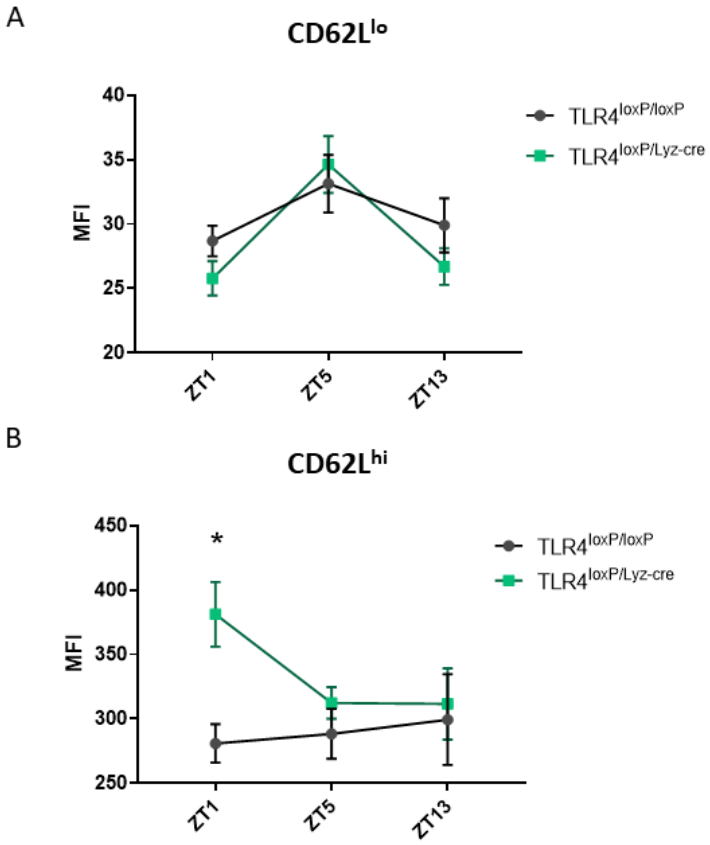


Figure 37. CD62L expression in neutrophils through time. *A. CD62L MFI in $CD62L^{lo}$ neutrophils. B. CD62L MFI in $CD62L^{hi}$ neutrophils (P<0.05, n=6). Two-way ANOVA analysis showed a trend to the difference in the genotype variable (P=0.07, F[1, 10]=4.016, n=6). Bonferroni post-hoc analysis showed a significative higher expression of CD62L at ZT1 (*P<0.05 vs $TLR4^{loxP/loxP}$, n=6). Data are mean±SEM.*

4.3.3. Effect of myeloid TLR4 absence in neutrophil complexity

Granularity and size are two intrinsic cell features that can be quantified by flow cytometry. Specifically, forward vs side scatter (FSC vs SSC) gating is commonly used to identify cells based on size and granularity respectively. Moreover, it has been recently described, by studying their light-scattering (SSC-A) values, that the granularity of human and murine neutrophils change over time (Adrover et al., 2020). In that study the authors describe that in mice, neutrophil granularity decreases over time (ZT1 vs ZT13) suggesting a progressive loss of granules as neutrophils circulated which could be related to their capacity to exert certain functions. We therefore checked if neutrophils lacking TLR4 were different in size and granularity from those that express TLR4, and whether this was related to their functional relevance. Two-way ANOVA analysis of SSC showed indeed a difference both in time ($P<0.05$, $F[1.605, 16.05]=34.49$) and genotype ($P<0.05$, $F[1, 10] = 8.418$) (Figure 38A). To analyze the size, we determined the FSC parameter and we found no differences between genotypes or time points (Figure 38B).

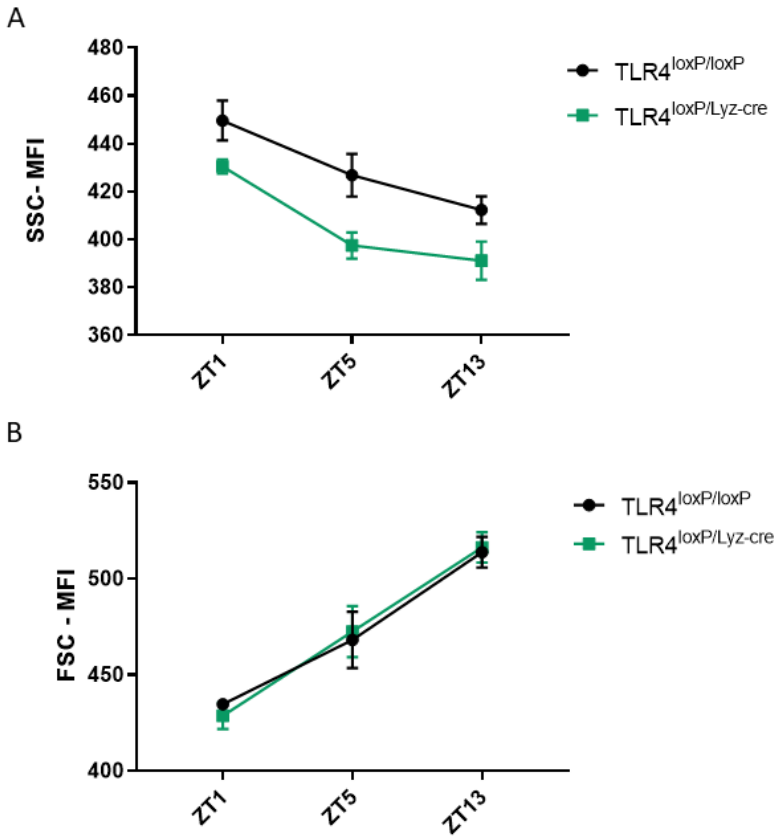


Figure 38. Quantification of neutrophil complexity by SSC and FSC analysis. *A. SCC-MFI values (granularity) at ZT1, ZT5 and ZT13. Two-way ANOVA analysis of the data showed a difference in both genotype (P<0.05, F[1.605, 16.05]=34.49) and time (P<0.05, F[1.605, 16.05]=34.49) (n=6). B. FSC-MFI values (size) at ZT1, ZT5 and ZT13. Data are mean±SEM.*

4.4. EFFECT OF SPECIFIC MYELOID TLR4 ABLATION IN NEUTROPHIL FUNCTIONS IN HOMEOSTASIS AND AFTER STROKE

Considering the data on the impact of myeloid TLR4 ablation on stroke outcome, and the depletion results showing that neutrophils account, mostly, for the neuroprotective phenotype associated to the TLR4$^{\Delta lyzM}$ genotype, we decided to study how TLR4 deletion affected neutrophil functions.

4.4.1. Study of neutrophil phagocytic activity before and after stroke in $TLR4^{loxP/loxP}$ and $TLR4^{loxP/LyzM\text{-}cre}$ mice

Neutrophils are professional phagocytes. They are endowed with the capacity to engulf and thereby eliminate pathogens or cell debris. We therefore asked if this ability was altered by the absence of TLR4. In order to do so, WBC from blood of $TLR4^{loxP/loxP}$ and $TLR4^{loxP/LyzM\text{-}cre}$ mice were extracted and exposed to carboxylate-modified polystyrene latex beads (Figure 39A). These beads have fluorescent properties and are small enough to be engulfed by neutrophils. Since they are fluorescent, by gating the sample for neutrophils and FITC signal using flow cytometry allowed us to discriminate between neutrophils that had engulfed beads ($Ly6G^+/FITC^+$) and those that had not ($Ly6G^+/FITC^-$) (Figure 39B).

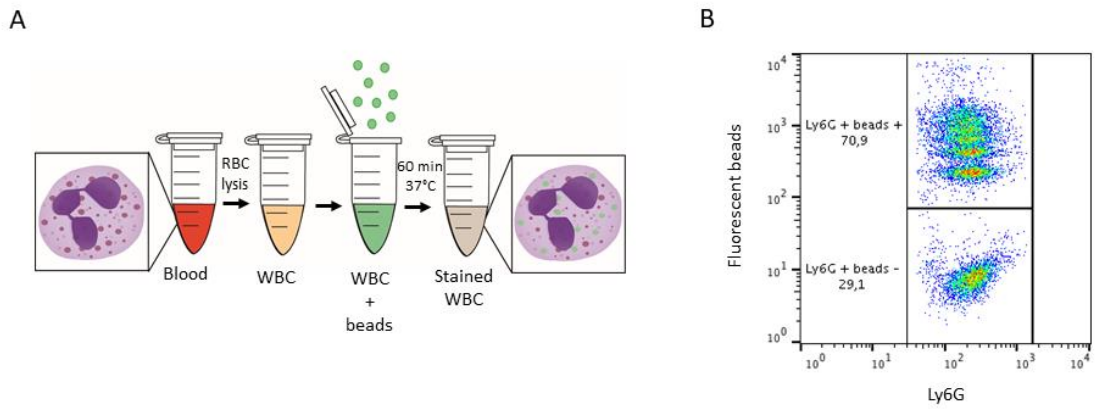


Figure 39. Neutrophil phagocytic activity. *A. Experimental design of phagocytic activity of myeloid cells assay. Blood is collected, RBC are lysed, and WBC incubated for 60 min at 37°C with the fluorescent beads. After incubation cells are washed and stained for Ly6G marker. B. Flow cytometry plot showing gated neutrophils ($Ly6G^+$ cells), positive and negative for FITC signal ($beads^+/beads^-$). Neutrophils in the upper quadrant are the ones that have engulfed the beads.*

Firstly, we studied the phagocytic activity of neutrophils in the **basal state**. Our results show that neutrophils lacking TLR4 showed an increased phagocytic activity, since the percentage of neutrophils with engulfed beads was higher in $TLR4^{loxP/LyzM\text{-}cre}$ mice than

in TLR4$^{loxP/loxP}$ mice (*P<0.05). However, 24 h and 48 h after ischemia there were no differences between genotypes (Figure 40A). Two-way ANOVA analysis revealed a significant effect of the time (P<0.05, F[2, 19]=12.92), and Bonferroni *post-hoc* test showed that the phagocytic activity of neutrophils from TLR4$^{loxP/LyzM\text{-}cre}$ mice decreases significantly over time (*P<0.05). Therefore, these results suggest that, at least in homeostasis, the lack of TLR4 renders neutrophils with a higher phagocytic activity which might result in pro-resolutive properties.

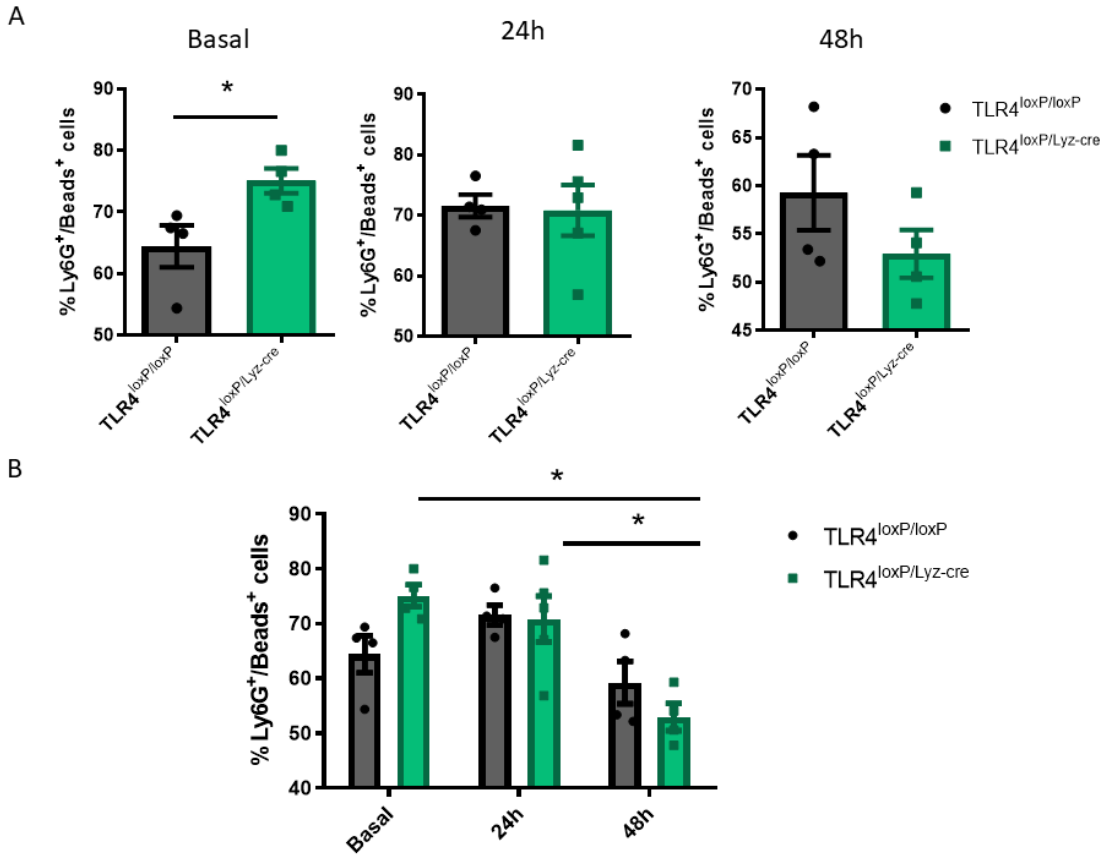


Figure 40. Analysis of neutrophil phagocytic activity. *A. Percentage of neutrophils that had engulfed beads in TLR4$^{loxP/loxP}$ mice and TLR4$^{loxP/LyzM\text{-}cre}$ mice at basal state, and 24 h 48 h after stroke (*P<0.05, n=4-5). B. Graphical representation of the data with all timepoints in the same graph. Two-way ANOVA analysis showed a significant effect of the time variable and the Bonferroni post-hoc showed a decrease in the phagocytic activity of neutrophils from TLR4$^{loxP/LyzM\text{-}cre}$ mice (*P<0.05, F[2, 19]=12.92, n=4-5).*

4.4.2. Study of neutrophil apoptosis in TLR4$^{loxP/loxP}$ and TLR4$^{loxP/LyzM\text{-}cre}$ mice

Neutrophils are cells preloaded with a very dangerous arsenal of destructive molecules. This fact makes apoptosis, which by definition is a controlled and programmed cell death, a key component in the inflammatory process. In this context, a possible explanation of the neuroprotective effect of neutrophils lacking TLR4 is that these neutrophils are able to die in a swift and controlled way. To explore this idea, we performed two different assays to determine the number of apoptotic cells both in blood and in the ischemic tissue. Firstly, we determined the total number of apoptotic cells by flow cytometry. This assay detects caspase 3 activity using a non-fluorescent substrate consisting of a fluorogenic DNA dye coupled to the caspase 3 recognition sequence which penetrates through the plasma membrane. In apoptotic cells, caspase 3 cleaves the substrate releasing the high-affinity DNA dye which migrates to the cell nucleus and stains DNA. This assay was performed in WBC from blood obtained in the basal state, and 24 h and 48 h after stroke (Figure 41A). Our results show no differences in apoptosis in neutrophils from TLR4$^{loxP/loxP}$ mice and TLR4$^{loxP/LyzM\text{-}cre}$ mice (Figure 41B). Two-way ANOVA analysis did reveal an increase in apoptotic neutrophils along time ($P<0.05$, $F[2, 10]$ 6.536) in both genotypes (*$P<0.05$ vs TLR4$^{loxP/loxP}$ basal; #$P<0.05$ vs TLR4$^{loxP/Lyz\text{-}cre}$ basal; \$$P<0.05$ vs TLR4$^{loxP/Lyz\text{-}cre}$ 24 h; $P=0.05$ TLR4$^{loxP/loxP}$ 48 h vs TLR4$^{loxP/loxP}$ 24 h) (Figure 38C).

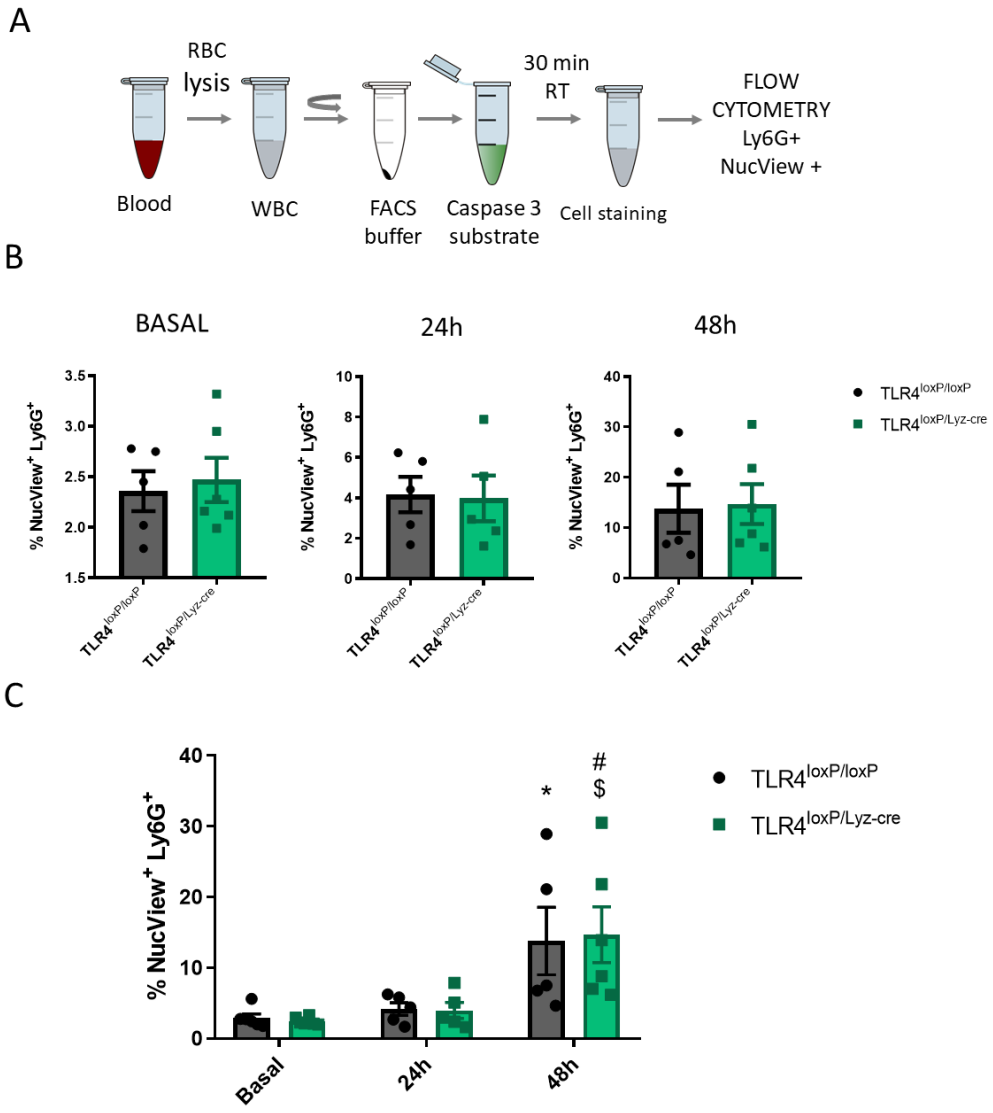


Figure 41. Apoptosis assay in blood neutrophils. *A. Experimental design: blood was collected, RBC lysed, WBC resuspended and finally caspase 3 substrate was added and incubated with the WBC for 30 min at RT. After incubation, cells were stained for Ly6G marker and samples were analyzed with a flow cytometer. B. Percentage of apoptotic neutrophils in blood (NucView^{+}/Ly6G^{+}). There was no difference between genotypes at the different time points. C. Two-way ANOVA analysis of the data showing a significative effect of time in both genotypes (Time: P<0.05, F[2, 10] 6.536; (*P<0.05 vs TLR4$^{loxP/loxP}$ basal; #P<0.05 vs TLR4$^{loxP/Lyz-cre}$ basal; $P<0.05 vs TLR4$^{loxP/Lyz-cre}$ 24 h; P=0.05 TLR4$^{loxP/loxP}$ 48 h vs TLR4$^{loxP/loxP}$ 24 h; n=5). Data are mean±SEM.*

Secondly, although we did not observe differences in apoptosis in blood neutrophils, we wanted to know if the same situation was occurring in infiltrated neutrophils into the brain tissue. To explore this possibility, we performed a TUNEL assay

in fixed brain slices obtained 48 h after stroke. This protocol identifies apoptotic cells by using the enzyme terminal deoxynucleotidyl transferase (TdT) to catalyze the incorporation of a fluorescent component (fluorescein-12-dUTP) at the free 3'-hydroxyl ends of the fragmented DNA, a staining that can then be combined with a specific marker for neutrophils (NIMP-R14). The total number of neutrophils and double positive cells were quantified and the percentage of apoptotic neutrophils (NIMP-R14^{+}/TUNEL^{+}) was calculated (Figure 42A). With this approach, we again failed to observe any differences in the percentage of apoptotic neutrophils between genotypes (Figure 42B). In summary, our results show that the neutrophilic phenotype does not seem to influence the apoptotic process. However, further experiments are required to establish the exact role of different neutrophil phenotypes on the apoptotic process, for example, studying other time points after ischemia.

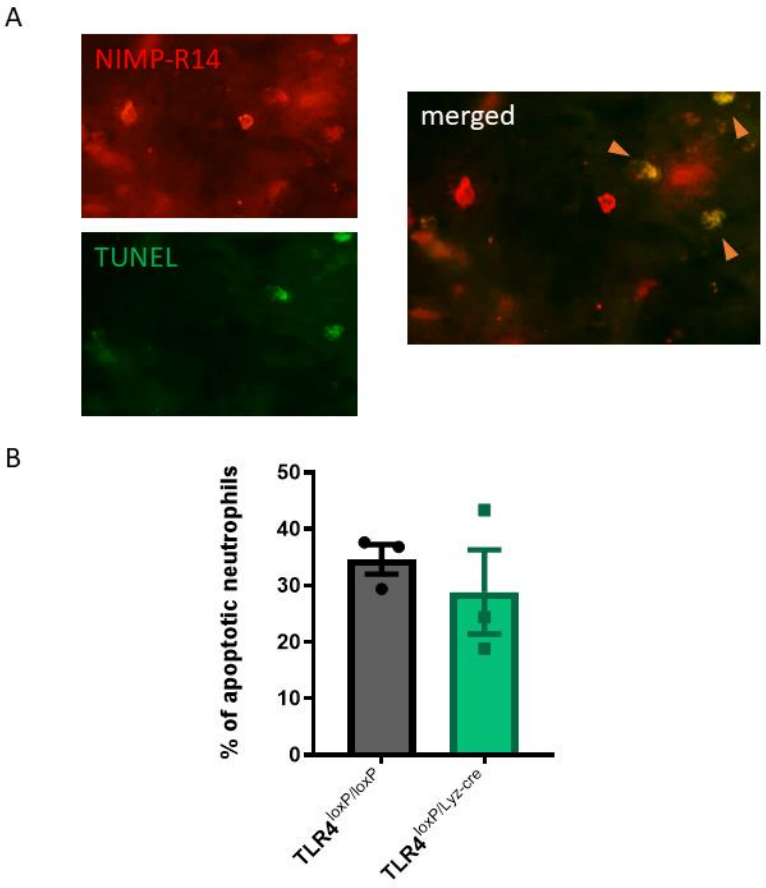


Figure 42. Apoptosis assay in infiltrated neutrophils. *A. TUNEL assay was performed in fixed brain slices and after they were stained with a neutrophil marker (NIMP-R14). Total number of neutrophils (NIMP-R14⁺ cells (top left)) was quantified. Double positive cells (NIMP-R14⁺/TUNEL⁺ cells (right)) were quantified as apoptotic neutrophils (orange arrowheads). B. Percentage of apoptotic neutrophils infiltrated in the ischemic tissue from TLR4$^{loxP/loxP}$ mice and TLR4$^{loxP/LyzM\text{-}cre}$ mice (n=3). Data are mean±SEM.*

4.4.3. Study of preferential phagocytosis of neutrophils by microglia in the ischemic core of TLR4$^{loxP/loxP}$ and TLR4$^{loxP/LyzM\text{-}cre}$ mice

Neutrophils must be promptly removed from the injury site to decrease damage associated to their inflammatory activity. Previous data from our laboratory showed that N2 neutrophils (NIMP-R12⁺/Ym1⁺) are preferentially phagocytosed by microglia in the ischemic core (Cuartero et al., 2013). To determine the effect of neutrophil TLR4 in this process, we measured neutrophil clearance by microglia engulfment with simultaneous

visualization of Iba1, NIMP-R14, and Ym1 in infarct cores of $TLR4^{loxP/loxP}$ and $TLR4^{loxP/LyzM\text{-}cre}$ mice. Confocal imaging showed, in agreement with the previous results of our group, that cytoplasm of microglia contained NIMP-R14 $^{+}$ cells (Figure 43A).

For quantification, as performed in the previous publications of the laboratory (Cuartero et al., 2013), we measured phagocytosis as percentage of microglia ($Iba1^{+}$ cells) engulfing neutrophils, and neutrophil phagocytic clearance as percent of total $Ym1^{+}$ or $Ym1^{-}$ neutrophils being engulfed by microglia. With this approach, we found no differences in the percentage of microglia containing neutrophils, which indicates that microglia are equally capable of phagocyting in both genotypes (Figure 43B). In contrast, in both genotypes, microglia preferentially engulfed $Ym1^{+}$ neutrophils at the ischemic core when compared with the $Ym1^{-}$ neutrophil subpopulation (*$P<0.05$). These results confirmed two hypotheses: firstly, that with this genetic model the microglia are functional and, secondly, that N2 neutrophils are engulfed preferentially to N1 by the microglia.

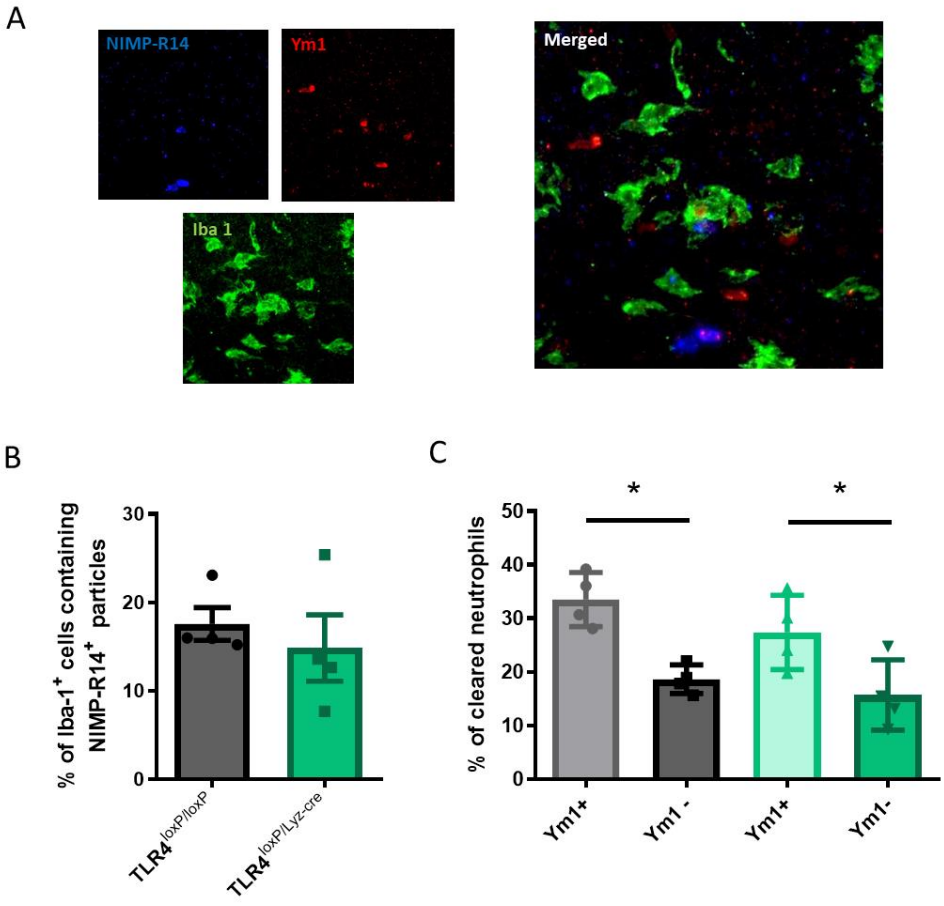


Figure 43. Preferential phagocytosis of N2 neutrophils by microglia. *A. Representative phagocytosis micrograph showing neutrophil staining (NIMP-R14$^+$ cells, blue), N2 marker (Ym1$^+$ cells, red) and microglia (Iba1$^+$ cells, green) and all merged channels showing an N2 neutrophil engulfed by microglia (top right). B. Percentage of phagocytic microglia in TLR4$^{loxP/loxP}$ mice and in TLR4$^{loxP/LyzM\text{-}cre}$ mice (n=4). C. Quantification of clearance of specific neutrophil population (Ym1$^+$/Ym1$^-$) (*P<0.05, n=4). Data are mean±SEM.*

4.4.4. Study of neutrophil ROS production in TLR4$^{loxP/loxP}$ and TLR4$^{loxP/LyzM\text{-}cre}$ mice

As explained in the Introduction section, the production of reactive oxygen species is a key functional response of granulocytes. It is crucial when contributing to host defense, but it can also result in collateral damage of tissues in a sterile inflammation situation. Briefly, the NADPH oxidase complex, which is dormant in quiescent cells, rapidly assembles when certain cell receptors are activated. In this situation, the enzyme

catalyzes the NADPH-dependent reduction of O_2 to form superoxide anions (O_2^-.) and ROS derived from this radical, including hydrogen peroxide (H_2O_2), hydroxyl radical (OH.) and hypochlorous acid (HclO). To quantify this process, DHR 123 (nonfluorescent) was added and, if needed, a stimulating peptide (WKYMVM, W-peptide for now on) to the cell suspension. Samples were incubated for 20 min at 37°C. Upon stimulation, DHR 123 is oxidized by H_2O_2 formed from superoxide, resulting in the formation of rhodamine (fluorescent dye) localized in the mitochondria. This oxidation process can be measured and quantified by flow cytometry by gating $Gr1^{hi}$ and Rho^+ cells (Figure 44A). We thus studied the neutrophil ROS production at a basal state, and 24 h and 48 h after the stroke (Figure 44B). We observed that at the basal state there was a significant decrease in ROS production between $TLR4^{loxP/loxP}$ and $TLR4^{loxP/LyzM\text{-}cre}$ neutrophils (*P<0.05). Also, there was a significant difference in W-peptide-induced neutrophil activation between genotypes (*P<0.05). At 24h we did not longer see a difference in ROS production between activated and non-activated neutrophils from $TLR4^{loxP/LyzM\text{-}cre}$ mice. Likewise, the difference in activated neutrophils between genotypes was no longer observable. At 48 h there were no differences either between genotypes or between activated and non-activated neutrophils, an effect that might be due to the DAMPS present in the plasma, that act as a stimulus activating neutrophils prior to the assay. Two-way ANOVA analysis of activated and non-activated neutrophils of both genotypes showed a significant effect of time (P<0.05). Also, in the activated groups, we observed differences between genotypes in the basal state (*P<0.05). Our data suggest that, after stroke, there is a rapid increase in the production of ROS, resulting in further tissue damage. Therefore, neutrophils lacking TLR4 could mitigate that damage and therefore contribute to the

neuroprotection since their ability to produce ROS is decreased in the first stage of the inflammatory process.

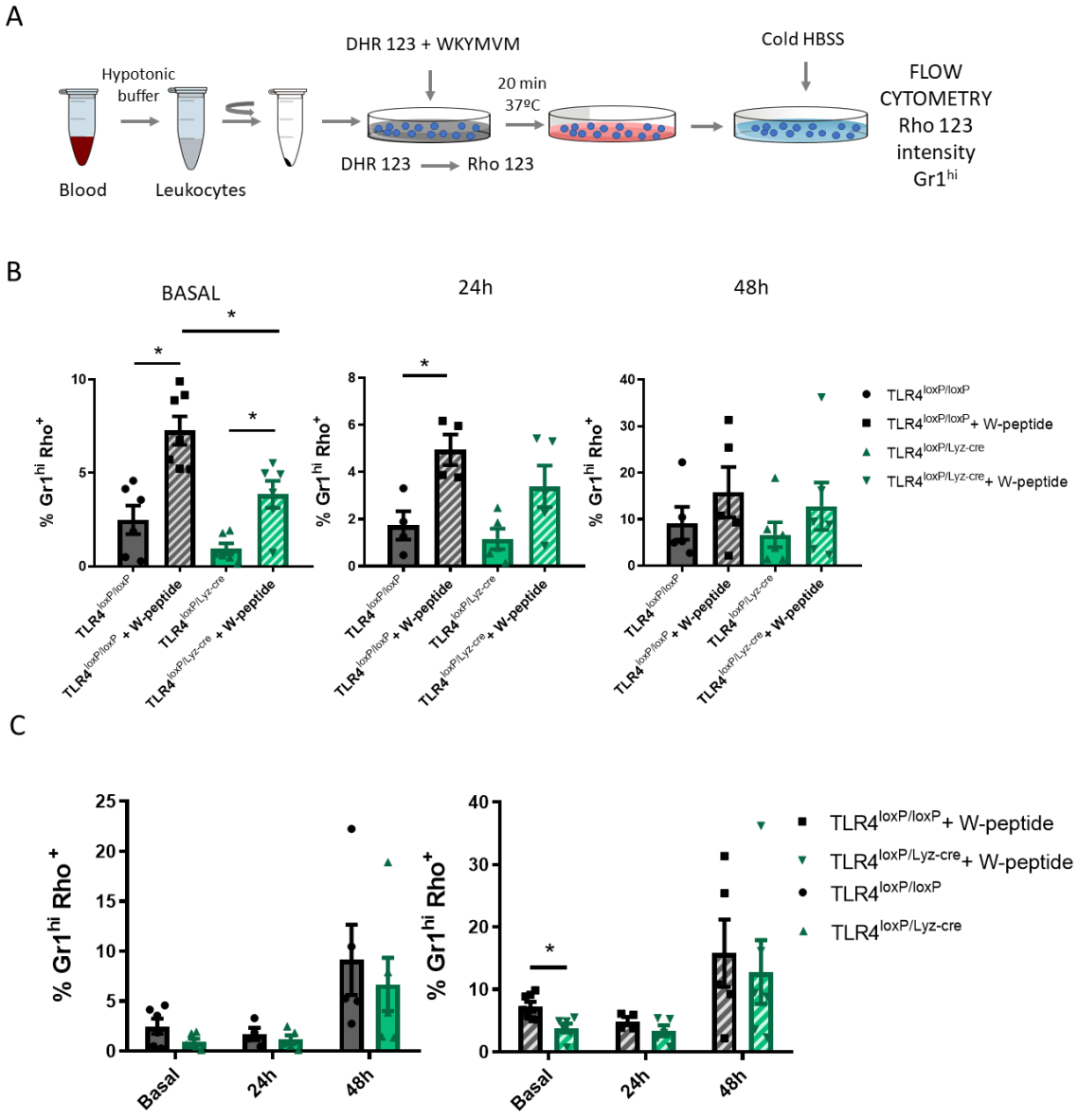


Figure 44. ROS production by neutrophils in the absence of TLR4. *A. Experimental design: blood was collected, RBC lysed, WBC resuspended and DHR123 and W-peptide (if necessary) were added. Samples were incubated for 20 min at 37°C. After incubation time, cold HBSS was added to stop the reaction. Cells were stained for Gr1 marker and washed. Samples were analyzed with the flow cytometer. B. Percentage of neutrophils producing ROS (% $Gr1^{hi}Rho^{+}$ cells) at basal state, 24 h and 48 h after the stroke with and without W-peptide activation (*P<0.05, n=4-6). C. Two-way ANOVA analysis of the data showing a significative effect of time in both genotypes (P<0.05) and a significative difference between activated neutrophils of both genotypes at basal state (*P<0.05, n=4-6).*

4.4.5. Study of NETosis in TLR4$^{loxP/loxP}$ and TLR4$^{loxP/LyzM\text{-}cre}$ mice

Upon activation, neutrophils can release extracellular trap and form NETs. As previously described, NETs are composed of chromatin decorated with granular proteins. We wondered whether the absence of TLR4 had any effect in the ability of neutrophils to undergo the NETosis process. To analyze NET formation, we tried both an *in vivo* and an *in vitro* assay. Firstly, we analyzed the ability of TLR4-lacking neutrophils to undergo NETosis *in vitro*. Briefly, we sorted the neutrophils from the blood and then those neutrophils were seeded and activated with PMA (20 nM). (Figure 45A). NETosis was quantified by counting the number of cells in which those three markers colocalized (NIMP-R14^{+}/C3-Histone^{+}/Elastase^{+}) (Figure 45B, white arrowheads and 63x micrographs) divided by the total neutrophil number (NIMP-R14^{+} cells) in both TLR4$^{loxP/loxP}$ and TLR4$^{loxP/LyzM\text{-}cre}$ mice. Interestingly, we found that neutrophils obtained in basal state from TLR4$^{loxP/LyzM\text{-}cre}$ were more prone to form NETs than those from TLR4$^{loxP/loxP}$ mice (Figure 45C, *P<0.05).

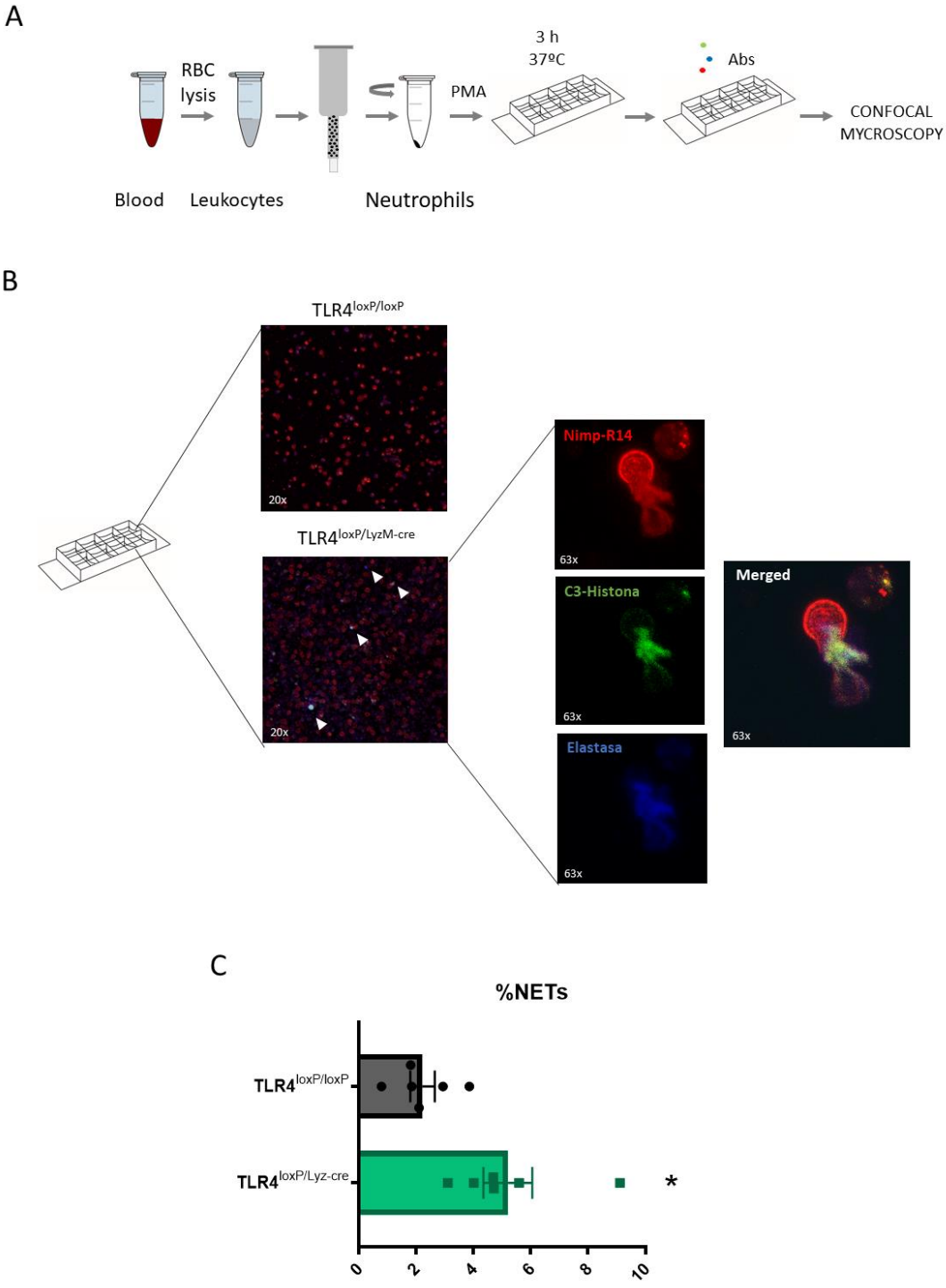


*Figure 45. In vitro NETosis in the absence of TLR4. A. Experimental design: blood was collected, RBC lysed, and neutrophils sorted and stimulated with PMA. Samples were incubated for 3 h at 37°C. After incubation time, cells were washed, fixed and stained for NIMP-R14, C3-Histone and Elastase. B. Micrographs of neutrophils treated with PMA from TLR4$^{loxP/loxP}$ and TLR4$^{loxP/LyzM\text{-}cre}$ mice. NETs are marked with white arrowheads. 63x zoomed images show a neutrophil extruding its content releasing the NET. C. Percentage of NETs (NIMP-R14^{+}/C3-Histone^{+}/Elastase^{+}) from TLR4$^{loxP/loxP}$ and TLR4$^{loxP/LyzM\text{-}cre}$ mice (*P<0.05, n=6).*

In order to see if this phenomenon was also occurring *in vivo* in the ischemic tissue, we stained fixed brain slices for the same markers as in the *in vitro* experiment (NIMP-R14, C3-Histone, Elastase) (Figure 46A, white arrowheads). In this case, we did not observe any differences in the percentage of NETs in the ischemic core between both genotypes (Figure 46B). While analyzing the images we observed that it was common to see elastase and C3-histone signal colocalizing without any trace of NIMP-R14. This is likely due to the dispersion of the neutrophil membrane when it is disrupted after the NET release. We also quantified those NET-like events to see if we could detect any differences, but we did not (Figure 46C). These results suggest that neutrophils lacking TLR4 are more prone to undergo NETosis in vitro after PMA stimulation although we could not observe this effect in vivo after stroke. Whereas it is possible that this process is unlikely to be involved in the neuroprotective effect that neutrophils lacking TLR4 display in this specific pathology, we cannot discard that NET content in blood does not reflect the *in vivo* NETosis process. Further studies are required to elucidate this issue.

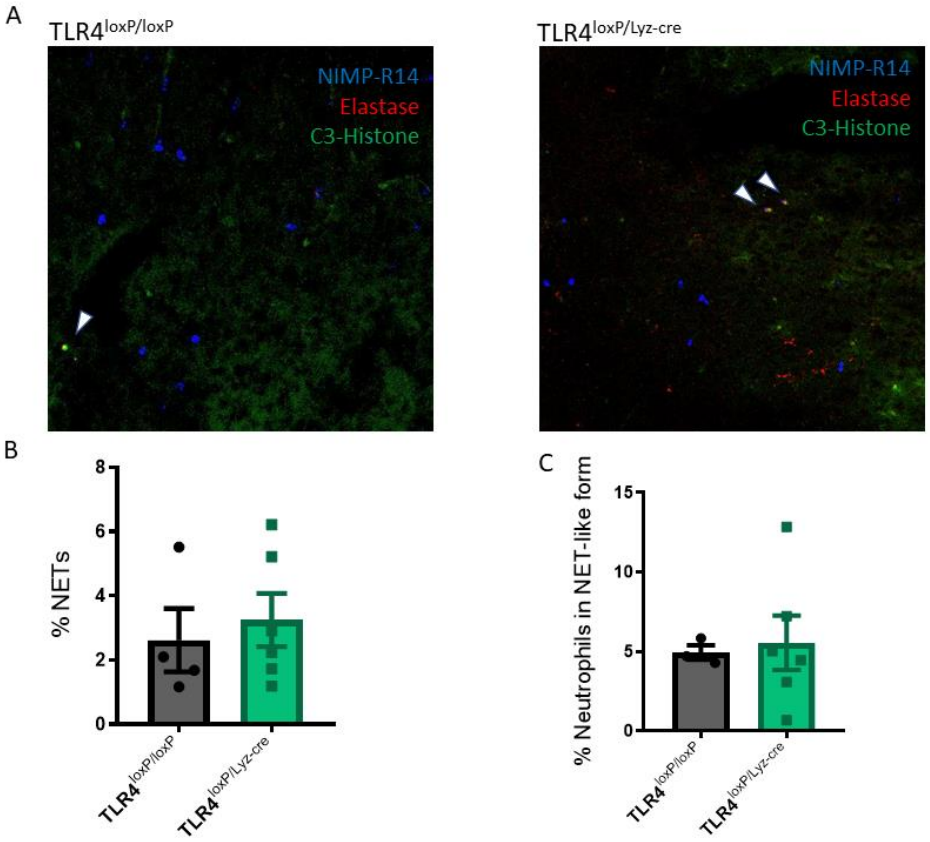


Figure 46. in vivo NETosis in the absence of TLR4 *A. Confocal images of fixed brain slices stained for NIMP-R14, C3-Histone and Elastase. NETs are marked with white arrowheads. B. Micrographs of neutrophils treated with PMA from TLR4$^{loxP/loxP}$ and TLR4$^{loxP/LyzM\text{-}cre}$ mice. NETs are marked with white arrowheads. B. Percentage of NETs (NIMP-R14^{+}/C3-Histone^{+}/Elastase^{+}) from TLR4$^{loxP/loxP}$ and TLR4$^{loxP/LyzM\text{-}cre}$ mice (n=4-6). C. Percentage of NET-like events (C3-Histone^{+}/Elastase^{+}) (n=4-6).*

4.4.6. Study of neutrophil migration in TLR4$^{loxP/loxP}$ and TLR4$^{loxP/LyzM\text{-}cre}$ mice

Our previous results showed an increased neutrophil density (neutrophils per mm^3) in the ischemic brain of TLR4KO mice. Following that thought we wondered whether TLR4-lacking neutrophils were more efficient infiltrating and migrating to tissues. Again, we addressed this question using both *in vitro* and *in vivo* determinations, in TLR4$^{loxP/loxP}$ and TLR4$^{loxP/LyzM\text{-}cre}$ mice. For the study of neutrophil migration *in vitro*, WBC were seeded in the top compartment of transwell inserts. Neutrophil migration was stimulated by the addition of CXCL1 (Figure 47A). Two-way ANOVA analysis of the percentage of

neutrophils that had migrated to the lower compartment showed a significant effect of time ($P<0.05$, $F(2, 25) = 10.75$) as well as a significant interaction between time and genotype ($P<0.05$, $F(2, 25) = 3.963$). Bonferroni *post-hoc* test showed a significant difference between the percentage of migrated neutrophils from $TLR4^{loxP/loxP}$ mice at 24 h and basal condition, and between 24 h and 48 h (Figure 47B, *$P<0.05$). This difference was not found in neutrophils from $TLR4^{loxP/LyzM\text{-}cre}$ mice. These results seem to indicate that neutrophils that do not express TLR4 have a lower ability to migrate when stimulated *ex vivo* with CXCL1.

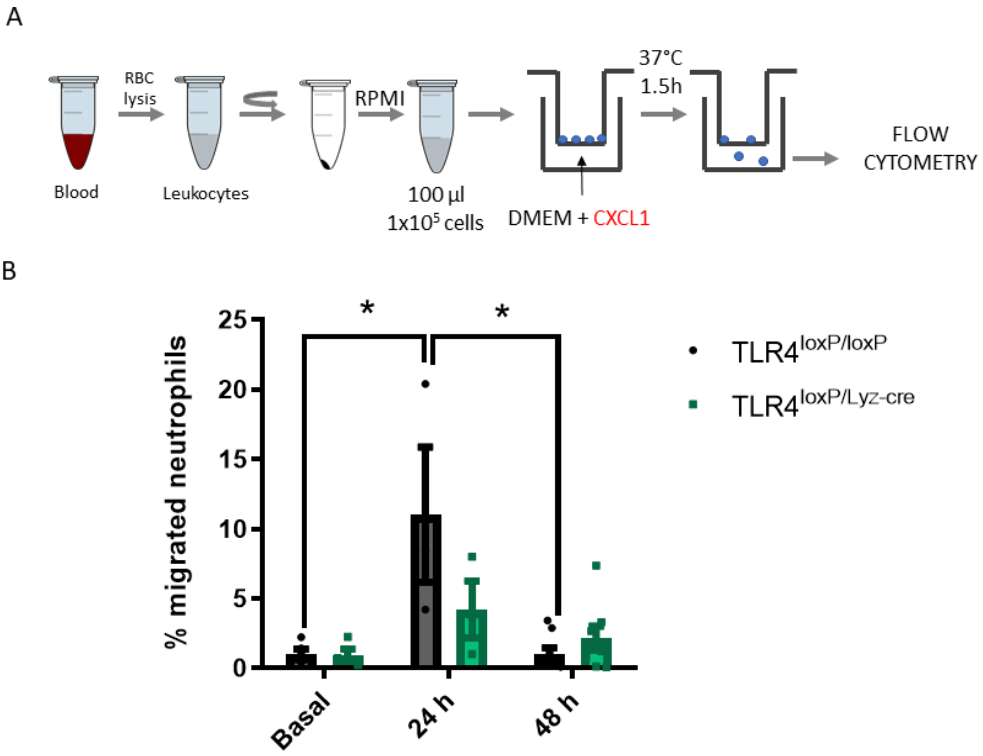


Figure 47. In vitro neutrophil migration assay. *A. Experimental design: blood was collected, RBC lysed, and WBC resuspended in RPMI at a concentration of $1x10^5$ cells per 100 µl. Cells were seeded in the top compartment of the Transwell insert. The lower compartment was filled with DMEM containing 0.05 ng per µl of CXCL1. Samples were incubated for 1.5 h at 37°C. After incubation time, cells that have migrated to the lower compartment were washed, stained for Gr1 and analyzed with a flow cytometer. B. Two-way ANOVA analysis of the data showing a significative effect of time in both genotypes ($P<0.05$, $F(2, 25) = 10.75$, n=3-8), a significative interaction between time and genotype ($P<0.05$, $F(2, 25) = 3.963$, n=3-8). Bonferroni post-hoc test showed a significative difference between the percentage of migrated neutrophils from $TLR4^{loxP/loxP}$ mice at 24 h and basal time and 24 h and 48 h (*$P<0.05$, n=3-8).*

We also explored neutrophil migration in an in vivo model. Firstly, we used the peritonitis model, which is especially relevant in the study of neutrophil infiltration since peritoneum is a compartment free of neutrophils in the basal state and therefore allows to analyze the differential infiltration efficiency of neutrophils of both genotypes. The peritonitis was induced by injecting zymosan in the peritoneum. To estimate the migration efficiencies of both types of cells, the ratio between cell in peritoneum and blood was compared. In this model, to confirm that the infiltration was due only to differences between genotypes and not to other physiological parameters, we used chimeric mice based on WT recipients of a BM mixture composed by 50% of DsRed BM and 50% $TLR4^{loxP/LyzM\text{-}cre}$ BM. With this strategy, we could analyze the behavior of both types of neutrophils in the same endothelium and under the same physiological conditions. As shown in Figure 48, neutrophils without TLR4 displayed a lower infiltration efficiency into the peritoneum (Figure 48, *P<0.05).

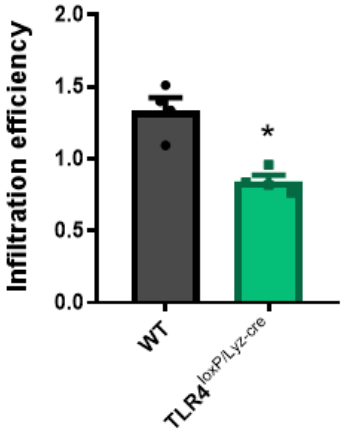


Figure 48. Neutrophil migration to the inflamed peritoneum. *Neutrophils without TLR4 display a lower infiltration efficiency (P<0.05, n=4).*

To further analyze this mechanism, we performed intravital microscopy of the inflamed cremaster muscle. This technique allows us to study three steps of the

infiltration process: rolling, adhesion, and neutrophil kinetics (speed, accumulated distance and directionality and Euclidean distance).

The analysis of the intravital images (Figure 49A) showed that neutrophils lacking TLR4 displayed a lower rolling and adhesion efficiency (Figure 49B, *P<0.05). Regarding the crawling step, the analysis of kinetics parameters did not show any significant difference between genotypes (Figure 49C and D). These results suggest that in an acute inflammation model, neutrophils lacking TLR4 seem to adhere and infiltrate less efficiently into the tissue. However, the local environment of the cremaster muscle and the inflammatory stimuli are vastly different from the brain environment. Thus, further intravital microscopy studies of the ischemic brain could be useful to fully dilucidate the infiltration mechanics of neutrophils.

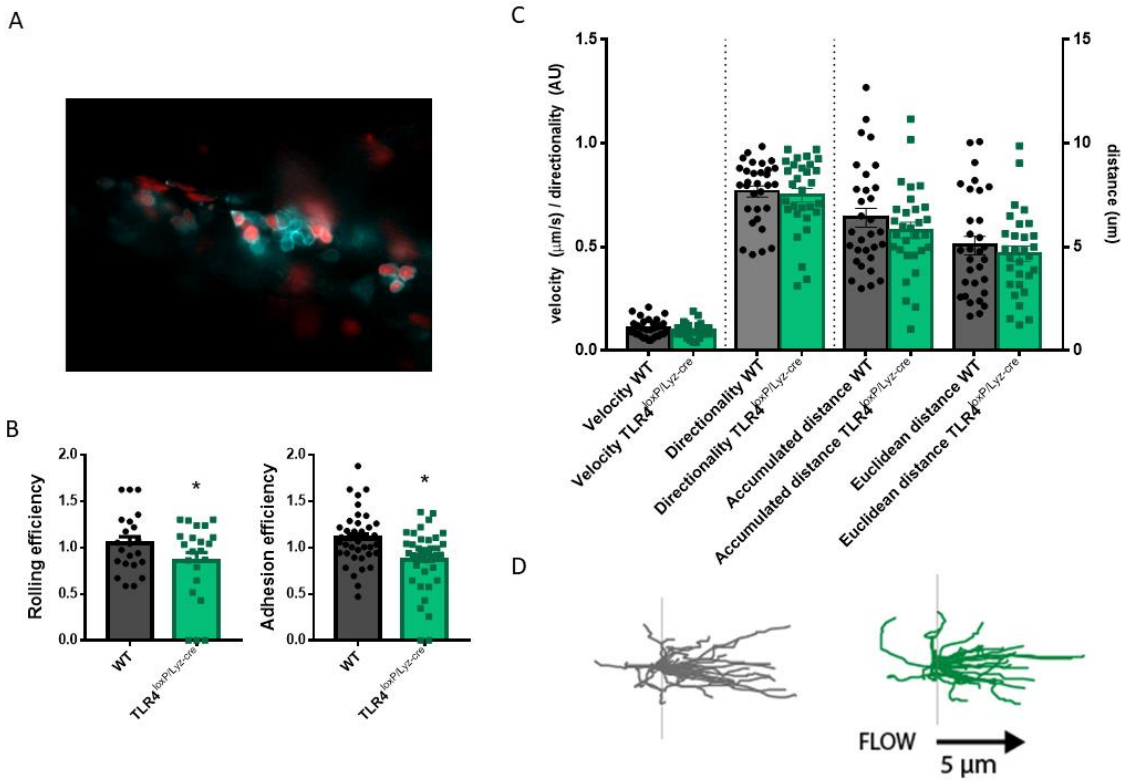
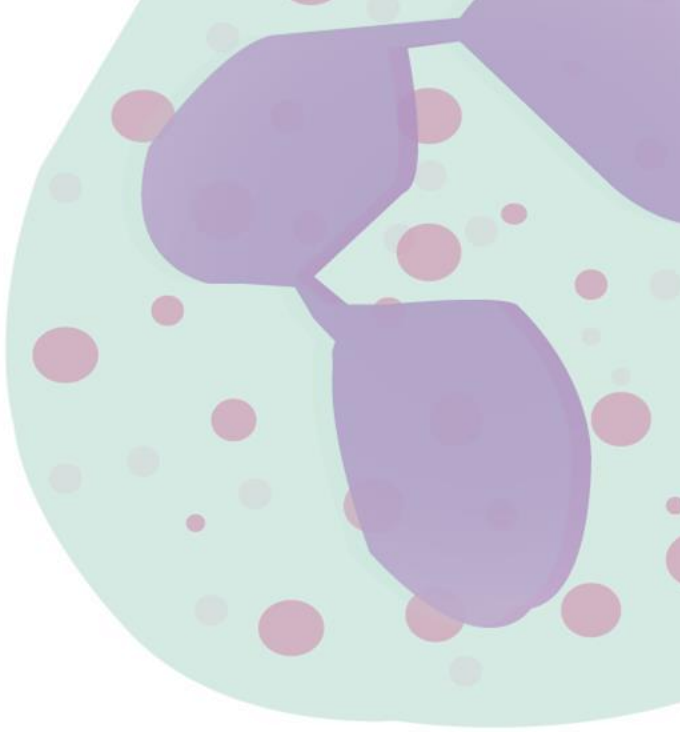


Figure 49. Study of neutrophil rolling, adhesion and crawling by intravital microscopy. *A. Representative image of the adhesion state taken with intravital microscopy. Cells from the DsRed BM donor are shown in red and neutrophils in blue (Ly6G antibody). Cells that only show blue are cells from TLR4$^{loxP/LyzM\text{-}cre}$ BM donor. B. Rolling and adhesion efficiency of neutrophils from DsRed and TLR4$^{loxP/Lyz\text{-}cre}$ mice (*P<0.05). C. Kinetic parameters of neutrophils with and without TLR4. D. Crawling pattern of neutrophils with (grey) and without (green) TLR4.*



DISCUSSION

5. DISCUSSION

Our laboratory has been long dedicated to deciphering the role of TLR4 in the pathophysiology of stroke. Specifically, we, as well as other groups, have focused (among other lines of research) in the role that TLR4 plays in inducing inflammation and tissue damage after stroke (Cao et al., 2007; Caso et al., 2007). However, the specific mechanisms by which TLR4 is deleterious are not fully understood. In this Doctoral Thesis, we show that TLR4 plays a crucial role in neutrophil polarization and function, in such a way that TLR4 absence induces neutrophil polarization towards an N2 phenotype, which is associated to neuroprotection. We have also found that neutrophil TLR4 absence modulates the induction of several pathways previously associated with the inflammatory process after ischemia, their migration into the parenchyma, and their maturation, all of which might underlie the functional outcome during stroke.

5.1. NEUTROPHIL CHARACTERIZATION AFTER ISCHEMIC STROKE: $TLR4^{+/+}$ AND $TLR4^{-/-}$ NEUTROPHIL TRANSCRIPTOME ANALYSIS

5.1.1. Role of TLR4 in N1/N2 neutrophil reprogramming after stroke: assessment and validation of global gene expression in WT and TLR4KO blood neutrophils

In order to explore the molecular mechanisms underlying the protective effects of N2 phenotype after stroke in the absence of TLR4, we performed a cDNA microarray analysis of isolated neutrophils from WT and TLR4KO mice. Gene Set Enrichment Analysis

revealed that neutrophils from TLR4KO mice differ from those from WT mice after MCAO since samples from both genotypes clustered together showing different transcriptomic signatures.

To link genes to specific pathways we evaluated key pathways and gene groups using bioinformatic approaches. GSA of the pathways enriched for the 500 genes more differentially expressed in TLR4KO neutrophils showed 3 major groups of altered pathways related to inflammation, circadian rhythms, neutrophil migration and cell death. According to these results, TLR4 might modulate the contribution of neutrophils to the inflammatory process after ischemia by regulating gene pathways. IκB kinase/NF-κB and TNFα/NF-κB pathways seem to be different between genotypes. In this context, many pieces of literature have reported LPS activation of NF-κB (Castro-Alcaraz et al., 2002; McDonald et al., 1997) so the downregulation of these routes in TLR4KO mice is in accordance with what the literature suggests. The alteration of these routes is also related to an induction of the apoptotic function of neutrophils and cell death programming (Castro-Alcaraz et al., 2002) which correlates with the observation of the cell death pathways alteration observed in the GSA. However, it is important to recall that transcriptomic results might differ from those obtained *in vivo* due to the influence and cooperation of other cells in several neutrophilic functions and the fact that not one but several pathways lead to the execution of a function, as we will explore in the apoptotic process further ahead. Another altered pathway is the IL6/Janus kinase/STAT3 which we found to be upregulated in neutrophils from TLR4KO mice. Of note, it has been described that IL-6 driven signaling via STAT3 modulates the inflammatory recruitment of neutrophils (Fielding et al., 2008) which means that the alteration of this pathway could

be crucial in neutrophil trafficking towards the damaged parenchyma after stroke. These results suggest that TLR4 modulates the contribution of neutrophils to the inflammatory process after ischemia, potentially modifying neutrophil trafficking and function.

In order to corroborate the different signatures between genotypes we conducted qPCR validation of some genes, specifically related to N2 phenotype such as Chil3, Arg1, Tnfaip3 and Per1. Chil3, also known as Ym1, is a chitinase-like protein recently described as a marker of N2 neutrophils (Cuartero et al., 2013; García-Culebras et al., 2019). Arg1, which codifies for the protein arginase 1, plays a role in the immune response of alternatively activated or M2 macrophages. Previous studies have shown that N2 neutrophils also express this molecule (Cai et al., 2019; Cuartero et al., 2013). Since we had observed that the inflammatory responses could be altered, we wondered whether Tnfaip3 expression was higher in TLR4KO neutrophils. Tnfaip3 has been studied given its role in the regulation of immune and inflammatory responses signaled by cytokines or TLR4 activation through terminating NF-κB activity. Therefore, to study this gene is of high interest since it has been shown that Tnfaip3 has an essential and direct involvement in the negative regulation of TLR4 activated routes (Boone et al., 2004). Lastly, since we observed that the circadian rhythm pathway was altered, we chose to analyze the expression levels of Per1, one of the genes that define the intrinsic clock and which plays a critical role in rhythm generation (Masubuchi et al., 2005). All of them were more expressed in TLR4KO neutrophils confirming our transcriptomic results.

5.1.2. Comparison of transcriptional signatures of non-aged and TLR4-/- neutrophils

Regarding the capacity of neutrophils to change their phenotype, one of the most accepted views proposes that there are different populations of neutrophils depending on their aging status (Adrover et al., 2019). This phenotypic change has been described to be driven not only by one unique process but by different mechanisms (Hellebrekers et al., 2018). Recently, it has been described that neutrophil aging is driven by external signals induced by microbiome-produced TLR4 agonists (Zhang et al., 2015). In this study, the authors induced a microbiome depletion by antibiotic administration, and after the treatment they observed a decrease in circulating aged neutrophils. Starting out from this premise, we wondered if our two types of neutrophils ($TLR4^{+/+}$ and $TLR4^{-/-}$) corresponded to the ones described as aged/non-aged by Frenette and cols. To that aim, we compared the 500 genes more overexpressed in TLR4KO neutrophils when compared to the WT ones, with the transcriptional changes observed in non-aged/aged neutrophils. Interestingly, we found that genes overexpressed in neutrophils from our stroke-exposed TLR4KO mice are significantly enriched in those overexpressed in the non-aged group. Consistently, Frenette's aged neutrophils, similar to the predominant population in our stroke-exposed WT animals, exhibit a higher expression of TLR4 and an upregulation of several inflammatory pathways. This correlates with our analysis showing that TLR4KO and non-aged neutrophils share some upregulated pathways such as negative regulation of immune effector process, regulation of inflammatory response and negative regulation of IL-6 production. Furthermore, they also found a significant reduction of aged neutrophils in TLR4-KO mice as we also observed. In their study, they found that

neutrophil pro-inflammatory activity correlates positively with neutrophil ageing. Taken all together, these data suggest that neutrophils without TLR4, as well as non-aged neutrophils, have an altered ability to participate in the inflammatory pathway.

However, as previously stated, neutrophil change of phenotype is not only driven by microbiome products. Recently, it has been described that neutrophils have an intrinsic timer that drives their aging process throughout the day following a circadian pattern (Adrover et al., 2019). In other to check if the neutrophil populations described in that study, fresh and aged were the same as the ones described by us, we again performed clustering and PCA analysis comparing our transcriptional data with theirs. In this case, we did not find any correlation between the transcriptional signatures. This is likely due to the fact that there is not a unique mechanism that drives the neutrophil phenotype change. From cancer to microbiome, circadian patterns and inflammation, there are multiple mechanisms that could lead to different transcriptional signatures of the neutrophil populations. The fact that TLR4KO neutrophils do not align with fresh neutrophils described by Hidalgo and cols. could suggest that there is some type of dysregulation in their circadian pattern. Even though there were no clear similarities in the transcriptional signatures of those subsets, certain pathways upregulated in the fresh subset were also upregulated in our TLR4KO neutrophils, such as negative regulation of immune effector process, regulation of the inflammatory response and negative regulation of IL-6 production pathways.

5.2. SPECIFIC MYELOID ABLATION OF TLR4 INDUCES NEUROPROTECTION AFTER STROKE

As previously stated, the absence of TLR4 mediates a neuroprotective effect due to the inhibition of the inflammatory response after stroke (Cao et al., 2007; Caso et al., 2007; Tang et al., 2008). Classically, it has been described that neutrophils are in part responsible for the ischemic damage after stroke, and therefore there is a positive correlation between the number of infiltrated neutrophils and the infarct volume (Jin et al., 2010; Segel et al., 2011; Yilmaz and Granger, 2010). However, we have recently described that TLR4 neuroprotective effect is concomitant to an increased density of infiltrated neutrophils into the ischemic core (García-Culebras et al., 2019). This fact had been previously suspected, since the absence of TLR4 showed an increased neutrophil accumulation in the ischemic brain (Kilic et al., 2008), that the authors speculated due to a futile inflammatory response produced as a compensatory mechanism counteracting the TLR4 absence. However, we showed that not only the absence of TLR4 produces an accumulation of neutrophils, but it also polarizes neutrophils towards a protective, N2 phenotype.

5.2.1. Role of neutrophilic TLR4 in the stroke injury

In order to fully demonstrate that the neuroprotection observed in the TLR4KO mice (where we had found an increase in the number of infiltrated neutrophils) was due to the lack of TLR4 in neutrophils, we generated mice that lack TLR4 in the myeloid lineage. This genetic model expresses the Cre recombinase enzyme under the Lysozyme M promoter (only expressed in myeloid cells). We first validated this genetic construction to ensure

that the TLR4 deficiency was specific for hematopoietic myeloid cell subsets. By analyzing the TLR4 expression in isolated microglia cells by PCR, we successfully proved that even though microglia share many features with other myeloid cells (given its mesodermal origin), it expresses TLR4 and, therefore, it was a suitable model to study the implication of neutrophil TLR4 in ischemic stroke. We then checked its impact on stroke outcome, finding that, after ischemic stroke, TLR4-deficient myeloid linage mice ($TLR4^{loxP/LyzM\text{-}cre}$) display significantly decreased infarct volumes than their respective controls ($TLR4^{loxP/loxP}$).

LyzM is expressed not only in neutrophils, but also in other myelomonocytic cells, such as monocytes. Studies have shown that, crossing LyzM-cre mice with GFP mice, the vast majority of cells expressing TLR4 were neutrophils. However, there were monocytes that also expressed the GFP (Faust et al., 2000). To further prove that the neutrophil TLR4 was the responsible for the observed neuroprotection, we depleted the neutrophil population and then performed the experimental ischemic stroke in $TLR4^{loxP/LyzM\text{-}cre}$ and $TLR4^{loxP/loxP}$ mice. Thus, we minimized the possibility that the lack of TLR4 in other myeloid subsets had an effect in our results. Again, as we had previously observed with the TLR4KO mice (García-Culebras et al., 2019), specific neutrophil depletion increased infarct volume. This result supports that the lack of neutrophil TLR4 is responsible for the observed neuroprotection, minimizing the contribution of other types of cells, although it is a possibility that we cannot discard completely.

Since we had previously observed that the complete absence of TLR4 increased the number of infiltrated neutrophils found in the parenchyma after stroke, we tested whether the specific ablation of TLR4 in neutrophils had a role in the neutrophil

infiltration in this setting. After generating chimeric mice in which half of the circulating neutrophil population expressed TLR4 and the other half did not, we observed that neutrophils lacking TLR4 were more present in the ischemic core. This results correlates with our previous findings that, in TLR4KO mice, there are more neutrophils in the ischemic brain (García-Culebras et al., 2019) and also suggests a higher ability of TLR4 lacking neutrophils to infiltrate into the ischemic brain tissue.

5.2.2. TLR4 absence in myeloid cells skews neutrophils towards an N2 phenotype

Considering that TLR4KO-induced neuroprotection is unexpectedly associated with an increased rate of neutrophil infiltration, and that TLR4 absence does not affect infarct volume after neutrophil depletion (as previously described in TLR4KO mice and described in this Doctoral Thesis in $TLR4^{loxP/LyzM\text{-}cre}$ mice), we hypothesized the existence of neutrophil subsets with different pathophysiological roles. In the last decade, there has been a proliferation of studies showing that, in addition to macrophages and lymphocytes, neutrophils are also capable of polarizing to different phenotypes. The first evidence of this fact was observed in systemic lupus erythematosus and other autoimmune conditions where a distinct subset of proinflammatory low-density granulocytes was described (Bennett et al., 2003; Denny et al., 2010). Shortly after, this concept was studied in the cancer field, where "tumor associated neutrophils" (TANs) could switch to a pro-tumorigenic phenotype characterized by the expression of arginase I, CCL2 and CCL5 (Fridlender et al., 2009). In the cerebrovascular disease context, our group had described that after stroke, the neutrophil population that infiltrates into the parenchyma is not homogenous. There are different neutrophil subpopulations that express markers traditionally associated to the macrophage alternative phenotype

(Cuartero et al., 2013). In this case, the activation of PPARγ mediated the neutrophil reprogramming towards an N2 phenotype (characterized by the expression of Ym1 marker, similarly to M2 macrophages) which occurred concomitant to a neuroprotective effect. The fact that skewing neutrophil polarity towards N2 phenotype is neuroprotective has also been very recently proposed by Lu and cols (2018). Although the approach in this study is not directly related to the genetic manipulation of TLR4, they induce N1 phenotype by IFNγ and LPS administration (and therefore stimulating TLR4) and N2 by TGFβ administration. In this study they found a negative correlation between the number of N2 neutrophils and the infarct volume (Cai et al., 2019), which corroborates our results. This protective phenotype is not only limited to the stroke pathology but it is also implicated in other pathologies that contribute to the cerebrovascular diseases such as atherosclerosis since they observed that rejuvenated neutrophils can reduce the pathogenesis of atherosclerosis (Geng et al., 2019) .

Taking all those evidences into account we hypothesized that the lack of TLR4 could induced neutrophil reprogramming towards N2 phenotype. Previously, we had found, by using stereological and cytometric analysis, a decreased number of N1 and an increased number of N2 neutrophils (as $Ym1^{+}$/NIMP-$R14^{+}$ cells) in the infarcted area of TLR4KO mice (García-Culebras et al., 2019). In this Doctoral Thesis we wanted to prove that the specific absence of TLR4 in neutrophils skews these cells towards an N2 phenotype. Indeed, after stroke, we found an increased number of N2 infiltrated neutrophils normalized by the infarct volume in $TLR4^{loxP/LyzM\text{-}cre}$ mice vs the control group. Also, we found an inverted ratio in the percentage of N1/N2 neutrophils in $TLR4^{loxP/LyzM\text{-}cre}$ mice where N2 neutrophils were predominant, supporting the hypothesis that it is neutrophilic TLR4, specifically, the

receptor responsible for N2 shifting ultimately associated with the observed neuroprotective effect.

5.3. ROLE OF TLR4 IN NEUTROPHIL DYNAMICS IN HOMEOSTASIS AND AFTER STROKE

5.3.1. Influence of myeloid TLR4 on neutrophil aging markers and its circadian rhythm

The results derived from our initial transcriptome analysis showed that neutrophils without TLR4 are comparable to non-aged neutrophils described by Frenette and col (Zhang et al., 2015). This study not only provided a transcriptomic profile of non-aged and aged neutrophils but also described surface markers (CD62L, CXCR4, CD11b) that can be used to identify those populations. Therefore, in order to analyze if our subsets match not only with the transcriptomic signature but also with the previously reported surface markers, we analyzed them in both genotypes. Our results showed that TLR4 lacking neutrophils have a higher expression of CD62L and a trend to a decrease in CD11b and CXCR4. Although these results are not fully conclusive, it is likely that an increase in sample size could contribute to clarify the expression of these markers. Nonetheless, this aligns with results from the transcriptomic analysis since this marker pattern is similar to the one corresponding to the non-aged group (Zhang et al., 2015). Moreover, we have observed that after the stroke this surface marker pattern is no longer observable. This suggests that the inflammatory response subsequent to the ischemia is strong enough to dysregulate the surface marker expression process and, very likely, to interfere with the polarization process.

As we have previously stated, the microbiome signaling is not the only regulator of the neutrophil plasticity. When studying this process, one of the main factors that has been described to influence neutrophil genotype is the circadian rhythm Even more, phenotype modulation is not the only change that neutrophils suffer through time. In addition to the proportion of fresh and aged neutrophils, the number of total neutrophils also varies during the day (Casanova-Acebes et al., 2013). With this idea in mind we wondered if the absence of TLR4 affected the regulation of the neutrophil circadian pattern. The analysis of the neutrophil kinetics in blood showed that neutrophils with TLR4 exhibited a typical circadian pattern. However, this motif was absent in TLR4-lacking neutrophils, for which their number did not oscillate through the day. This result suggests that the absence of TLR4 prevents neutrophils from their typical circadian fluctuation.

Since the absolute count of neutrophils was altered, it is plausible that the proportion of fresh and aged subsets was also modified. It has been reported that all neutrophils are $CD62L^{hi}$ at ZT13 whereas at ZT5 there is a mixture of $CD62L^{hi}$ and $CD62L^{lo}$ neutrophils. We found that, whereas neutrophils with TLR4 showed the canonical pattern, there were more “young” neutrophils in blood at ZT1 in $TLR4^{loxP/LyzM\text{-}cre}$ mice, as shown by a change in the expression of $CD62L^{hi}$ at ZT1 in TLR4-lacking neutrophils. In summary, the absence of TLR4 abrogates the circadian pattern displayed by neutrophils under physiological conditions. This observation could be consistent with a phenotype associated with neuroprotection, since the presence of aged neutrophils in physiological conditions was concomitant with a poor outcome when mice were subdued to a vascular ischemia (Adrover et al., 2019).

5.3.2. Influence of TLR4 in neutrophil intrinsic features

We also wondered whether some intrinsic properties of the neutrophils were also altered by the absence of TLR4. Two of the main characteristics used to describe and discriminate the different types of cells are size and complexity. In fact, those are the two characteristics used when gating cell population by flow cytometry. Recently, it has been described that, as well as neutrophil count and surface markers, the granularity (complexity) of both murine and human neutrophils changes over time (Adrover et al., 2020). However, in our experiments, we observed no difference in cell size between genotypes. Regarding complexity, we have found that neutrophils with TLR4 are more complex and that the complexity in both genotypes decreased over time. Hypothetically, this difference of complexity would entail a differential capacity to execute certain functions. In this context, it has been suggested that the progressive discharge of granules, which decreases the complexity, could correlate with a partial loss of their arsenal and consequently, with their capacity to elicit organ damage (Adrover et al., 2020). Since we have observed that neutrophils without TLR4 are less complex we could hypothesize that indeed they could be less harmful. In order to explore this possibility, we performed a thorough study of the different neutrophil functions in both genotypes.

5.4. ROLE OF TLR4 IN NEUTROPHIL FUNCTION IN HOMEOSTASIS AND AFTER STROKE

Considering that TLR4-lacking neutrophils are, at least, partially responsible for the neuroprotection observed in $TLR4^{loxP/LyzM\text{-}cre}$ mice, we decided to globally assess the functionality of the TLR4-lacking neutrophil, and how this affected the outcome after

stroke. In order to do so, we studied several parameters associated with neutrophil function such as phagocytic activity, apoptosis, microglia engulfment, ROS production, NETosis and neutrophil migration. Also, we asked whether those functions were in any way altered by the ischemic insult, for which we studied them in the basal state and after the stroke.

5.4.1. Role of TLR4 in neutrophil phagocytic activity before and after stroke

Firstly, we studied the phagocytic activity of neutrophils. Since this function could be a key player in the resolution of the inflammatory process by eliminating cell debris produced in the ischemic tissue we wanted to know if it was altered by the lack of TLR4 or the ischemic process itself by detecting the percentage of neutrophils that had phagocytosed fluorescent beads by flow cytometry. In the basal state we observed that there is a higher percentage of phagocytosing neutrophils in the TLR4-lacking genotype. However, at 24 and 48 hours after ischemia we did not observe any significant differences between genotypes. Moreover, when comparing the data altogether we observed that the phagocytic activity decreased significantly in $TLR4^{loxP/Lyz\text{-}cre}$ neutrophils. These results suggest, that even there was no difference in the phagocytic activity between genotypes at 24 h, the difference observed in the basal state may contribute to the pro-resolving properties of TLR4-lacking neutrophils in the very early stages of the inflammatory process. Further experiments would be required in order to analyze the phagocytic activity at earlier time points after the stroke, when the phagocytic activity could be crucial to limit the expansion of the inflammatory response.

The phagocytic activity of neutrophils after stroke has been evaluated in stroke patients as the ability of neutrophils to engulf fluorescent bacteria (Ruhnau et al., 2014).

In this study, the percentage of neutrophils that undergo phagocytosis was similar to ours. However, they did not see the decrease in the phagocytic activity over time that we observe in the neutrophils without TLR4. Also, it has been observed in other pathologies, such as heat stroke, that the ability of phagocytose correlates negatively with CD11b expression (Lu et al., 2004), which agrees with our results, where we see that neutrophils without TLR4 tend to express less CD11b in the basal state, as we have previously commented. Moreover, it is known that neutrophils that had phagocytosed elements such as apoptotic cells or platelets seem to be fixated in an unresponsive state, since they become unable to respond to further inflammatory stimuli and consequently, fail to release their granular content or to generate NETs (Manfredi et al., 2018). The fact that in the very acute phase of the inflammatory process TLR4-lacking neutrophils are more capable of phagocytose and consequently be induced into a "calming state" could potentially limit the collateral damage caused by the neutrophils and contribute to their neuroprotective role.

5.4.2. TLR4 influence on neutrophil apoptosis in homeostasis and after stroke

We also studied the ability of neutrophils to undergo the apoptotic process. This particular process is of interest since it is key in the disarming process of a cell that contains a very powerful arsenal of molecules. Previous studies have shown that TLR4 stimulation induces an increase in the lifespan of the neutrophils (Goshima et al., 2004; Sabroe et al., 2003). This observation could be linked to a decrease in the apoptotic process. We therefore measured the percentage of apoptotic neutrophils in blood in the basal state, and 24 and 48 hours after stroke. We did not observe any differences in

apoptosis in blood neutrophils at any given time point. When comparing both genotypes at all time points studied, we observed an increase in the percentage of apoptotic neutrophils in both genotypes at 48 h. At first this could be conflicting with the preexisting literature. However, these discrepancies are likely due to the fact that all prior studies were conducted by administering an external stimulus such as LPS, which is much stronger than the weak TLR4 activation that can occur in a basal state (probably just given through the microbiome). Another point to consider is that the increase in neutrophil survival may be an artifact resulting from the presence of monocytes demonstrated in the techniques reported to obtain highly purified neutrophils, as it has been shown that variations in apoptosis rates after TLR4 activation were due to monocyte contamination (Sabroe et al., 2003). Since, in our model, monocytes express TLR4 (at least, the majority of them) and all the assays were performed with WBC and not with highly purified neutrophils, additional experiments with highly purified neutrophils are required to fully understand exclusively the role of the neutrophilic TLR4. Also, we wanted to know if the same situation was occurring in a different scenario, such as the injured brain tissue. In order to do so, we performed the TUNEL assay to analyze the percentage of apoptotic infiltrated neutrophils. In this case, again, we did not observe any differences in the percentage of apoptotic neutrophils. Taking all these data into account we can conclude that the neutrophilic phenotype does not influence the apoptotic process. Nonetheless, more experiments are necessary to elucidate the role of the different neutrophil phenotypes on the apoptotic process.

5.4.3. Preferential phagocytosis of N1/N2 neutrophils by microglia in the ischemic core

Previous studies from our laboratory established that, after rosiglitazone treatment, N2 neutrophils (NIMP-R14^{+}/Ym1^{+}) are preferentially cleared by microglia in the injured tissue (Cuartero et al., 2013). This observation was later corroborated by other groups that also described that N2 phenotype promotes self-clearance of neutrophils and benefits inflammatory resolution after stroke (Cai et al., 2019). Since neutrophils carry a huge amount of detrimental cargo, they must be removed from the injured tissue as soon as possible in a safe and controlled way. One way to clear the infiltrated neutrophils is their engulfment by the microglia. In order to explore whether neutrophilic TLR4 is implicated in this clearance process we studied this mechanism by co-visualization of Iba1, NIMP-R14 and Ym1 in the infarcted tissue from mice of both genotypes, following the previously demonstrated premise that microglia express TLR4 in both genotypes. This fact is especially relevant since the absence of TLR4 has been described to decrease the phagocytic activity of microglia (Rajbhandari et al., 2014). Hence, our previous PCR results were functionally validated. We found that, in both genotypes, the percentage of microglia containing neutrophils was the same, which corroborates that, in this genetic model, the phagocytic activity of the microglia is intact. Also, we again confirmed that N2 neutrophils are engulfed preferentially to N1 by the microglia, as previous studies stated (Cuartero et al., 2013). Considering that dying neutrophils ultimately disintegrate potentially liberating their cargo and contributing to the tissue damage, and that phagocytosis promotes secretion of anti-inflammatory

mediators, an increase in N2 neutrophils engulfment could contribute to the resolution of inflammation and brain recovery after stroke.

5.4.4. TLR4 contribution to neutrophil ROS production in homeostasis and after stroke

As previously stated, neutrophils are activated by DAMPs and consequently they produce ROS, which could produce additional tissue damage in a sterile inflammation context (Prince et al., 2011). In order to quantify this process, we measured the ROS production with and without adding a stimulating peptide (W-peptide) to WBC. We observed that neutrophils without TLR4 produced significantly less ROS in the basal state, in agreement with the reported involvement of TLR4 in eliciting oxidative burst in neutrophils when induced by LPS (Remer et al., 2003). In contrast, 24 h after stroke, W-peptide stimulation failed to increase ROS production. Remer et al showed that TLR4 knockout mice, which failed to produce ROS in the presence of LPS, were able to produce ROS when activated by PMA. The apparent discrepancy could be due to the difference in the stimuli, which in their case was PMA, and in our study was W-peptide. In alignment with our hypothesis, Frenette and cols, whose study was used in the first part of this Doctoral Thesis to establish a comparison with control and old neutrophils, described that aged neutrophils exhibited significantly increased ROS production (Zhang et al., 2015). Since aged neutrophils are transcriptionally similar to $TLR4^{loxP/loxP}$ mice this result completely endorses our results. This mechanism has also been studied in myocardial infarction. In this pathology, neutrophils treated with metoprolol are less capable of producing ROS which contributes to the protective effect of metoprolol treatment

(García-Prieto et al., 2017) which also aligns with our hypothesis. In spite of the abundant studies of neutrophil ROS production, in general, ROS studies are hampered by diverse technical issues. In patients, it has been reported that stroke produces a decrease in ROS production when analyzed without any external stimulation, and no changes between control individuals and patients when stimulated by the chemotactic peptide fMLP, which is a classic chemotactic agonist that induce chemotaxis, degranulation and production of ROS that, when used *in vitro,* it is known to produce inflammatory changes in neutrophils (Videm and Strand, 2004). However, we observed an increase in the oxidative burst function over time in both genotypes. This disparity could be due to the non-specificity of traditional probes which are not specific for a single molecule, not targetable to a particular intracellular compartment and, in general, not capable of being used *in vivo* (Amulic et al., 2012). Further experiments would be necessary to complete and validate our results on ROS production. New approaches could be used, such as redox-sensitive fluorescent protein-based probes (i.e. roGFP or HyPer), transcription profiling of superoxide or hydrogen peroxide-sensitive genes or detection of products of reactive oxygen using mass spectrometry (Murphy et al., 2011).

In summary, the lack of TLR4 in neutrophils could mitigate the damage caused by neutrophils and contribute to neuroprotection since their ability to produce ROS is decreased in the early stages of the inflammatory process.

5.4.5. Role of TLR4 on NETosis *in vitro* and in infiltrated neutrophils after stroke

One of the main functions of the neutrophil is its ability to form NETs. When activated, neutrophils are able to extrude large macromolecular structures that comprise

neutrophil DNA, citrullinated histones, and an array of active proteases. We pondered if neutrophil TLR4 could influence the ability to undergo this process. In order to do so, we performed analysis both *in vivo* and *in vitro*. Firstly, we stimulated isolated neutrophils with PMA, and quantified by confocal microscopy the percentage of neutrophils that did NETosis. Surprisingly, we found that neutrophils without TLR4 are more prone to undergo NETosis. *A priori*, this result could be disconcerting. It has been reported that NET formation increases in aged neutrophils when stimulated with LPS (Zhang et al., 2015). Also, in lung injury, NETs markers were significantly lower in TLR4KO mice than in WT mice (Li et al., 2017). In both studies, a possible confounding factor could be the role of the platelet TLR4. It is known that platelet TLR4 activates the neutrophil to produce NET and therefore it is a threshold switch for this process (Clark et al., 2007). Since, in both cases, platelets are genetically equal to neutrophils (both express TLR4 the exact role of each one), cannot be discriminated. Very recently, a new study has proposed that Bmal1$^{\Delta N}$ mice (Bmal is only lacking in neutrophils which confers a constitutive young state in neutrophils) show a higher proportion of NET-forming cells than CXCR$^{\Delta N}$ ones (CXCR4 is only lacking in neutrophils which confers a constitutive aged state in neutrophils) when stimulated with PMA (Adrover et al., 2020) , as we did, which aligns with our results. Another point to discuss in this matter is the necessity of ROS production in order to form a NET. ROS is needed to promote the release of the azurosome complex, with contains, among others, MPO and NE, which will degrade the acting-based cytoskeleton (Manfredi et al., 2018). Since TLR4 lacking neutrophils produce less ROS in comparison with those that express TLR4, *a priori* less NETosis would be expected. There are different reasons to argue this point. Firstly, neutrophils without TR4 produce significantly less ROS than

neutrophils with TLR4 but they are still able to produce some which could be enough to start this process. Secondly, there are independent pathways leading to the generation of NETs. There is a ROS-independent pathway which is the first to be activated in neutrophils when they are challenged by activated platelets and apoptotic cells (Manfredi et al., 2018). Therefore, it is plausible to think that even when TLR4-lacking neutrophils produce less ROS they could produce NETs in more quantity that neutrophils with TLR4.

All these results pose some questions; since this phenomenon is quite variable depending on the stimuli provided, we wanted to elucidate what was occurring *in vivo* in the ischemic tissue. In order to do so, we analyzed with confocal microscopy the percentage of neutrophils undergoing NETosis 48 h after stroke. In this case, in contrast with *in vitro* results, we did not observe any differences between genotypes. This difference could be due to various reasons. On the one hand, we have previously seen that neutrophils lacking TLR4 have an increased phagocytic activity in the basal state. Neutrophils that have engaged in a phagocytic process have its MPO and NE content sequestered within the phagolysosome and, therefore they are less able to reach the cytoskeleton and the nucleus, which is a critical checkpoint in NET formation (Branzk et al., 2014; Metzler et al., 2011). Also, as we were affirming previously, in our genetic model platelets express TLR4 and, for that reason, platelets may be playing a role as both activating neutrophils and switching them off. Neutrophils that have phagocytosed activated platelets seem to be driven to an unresponsive state, and they become unable to respond to further inflammatory stimuli failing to generate NETs (Manfredi et al., 2015). In summary, the NETosis process seems unlikely to be involved in the neuroprotective effect that neutrophils without TLR4 show in stroke but we could

hypothesize that the reduced NETosis due to their previous phagocytic activity might mitigate this damaging function. Also, we cannot obviate that NET content in blood was not measured for technical reasons and consequently further studies are required to clarify this issue.

5.4.6. Implication of neutrophilic TLR4 in neutrophil migration

Since our previous results showed an increased neutrophil density in the injured brain of TLR4KO mice, we wanted to know if this fact was due to their increased ability of migration and infiltration into the tissues. In this case, we also tackled this issue with *in vitro* and an *in vivo* approaches. Firstly, for the *in vitro* study we used a transwell chamber system in which neutrophils were seeded and stimulated with CXCL1. We observed a significant difference both in time and genotype when comparing all data together, as neutrophils expressing TLR4 were more able to migrate through the insert, being that migration significantly higher at 24 h after the stroke declining to the basal level 48 h after the ischemia. This is in agreement with evidence in the literature showing that crosstalk between TLR4 and chemokine receptors in PMNs results in marked augmentation of chemokine driven PMN migration (Fan and Malik, 2003) and that TLR4, as well as TLR2, is crucial for neutrophil migration (Castoldi et al., 2012).

However, as we have been affirming during this whole dissertation, the *in vitro* context is not comparable with the *in vivo* context. That is the reason why we also carried out several *in vivo* experiments to complete the whole picture in collaboration with Dr. Hidalgo (CNIC, Madrid, Spain). Firstly, we performed the study of neutrophil migration in a peritonitis model. This model is especially interesting in this setting because the

peritoneum is constitutively a compartment free of neutrophils in physiological conditions, making extremely easily to study neutrophil infiltration. Moreover, by using a chimeric approach, it is possible to determine the differential neutrophil migration efficiency of two different genotypes in the same environment. Using this setting, we found that neutrophils without TLR4 displayed a lower infiltration efficiency into the peritoneum when injected with zymosan, demonstrating that neutrophil TLR4 is implicated in neutrophil migration towards inflamed tissues in a sterile inflammation context.

In order to go one step further and to have a complete view of the whole infiltration process (rolling, adhesion and neutrophil kinetics), we performed intravital microscopy of the inflamed cremaster muscle. The analysis of the intravital footage showed that neutrophils without TLR4 have a lower rolling and adhesion efficiency, showing the role of neutrophil TLR4 in this scenario. The knowledge of the influence of TLR4 in the neutrophil recruitment process has been vastly explored focusing on endothelial TLR4 (Zhou et al., 2009). However, little was known about the implication of the neutrophilic TLR4 itself. One of the existing few studies states that the release of myeloid-related proteins (MRPs) 8 and 14 activates a TLR4-mediated pathway that reduces leukocyte rolling velocity and stimulates adhesion (Pruenster et al., 2015), which aligns with our results. Regarding the crawling step, the kinetics parameters did not show any significant differences between genotypes. In conclusion, all these results point towards the idea that, in an acute inflammation model, TLR4 is vital for neutrophils to infiltrate into the tissue. In the same manner that the *in vivo* context is widely different from the *in vitro* situation, the cremaster physiology is drastically different than the brain. Our results in

experimental stroke support the idea that the lack of TLR4 increases the neutrophil infiltration into the brain tissue, as opposed to our observations in the cremaster. This discrepancy could be due to several reasons. To start with, the stimuli involved are completely different in this model from the ischemia context. Also, it is necessary to take into account that one of the main structures that intervene in the neutrophil infiltration process is the blood-brain barrier (even if it is disrupted, as happens in stroke). Since the cremaster muscle does not have the same vascular structure, there is a possibility that these processes occur in a different manner in the brain environment. Moreover, other mechanisms could be involved in this process, such as neutrophil clearance and survival within the tissue, that should be studied in depth. In conclusion, further intravital microscopy studies of the ischemic brain are necessary to fully comprehend the neutrophil migration process in the stroke pathology.

In summary, in this Doctoral Thesis we have demonstrated that TLR4 enacts a crucial role in neutrophil polarization since its absence skews neutrophil polarization towards an N2 phenotype and modulates the induction of different pathways associated with the inflammatory process in homeostasis and after ischemia. Furthermore, neutrophils without TLR4 are similar transcriptionally to the non-aged neutrophil phenotype described in Frenette and col's previous studies (Zhang et al, 2015). With loss-of-function approaches we have proven that neutrophil TLR4 is specifically involved in neutrophil dynamics under physiological conditions as well as in stroke-induced tissue damage. In addition, we have shown that the lack of TLR4 in neutrophils limits the ROS production by neutrophils potentially contributing to the neuroprotective effect, supporting the key inflammatory role of the neutrophil TLR4 receptor. Although,

undoubtedly more studies are needed to fully elucidate the role of neutrophilic TLR4 in the stroke pathology, this Doctoral Thesis contributes to the idea that TLR4, especially when targeted in specific cell types, could be a potential target to develop neuroprotective strategies.

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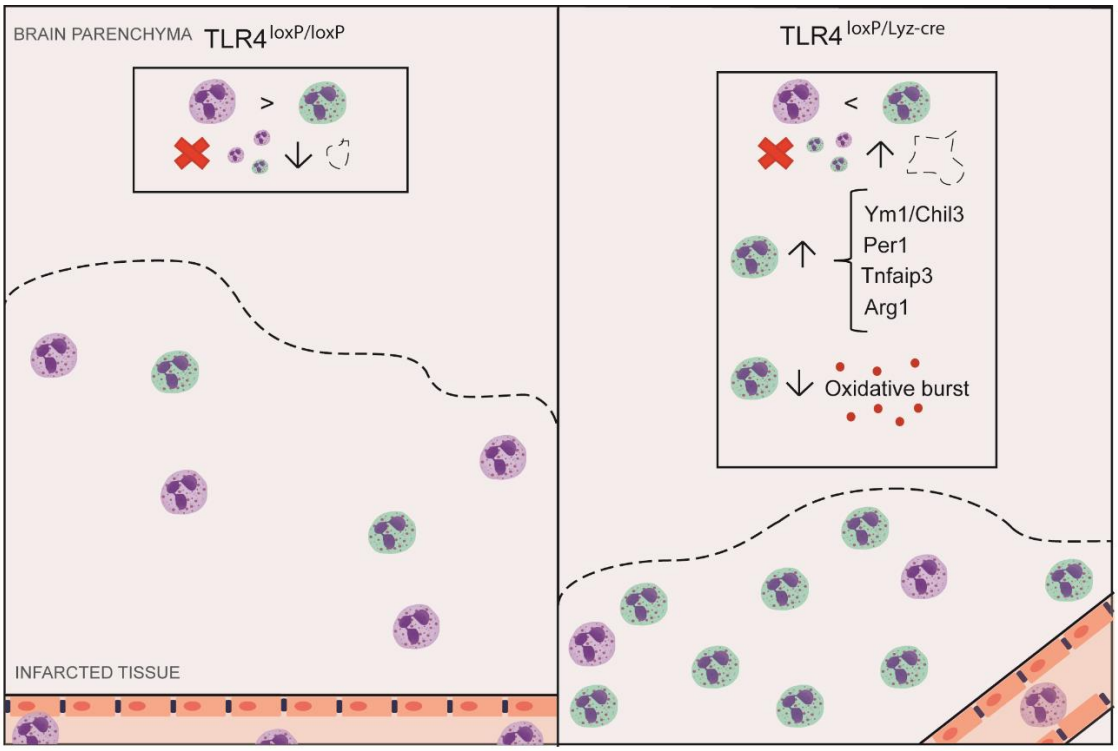
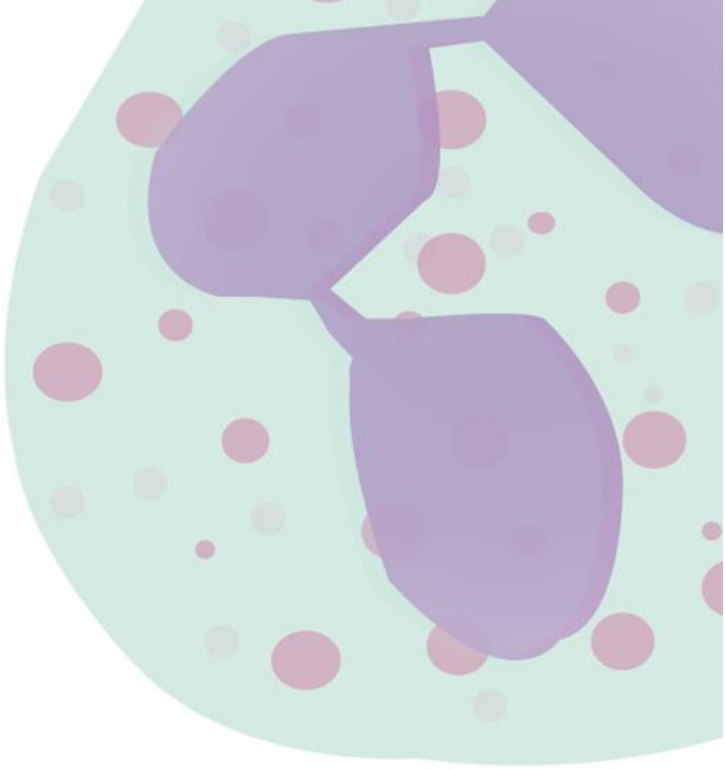
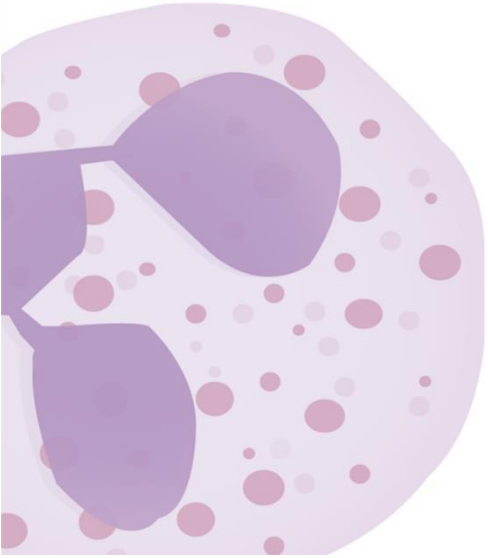


Figure 50. Graphical abstract of this Doctoral Thesis. *The lack of TLR4 in neutrophils induces neuroprotection after stroke and skews neutrophil phenotype towards N2. TLR4 absence also influences the ability of neutrophils to exert certain functions, i.e. oxidative burst.*



CONCLUSIONS



CONCLUSIONS

1. The transcriptomic analysis of mRNA from neutrophils expressing and not expressing TLR4 reveals that neutrophil TLR4 modulates the contribution of neutrophils to ischemia-induced inflammation, migration to the parenchyma and functional priming.
2. Neutrophils without TLR4 were validated as an N2 population since they show an upregulation of genes used for N2 neutrophil characterization (such as Chil3, Arg1, Tnfaip3 and Per1).
3. Neutrophils lacking TLR4 are transcriptionally similar to non-aged neutrophils described in the literature and they show an alteration in the ability to participate in the inflammatory response.
4. Myeloid-specific TLR4 deficiency is involved in neuroprotection after stroke since the infarct volume in mice whose neutrophils do not express TLR4 is significantly smaller. This fact is concomitant with an increased infiltration of TLR4-lacking neutrophils into the ischemic tissue.
5. In homeostasis, the absence of TLR4 keeps neutrophils in a steady youth status that is dysregulated, at least in part, after an ischemic insult. Lack of TLR4 also prevents neutrophils from their normal circadian fluctuation and alters the intrinsic properties of the cell as its complexity, which is reduced in TLR4 lacking neutrophils.
6. TLR4-lacking neutrophils show a higher phagocytic activity, with might result in pro-resolutive properties, at least in homeostasis.
7. Neutrophil apoptosis is not affected by the absence of myeloid TLR4.

8. N2 neutrophils are preferentially engulfed by the microglia when compared with N1 neutrophils.
9. Neutrophils lacking TLR4 produce less ROS in the first stage of the inflammatory process, which could contribute to the neuroprotection associated to this subset.
10. Neutrophils without TLR4 are more prone to undergo NETosis *in vitro* but not *in vivo* after stroke.
11. In summary, TLR4 plays a crucial role in neutrophil polarization, since its absence skews neutrophil polarization towards an N2 phenotype which resembles the non-aged neutrophil phenotype described in the literature. Moreover, neutrophilic TLR4 is involved in stroke-induce damage contributing, among other functions, to ROS production. In this sense, neutrophilic TLR4 could represent a potential target to develop neuroprotective strategies.

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