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# Role of PAR2 in M1-like macrophages associated with RA

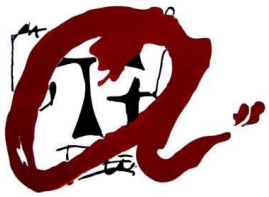
This dissertation has been done from the external internship carried out at the University of the West of Scotland (UWS)

Undergraduate Dissertation

Degree of Biotechnology

Supervised by Dr. Anne Crilly

Mentored by Dr. Maria Montserrat Poblet Icart



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# Center data

This undergraduate dissertation has been done from an external placement in the Center for Musculoskeletal Science (CMS) from January to June. The group belong to the University of the West of Scotland (UWS), located in Paisley, the United Kingdom. The project has been supervised by Dr. Anne Crilly.

The research of this group is mainly focused on the pathologic mechanisms involved in arthritis at the cellular, genetic and functional level. One of the main lines of research focuses on the participation of protease-activator receptor 2 (PAR2) in inflammation produced by arthritis. This protein modulates inflammatory responses and has been found to be overexpressed in patients with rheumatoid arthritis.

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# Abstract

Rheumatoid Arthritis (RA) is an autoimmune disease characterised by chronic inflammation in the joints. M1-like macrophages have been associated with the synovial inflammation observed in the pathology due to the release of pro-inflammatory cytokines. Nowadays, the main treatments for RA target the inflammatory pathways. While several therapies with different mechanisms of action exist, they do not work in all patients. Also, RA patients sometimes develop antibodies against these drugs.

Protease-activated receptor 2 (PAR2) has been proposed as a possible therapeutic target since the receptor has been linked to the severity of RA. However, the mechanism by which PAR2 drives RA pathology is unclear and until recently, a suitable inhibitor has not been available. The aim of this study was to characterise the function of PAR2 in M1-like macrophages and investigate its possible inhibition with the recently reported PAR2 inhibitor, AZ8838.

THP-1 cells were differentiated with phorbol-12-myristate-13-acetate (PMA) to an M1-like macrophage phenotype. The cells were stimulated with several molecules (lipopolysaccharide (LPS) and the PAR2 activating peptides FLIGRLO-$NH_2$ and SLIGKV-$NH_2$) in the presence or absence of AZ8838. Culture supernatants were collected after 24 hours. ELISA was used to measure the cytokine profile, with changes in cell morphology also analysed. Cell viability was investigated using an MTT assay.

PMA differentiated macrophages released high amounts of pro-inflammatory cytokines related to RA (IL-6, IL-8 and TNFα). Moreover, the activation of PAR2 enhanced the inflammatory response as evidence by IL-6 secretion, suggesting that the inhibition of PAR2 in M1-like macrophages is a potential treatment for this disease. However, AZ8838 was not able to inhibit PAR2 activity at the concentrations studied. Therefore, different concentrations of AZ8838 and macrophage PAR2 activators should be tried in future investigations. Finally, neither AZ8388 or any of the stimulators used affect the viability of the cells.



## Key concepts

*PAR2; Rheumatoid Arthritis; Macrophages; PMA; Cytokines; Inflammation*

# Abbreviations

| | | | |
|---|---|---|---|
| **AIA** | Antigen-induced arthritis | **JAKs** | Janus Kinases |
| **ADA** | Anti-drug antibodies | **MHC-II** | Major histocompatibility complex class II |
| **CIA** | Collagen-induced arthritis | **MTT** | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| **DAS28** | Disease Activity Score 28 | **O.D.** | Optical density |
| **DMARDs** | Disease-modifying antirheumatic drugs | **OC** | Osteoclast |
| **DMSO** | Dimethyl sulfoxide | **PAR-AP** | PAR-activating peptides |
| **ELISA** | Enzyme-linked immunosorbent assay | **RA** | Rheumatoid arthritis |
| **ENMD-1068** | 6-Amino-1-[4-(3-methyl-1-oxybutyl)-1-piperazinyl]-1-hexanone hydrochloride | **RP** | Reverse peptide |
| **FBS** | Foetal bovine serum | **SLIGKV-$NH_2$** | SLIGKV-amide |
| **FLIGRLO-$NH_2$** | 2-Furoyl-LIGRLO-amide | **THP-1** | Human leukaemia-derived monocytic cell line |
| **GB88** | 5-isoxazoyl-Cha-Ile-spiro[indene-1,4-piperidine] | **TNFα** | Tumour necrosis factor alpha |
| **GPCR** | G-protein-coupled receptor | **TLR** | Toll-like receptor |
| **GM-CSF** | Granulocyte-macrophage colony-stimulating factor | **TMB** | 3,3',5,5'-Tetramethylbenzidine |
| **HLA** | Human leukocyte antigen | **PBMCs** | Peripheral blood mononuclear cells |
| **HRP** | Horseradish peroxidase | **PBS** | Phosphate-buffered saline |
| **IFNγ** | Interferon gamma | **PMA** | Phorbol-12-myristate-13-acetate |
| **IL-X** | Interleukin-X | **UK** | United Kingdom |

# Introduction

## Rheumatoid arthritis

Rheumatoid Arthritis (RA) is an autoimmune disease that causes chronic inflammation in the joints leading to progressive articular damage. Both cartilage and bone can be affected resulting in disability (Smolen *et al.*, 2016). According to the National Rheumatoid Arthritis Society, about 1% of the population has RA in the United Kingdom (UK), equating to more than 400,000 people. However, the population affected in Spain is around 0.5%, some 200,000 people (Coordinadora Nacional de Artritis, 2019).

Acute inflammation usually occurs as a response to an injury or infection and ends when damage is resolved. In some cases, this inflammation may be uncontrolled and becomes chronic, leading to the development of some diseases such as RA. In this particular disease, synovial tissue inflammation is a feature which is perpetuated by a number of pro-inflammatory mechanisms and regulatory pathways (Alivernini *et al.*, 2018).

Although the origin of the disease is still unknown, some risk factors may contribute to its development. Over 100 loci have been associated with RA, most of which implicate immune mechanisms and many of these loci are genes encoding the major histocompatibility complex class II (MHC-II), called human leukocyte antigen (HLA) in humans, especially the HLA-DRB1 gene on chromosome 6 (Bax *et al.*, 2011). Additionally, together with the genetic background, some environmental effects may help the development of the disease, including smoking, vitamin D deficiency, obesity or a perturbed gastrointestinal microbiome among others (McInnes and Schett, 2017).

### Synovial inflammation in rheumatoid arthritis

The main characteristic feature in RA is joint swelling reflecting synovial inflammation caused by an immune inflammatory response. Synovial fluid is found inside the synovial joints and has the main function of reducing the friction between articular cartilage during movement in order to avoid damage. However, the composition of this fluid is altered in some diseases such as RA. According to Smolen *et al.* (2016), the composition of the synovium in RA includes many innate and adaptive immune cells such as monocytes, dendritic cells, B cells or T cells. These cells modulate the expression of several inflammatory associated molecules including cytokines and chemokines.

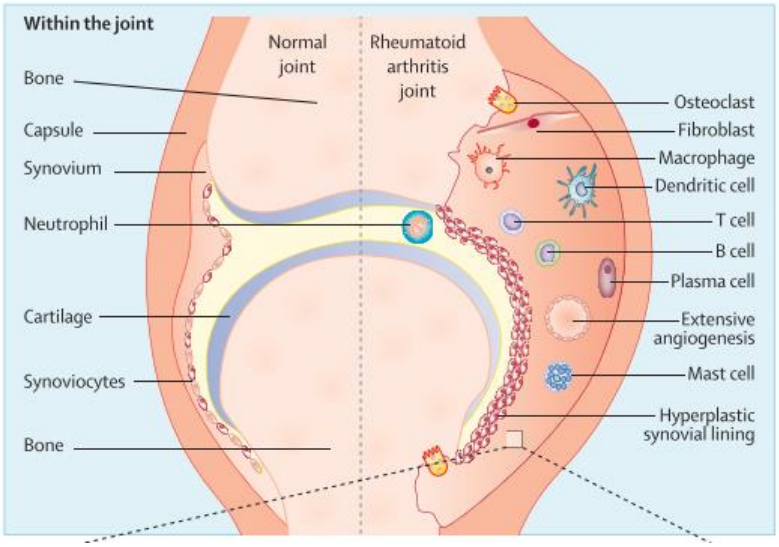


***Figure 1.*** Image representing a normal joint (healthy) and an inflamed joint characteristic of RA (Extracted from Smolen *et al*., 2016).

## Macrophages in rheumatoid arthritis

Macrophages play a key role in the initiation, development and resolving of an inflammatory response through the release of cytokines and enzymes involved in this process. Importantly, macrophages can polarize into two subsets with different physiological functions: namely M1 and M2 macrophages. On one hand, M1 macrophages induce inflammatory responses releasing reactive oxygen species (ROS) and high levels of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα), interleukin 6 (IL-6) and interleukin 12 (IL-12). On the other hand, M2 cells are characterised by secretion of anti-inflammatory cytokines like interleukin 10 (IL-10). Although both macrophages coexist, an imbalance between them is associated with some pathologies (Martinez and Gordon, 2014).

Macrophages are commonly found in the healthy joint, however, they become activated in the inflamed joint and regulate the secretion of pro-inflammatory cytokines driving joint destruction (Kennedy *et al*., 2011). Despite the fact that macrophages in RA have not been clearly classified in M1 or M2, they seem to be close to M1 functions. In fact, Milman *et al.* (2010) demonstrated that interferon gamma (IFNγ) levels were increased in 57 RA patients and correlated with disease severity. IFNγ is difficult to detect in RA tissues and fluid, however, it may be involved in macrophage differentiation in early RA

due to it has been reported to contribute in the polarization into M1 macrophages (Biswas and Mantovani, 2010). In addition, M1 macrophages release high amounts of TNFα, which has been associated with RA due to its potential to degrade cartilage and bones (Kennedy *et al*., 2011).

Another study demonstrating the importance of M1 macrophages in RA was performed by Fukui *et al.* (2018). In this case, peripheral blood mononuclear cells (PBMCs) were isolated from RA patients and healthy donors in order to investigate the relationship between M1/M2 subsets and osteoclastogenesis. Osteoclasts (OCs) are found in synovitis and have been shown to cause bone resorption[1], a hallmark of RA. The results demonstrated that RA patients with severe disease had higher M1/M2 ratios which may increase osteoclastogenesis.

### Cytokines related to rheumatoid arthritis

The synovial inflammation produced in RA patients is regulated by a network of inflammatory cytokines and chemokines, many of them released by macrophages. Moreover, many clinical studies have shown variants in the cytokine profile of an RA patient compared to a healthy patient. These small molecules lead to the induction or aggravation of the inflammation by activating endothelial cells and stimulating leucocyte infiltration. To date, TNFα and IL-6 have been demonstrated as being essential for disease, however, other cytokines have been shown to have an important role in RA in the last several years (McInnes *et al.*, 2015; Smolen *et al.*, 2016).

It has been known for over 20 years that TNFα has a pivotal role in RA pathophysiology time ago (Brennan *et al.*, 1992). This cytokine mediates leukocyte activation and migration, endothelial activation, chemokine expression and contributes to the activation of osteoclasts, among other pro-inflammatory effects (McInnes *et al.*, 2015).

Apart from TNFα implication, IL-6 and IL-8 have also been linked with the disease due to the detection of high levels in synovial fluid samples from RA patients (An *et al*., 2018). Ohshima *et al*. (1998) investigated the involvement of IL-6 in the pathology of RA comparing IL-$6^{-/-}$ mice and IL-$6^{+/+}$ after antigen-induced arthritis (AIA). Safranin O

[1] Bone resorption is the process in which osteoclasts break the bone and release minerals.

staining[2] showed well-preserved cartilage in IL-$6^{-/-}$ mice which was completely destroyed in wild-type, suggesting a pivotal role for this cytokine in RA. Moreover, IL-8 is a pro-inflammatory cytokine that activates neutrophils promoting its degranulation and releasing superoxide and lysosomal enzymes associated with several inflammatory diseases (An *et al.*, 2018).

Other cytokines have been shown to be involved in RA pathogenesis. These include granulocyte-macrophage colony-stimulating factor (GM-CSF) and several members of interleukin 1 (IL-1) family including IL-1α, IL-1β and interleukin 18 (IL-18). Focusing in the adaptive immune response, interleukin-17A (IL-17A) promotes the release of pro-inflammatory cytokines (including TNFα, IL-6, IL-1 and GM-CSF), as well as activates other inflammatory mechanisms, suggesting also playing a role in the disease (McInnes *et al.*, 2015).

### Treatment for rheumatoid arthritis

As evidenced from current literature (discussed above), inflammation is the main cause of the clinical events that are present in RA. For this reason, it is not illogical to think that most treatments are aimed at its reversal (Smolen *et al.*, 2016). Nowadays, treatments for RA, as well as other diseases considered autoimmune, target inflammatory mediators or its signalling pathways. These therapies help to retard the progression and to reduce the symptoms but not to cure the disease (Alivernini *et al.*, 2018).

Glucocorticoids are widely used and offer a fast response reducing the inflammation but are associated with long-term side-effects. On the other hand, there is a group of drugs called disease-modifying antirheumatic drugs (DMARDs), which also target the inflammation and preserve the joints. Two major classes of DMARDs exist: conventional synthetic DMARDs and biological DMARDs. Some of the most commonly used synthetic DMARDS are methotrexate and sulfasalazine that are usually taken in combination with glucocorticoids. Biological DMARDs are usually reserved for severe cases when other medications have not produced good results. Many of the biological therapies are monoclonal antibodies which block cytokine receptors or interfere in several signal

[2] Safranin O is a biological stain usually used to detect cartilage.

pathways to avoid the pro-inflammatory response of these processes (Smolen *et al.*, 2016).

According to Smolen *et al.* (2016), several modes of action of these biological therapies are currently approved for the treatment of RA such as TNF inhibition, IL-6 receptor inhibition, T-cell co-stimulation blockade or B-cell depletion.

Anti-TNF therapy is the most studied due to the pivotal role of TNFα in the disease, however, although this drug promises high efficacy, the downside is occurrence of adverse events in some patients (Singh *et al.*, 2014). Several anti-TNF therapies have been developed such as infliximab, etanercept or adalimumab. A clinical study performed by Santos-Moreno *et al.* (2016) investigated the response at 36 months in a cohort of patients with RA treated with these three anti-TNF therapies. The study revealed that these treatments reduce the activity and disability caused by the disease due to the Disease Activity Score 28 (DAS28)[3] was reduced in all treatments.

This reduction in the activity of the diseases is because anti-TNF therapy produces changes in cellular and phenotype composition, stromal cell activation, cytokine and chemokine expression, and activation of regulatory pathways (e.g. IL-10), among others. Similar effects have been observed in IL-6 receptor inhibition studies. Moreover, IL-6 is also acting as a key molecule driving the T cell differentiation into Th17 cells, which have been suggested to be mediate several organ-related autoimmune diseases (McInnes *et al*., 2015; Sonderegger *et al.*, 2008). Tocilizumab is a humanized anti-IL-6 antibody which has been indicated for the treatment of moderately to severe RA (Drugbank, 2019).

New therapies targeting other cytokine pathways are being developed such as mavrilimumab which binds to the GM-CSF receptor α. However, at the moment, there are not agents targeting this cytokine which have been approved for clinical use. Janus Kinases (JAKs) inhibitors such as tofacitinib have been created due to several cytokine receptors act via these proteins. Also, modulation of T-cell activation has been studied (abatacept). Another therapies that have been successful in clinical studies is the IL-17A inhibition (McInnes and Schett, 2017).

[3] DAS28 is an index which considers 28 joints in order to calculate the activity of RA.

**Table 1.** Treatments for RA (Drugbank, 2019; Smolen *et al.*, 2016).

| | | |
|---|---|---|
| **Conventional synthetic DMARDs** | | |
| **Methotrexate** | Small chemical | Block synthesis of pyrimidines and purines |
| **Sulfasalazine** | Small chemical | Immunomodulatory effect |
| **Biological DMARDs** | | |
| **Adalimumab** | Human monoclonal antibody | TNFα inhibitor |
| **Infliximab** | Chimeric monoclonal antibody (human and murine) | TNFα inhibitor |
| **Etanercept** | Dimeric fusion protein | TNFα inhibitor |
| **Tocilizumab** | Humanized monoclonal antibody | IL-6 inhibitor |
| **Mavrilimumab** | Human monoclonal antibody | GM-CSF inhibitor |
| **Abatacept** | Soluble fusion protein | T-cell activation inhibitor |
| **Targeted synthetic DMARDs** | | |
| **Tofacitinib** | Small chemical | JAK inhibitor |

Even though the biological therapies have been a breakthrough in the treatment of autoimmune diseases such as RA, not all the patients respond to these drugs in the same way. It must be considered that RA may present different inflammatory signalling pathways in each patient and the proper treatment for a patient may not be right in another. This highlights the importance to develop new therapies with alternative mechanisms of action (Alivernini *et al.*, 2018). Moreover, in some cases, a lack of treatment efficacy occurs due to the patient develop anti-drug antibodies (ADA). For example, Quistrebert *et al.* (2018) reported in their study that 20-30% of patients treated with adalimumab or infliximab developed ADA in less than 18 months. Finally, it is important to take into account the side-effects of these treatments such as the high risk of infection (Santos-Moreno *et al.,* 2016).

## Protease-activated receptor 2

Protease-activated receptors (PARs) are a family of G-protein-coupled receptors (GPCRs) mainly activated through proteolytic cleavage in the N-terminus. This cleavage unmasks a tethered ligand that binds to the external domain of the receptor in order to activate it (Cheng *et al.*, 2017). In the case of PAR2, this receptor can be activated by serine proteases such as trypsin, releasing the ligand SLIGKV in humans and SLIGRL in mice. However, synthetic peptides called PAR-activating peptides (PAR-AP) which mimic

or are similar to the natural ligand sequence can bind to the extracellular domain activating the receptor without cleavage (Maruyama *et al.*, 2017).

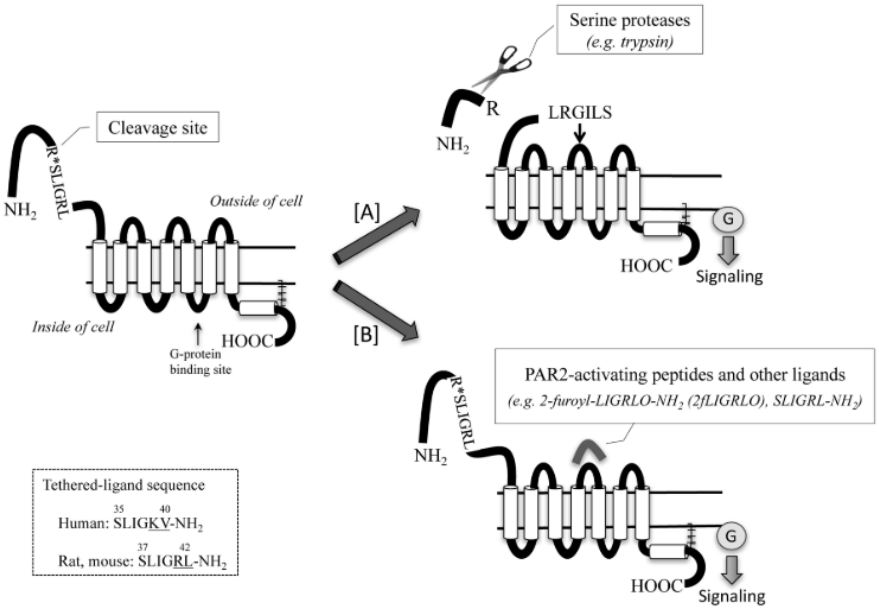


***Figure 2.*** PAR2 activation. [A] shows the activation by proteolytic cleavage (trypsin) while [B] represents the activation by PAR-AP (Extracted from Maruyama *et al.*, 2017).

PARs are expressed in macrophages and have been associated with a wide range of diseases such as cancer and inflammatory conditions. According to Ramachandran *et al.* (2012), there is evidence indicating that these proteins are involved in inflammatory diseases. In addition, joint pathology has been seen to be abrogated in $PAR2^{-/-}$ mice (Crilly *et al.*, 2012). Additionally, Steven *et al.* (2013) reported that PAR2 activation by PAR2-AP SLIGKV increases cell surface expression of this receptor in macrophages. Moreover, this activation enhanced the pro-inflammatory features of M1 macrophages, increasing TNFα secretion and reducing IL-10. Therefore, data suggest that targeting PAR2 is a potential therapeutic strategy for inflammatory diseases.

Additionally, crosstalk between PAR2 and toll-like receptor 4 (TLR4)[4] has been reported before. For example, Bucci *et al.* (2012) showed that lipopolysaccharide (LPS) injection, which is a TLR4 agonist, increases PAR2 expression in vascular tissue. Furthermore, TLR4

[4] TLRs are a class of receptors that recognize pathogens and initiate an immune response. They are expressen in macrophages.

inhibition reduces PAR2-AP-induced vasorelaxation and PAR2 showed a reduced expression in TLR4$^{-/-}$ compared to wild-type mice. Other studies, such as Rallabhandi *et al.* (2008) reported that TLR4 and PAR2 have a synergistic response, enhancing the inflammatory immune response.

In the present study, several molecules have been used in order to activate macrophages. LPS has been used as a well-known activator of macrophages. On the other hand, a synthetic ligand mimicking the original ligand sequence has been used to activate PAR2 specifically (SLIGKV-$NH_2$). Finally, 2-Furoyl-LIGRLO-amide (FLIGRLO-$NH_2$) is another potent selective agonist of the PAR2 receptor which consists of a modified natural ligand sequence (McGuire *et al.*, 2004).

### PAR2 in rheumatoid arthritis

As it has been previously explained, RA is mainly characterised for synovial inflammation. The synovial microenvironment contains many inflammatory cells secreting proteinases that can act as PAR2 activators such as mast cell tryptase, trypsin or neutrophil proteinase 3. These founds suggests that this receptor may have a role in regulating immune responses and, consequently, in RA (Steven *et al.*, 2013).

In fact, Ferrell *et al.* (2003) have already demonstrated that PAR2 plays a pivotal role in chronic inflammation. PAR2 overexpression was observed in synovium and peripheral tissues 2 weeks after induction of inflammation in mice. In addition, joint swelling and hyperemia were inhibited in PAR2$^{-/-}$ monoarthritis mice model of chronic inflammation. These results correlate with another study developed by Crilly *et al.* (2012), using Collagen-Induced Arthritis (CIA) murine model. In this case, PAR2$^{-/-}$ mice developed arthritis with the same incidence as wild-type animals but with less severity. In the same previous study, cytokines and chemokines analysis of lymph node cell suspensions incubated with collagen showed a lower concentration of inflammatory cytokines namely interferon-gamma (IFNγ) and IL-17 in PAR2$^{-/-}$ mice. Additionally, PAR2$^{-/-}$ mice showed reduced levels of IL-1β and IL-6, important interleukins in Th17 polarisation. Furthermore, IL-12 and TNFα were also reduced (Crilly *et al.*, 2012). These data suggest a key role of PAR2 in the inflammation produced in RA.

## PAR2 inhibition

With the current knowledge, PARs inhibition suggests a potential new therapy for some diseases. In fact, the first marketed drug targeting a PAR receptor has already been developed and is called vorapaxar, a PAR1 antagonist used to prevent thrombosis (Cheng *et al.,* 2017). Nevertheless, it seems that the discovery of PAR2 antagonist is being more difficult.

Until today, some molecules have been studied as PAR2 antagonists, but they have shown weak results. Some of the antagonists studied are ENMD-1068 (6-Amino-1-[4-(3-methyl-1-oxybutyl)-1-piperazinyl]-1-hexanone hydrochloride) and GB88 (5-isoxazoyl-Cha-Ile-spiro[indene-1,4-piperidine]). According to Lohman *et al.* (2012), large doses of ENMD-1068 are needed in order to observe an anti-inflammatory activity of this treatment *in vivo*. On the other hand, orally GB88 ameliorated the signs associated with CIA in rats, reducing mast cell degranulation and macrophage accumulation in diseased joints. Despite the promising results, it seems that GB88 is still a weak PAR2 antagonist (Cheng *et al.,* 2017).

Cheng *et al.* (2017) proposed in *Nature* journal two novel antagonists of PAR2, among them, AZ8838, which is the antagonist used in the present project. This molecule binds in an occluded pocket near the extracellular surface of the receptor. Its binding may prevent structural rearrangements required to activate the receptor and its signalling pathways.

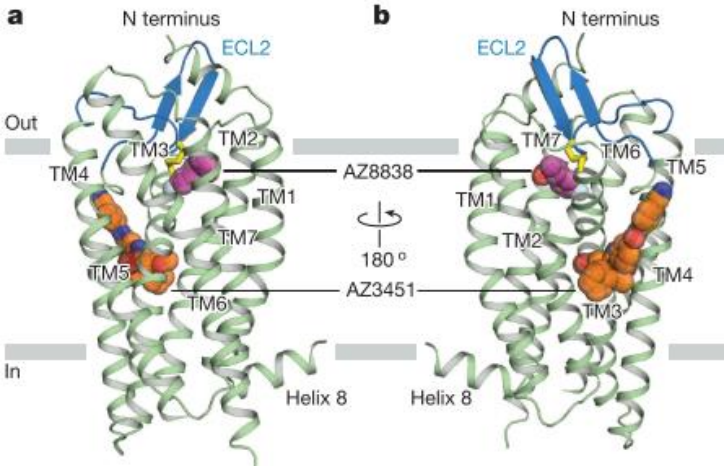


***Figure 3.*** Structure of PAR2 in cartoon representation with the antagonist AZ8838 in magenta (Extracted from Cheng *et al.,* 2017).

## Cell line and reagents used

In this study, the role of PAR2 has been studied in macrophages and, for that, the human leukaemia-derived monocytic cell line (THP-1) has been used. This cell line is a common model for *in vitro* studies investigating primary human macrophages due to the experimental limitations involved in the use of tissue macrophages. For example, primary tissue macrophages cannot be expanded *ex vivo* and have a limited life span in cell culture. Furthermore, the process to obtain them is quite invasive by donors and this causes a limited cell number (Daigneault *et al.*, 2010; Lund *et al.*, 2016).

Additionally, phorbol-12-myristate-13-acetate (PMA) has been the stimulus used in order to differentiate monocytes into macrophages. According to Lund *et al.* (2016), THP-1 differentiated with PMA acquire a phenotype that mimics the primary human macrophages in many aspects. Once the cells have been differentiated, the exposure to pro-inflammatory stimuli such as LPS results in the macrophages acquiring typical features of the M1 phenotype. Therefore, cells increase their adherence and secretion of pro-inflammatory mediators such as TNFα or superoxide (Lund *et al.,* 2016).

Nevertheless, although THP-1 cell differentiation using PMA is widely used, there is no specific protocol for the procedure. As a matter of fact, macrophages can show small differences depending on the concentration of PMA used and the period of rest after the PMA exposition (Lund *et al.,* 2016).

# Hypothesis and objectives

PAR2 has been reported having an important role in the development of inflammation. Our hypothesis is based on the fact that PAR2 is overexpressed in RA and may contribute to the release of pro-inflammatory cytokines by M1 macrophages. Therefore, the aim of the study is to determine the role of this protein in M1-like macrophages and its possible inhibition with AZ8838 as a treatment for the disease. Cell line THP-1 will be differentiated with PMA in order to obtain M1-like macrophages and the effect of the PAR2 inhibitor, AZ8838, determined.

Secondary objectives for the study are presented below:

- Study the cellular growth of THP-1 cells.
- Determine the morphological characteristics of THP-1 cells in response to PMA.
- Determine the best concentration of PMA to differentiate the THP-1 cells.
- Determine the best concentration of LPS to stimulate the THP-1 cells.
- Determine the cytokine profile released by M1-like macrophages.
- Determine the effects of the PAR2 activation using different molecules: LPS, FLIGRLO-$NH_2$ and SLIGKV-$NH_2$.
- Study the cellular viability in response to different PAR2 activators and the inhibition of PAR2.

# Materials

## Reagents

- THP-1 cell line
- RPMI 1640 Medium + GlutaMAX™
- Fetal calf serum (FCS)
- Penicillin-streptomycin
- Trypan blue
- Phorbol 12-myristate 13-acetate (PMA)
- Lipopolysaccharide (LPS)
- TNFα ELISA human kit (eBioscience)
- IL-6 ELISA human kit (Invitrogen)
- IL-8 ELISA human kit (Invitrogen)
- Phosphate buffered saline (PBS)
- Tween 20
- MTT assay kit (Thermo Fisher)
- Dimethyl sulfoxide (DMSO)
- Ethanol 70%

## Materials

- Flasks (several volumes)
- Eppendorf
- Vials (several volumes)
- Falcon tubes (several volumes)
- Tips (several volumes)
- Pipettes (several volumes)
- Hemocytometer (Neubauer chamber)
- 96-well plates for cell culture
- 6-well plates for cell culture
- 96-well ELISA plates (Immulon 4)

## Machines

- Microscope
- Centrifuge
- Incubator
- Biosafety hood
- Plate reader (BioTek)
- Freezer -20ºC
- Fridge 4ºC

## Informatic software

- BioTek Gen5
- Microsoft Office Excel
- GraphPad InStat 3
- GraphPad Prism 8
- ImageJ

# Methodology

## Summary of procedures

In order to be able to perform this project, the THP-1 cell line has been used. After a time of acclimation and cell growth, the cells have been stimulated with PMA. Once differentiated, the cells have been activated and ELISA assay has been used to measure the cytokine concentration. This study has been done in the absence of a PAR2 inhibitor to determine the role of PAR2 in PMA-induced macrophages and the better activation conditions and in the presence of AZ8838 to investigate its effect in the cytokine profile released. MTT assay has also been performed to study the cell viability.

Therefore, the project can be split into two parts. A summary of the procedures that have been performed in order to generate the growth curve and determine the cytokine profile in response to several PMA concentrations is shown in Figure 4. On the other hand, Figure 5 presents a summary of the investigation using the PAR2 inhibitor (AZ8838).

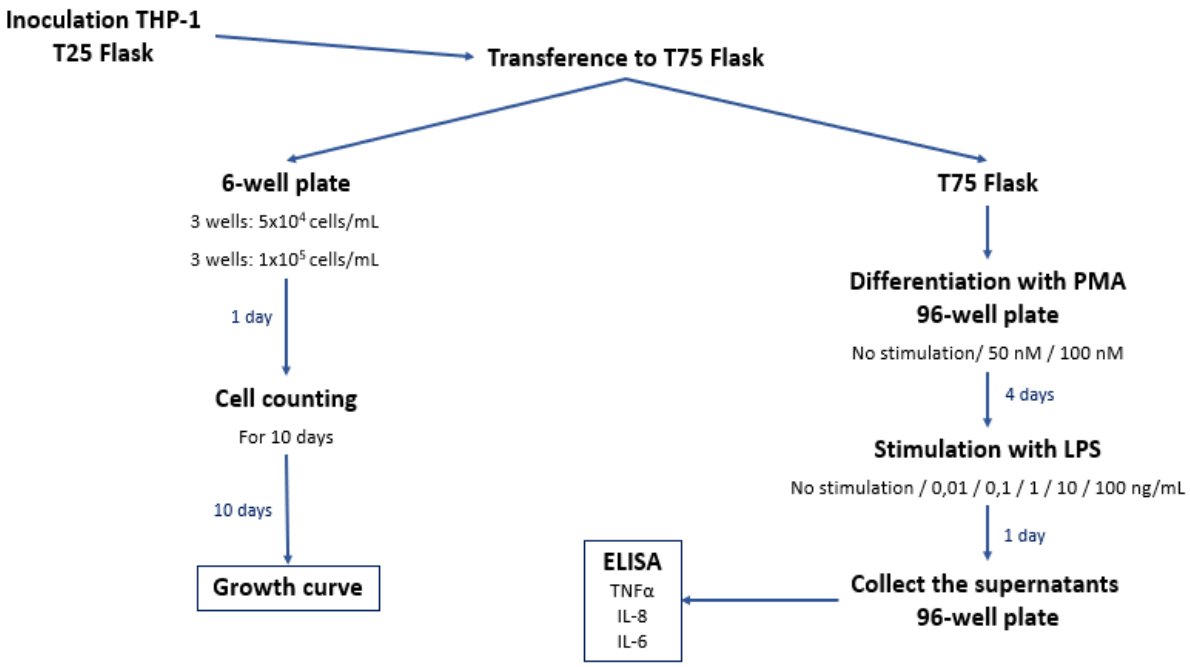


***Figure 4.*** Diagram of the processes performed in THP-1 cell line without PAR2 inhibitor.

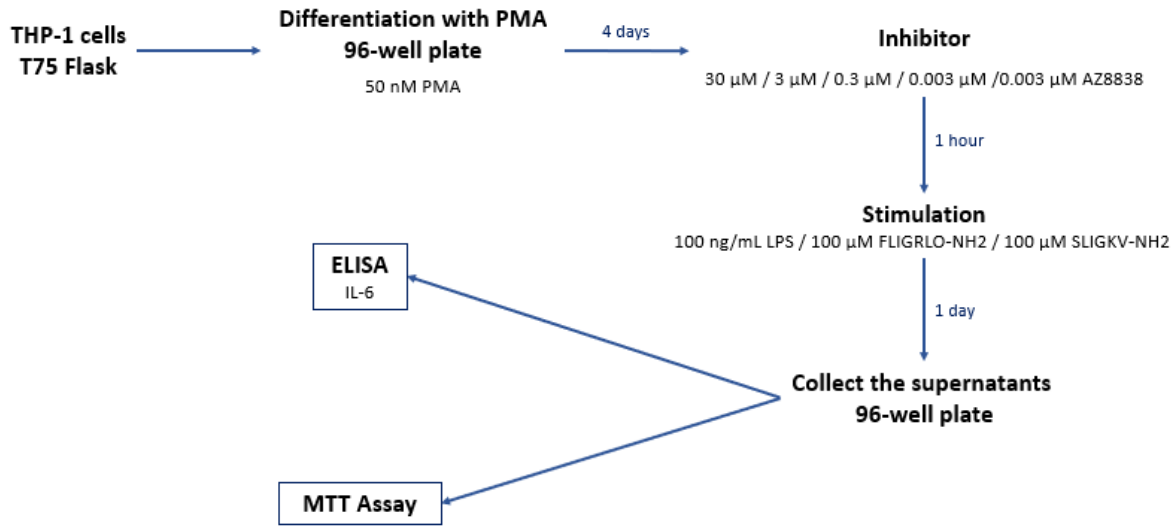


***Figure 5.*** Diagram of the processes performed in THP-1 cell line to study the effect of AZ8838.

## Cell culture

In order to carry out the study, THP-1[5] cell line was used. For cell culture, the proper procedures of cell cultures were followed to avoid contamination and create an adequate environment for cell growth and development. Therefore, the steps were performed in a class II hood which is a laminar vertical airflow hood that provides the aseptic environment necessary for cell culture experiments. The hood was routinely cleaned with 70% ethanol before and after each use, along with all equipment being used. Ultraviolet light was also used to eliminate all the possible contamination in an efficient way.

THP-1 cells were initially cultured in 10 mL of complete medium. The medium used was RPMI 1640 Medium/Glutamax supplemented with streptomycin and penicillin (1%) to prevent bacterial contamination. Before use, foetal calf serum (FCS) was also added giving a final concentration of 10%. RPMI 1640 was originally developed to culture human leukaemic cells, furthermore, GlutaMAX™ is an L-alanine-L-glutamine dipeptide that substitutes L-glutamine, an essential nutrient for cell growth (Thermo Fisher, 2019).

The cells were cultured using a T25 flask at 37º C and 5% $CO_2$ for 3 days. Approximately 72 hours after incubation, the cells were transferred into a bigger flask (T75 flask) with

[5] THP-1 cells were given by Ms Lynette Dunning from the University of the West of Scotland, Paisley.

20 mL of fresh medium. THP-1 cells are suspension cells and, therefore, the flask content had to be centrifuged at 1200 rpm for 6 minutes and were then then resuspended with fresh medium before being transferred to a fresh new flask.

The cells were routinely cultured at 37º C and 5% $CO_2$ until the end of the experiment using T75 flasks and 20-25 mL of supplemented medium. Cells were checked almost every day and the medium replaced with fresh medium two times a week.

## Cell counting

After seven days of culturing, THP-1 cells were recollected, and 5 mL were placed in each well of a 6-well plate (Plate 1, Figure 6). Two different concentrations were applied: 3 wells for the highest concentration (1x$10^5$ cells/mL) and other 3 wells for the lowest concentration (5x$10^4$ cells/mL).

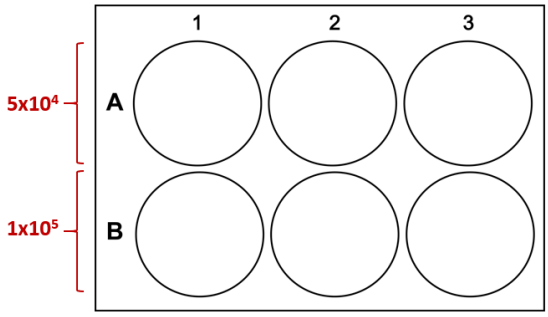


***Figure 6.*** Plate 1. 6-well plate diagram with the two different concentrations used (5x$10^5$ and 1x$10^5$ cells/mL).

Plate 1 was used to check the THP-1 cell growth. The cells were counted over a period of 10 days: day 1, 2, 3, 4, 7, 8, 9 and 10. In order to be able to do this counting, the cells were previously diluted with trypan blue reagent and placed into the Neubauer chamber. Trypan blue is a dye exclusion method in which dead cells are permeable to this dye while living cells are not, this fact allows assessing the cell viability. Depending on the day and the number of cells, different dilutions were used to perform the cell counting properly (1:2 or 1:3).

The microscope was used to count the cells with the 10x objective. Four squares of 1 $mm^2$ were counted to do the average of the cell number. The total volume of each one is 0.1 µL and, therefore, the number of cells must be multiplied per 10,000 to get the cells for each mL. Moreover, it should be considered if previous dilutions of the sample

have been made before to calculate the initial cell concentration. The formula used for this calculation is shown below:

$$\frac{Cells1+Cells2+Cells3+Cells4}{4} \; x\, 10^{4} \; x \; (dilution\ factor) = Concentration\ (cells/mL)$$

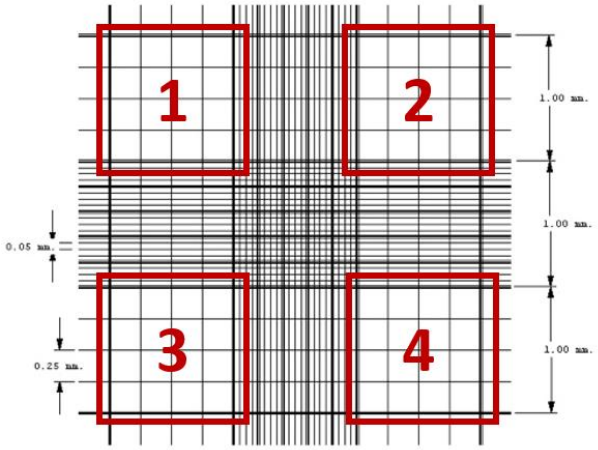


***Figure 7.*** Diagram of the hemocytometer (Neubauer chamber). The 1, 2, 3 and 4 squares were counted to get the number of cells.

## PMA differentiation and LPS stimulation without inhibitor

In order to perform this part of the experiment, a 96-well plate (Plate 2, Figure 8) was used with a final cell concentration of $5x10^5$ cells/mL in each well. Two different PMA concentrations were performed: 50 and 100 nM. Moreover, some cells were not stimulated to be used as a control.

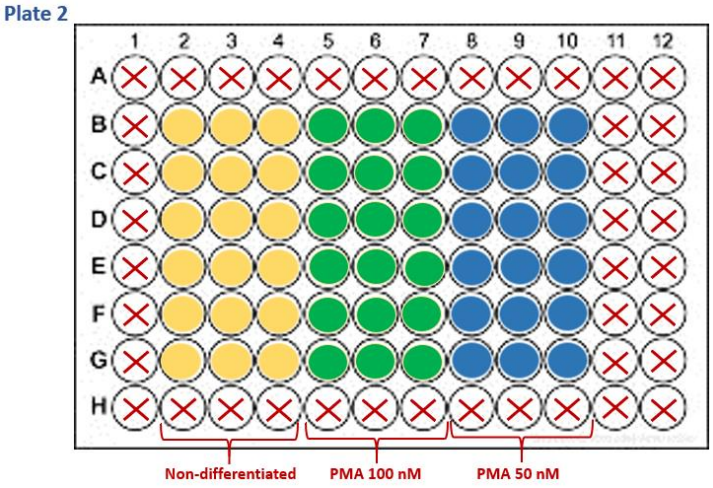


***Figure 8***. Plate 2. Diagram of the 96-well plate used for the differentiation of the THP-1 cells with three different PMA concentrations (0, 50 and 100 nM).

Plate 2 was incubated for 3 days at 37º C and 5% CO2. After 72 hours of incubation, the PMA was removed and replaced by fresh medium to allow the cells to rest. The cells were incubated for 24 hours before the LPS stimulation.

Plate 2 was used for the stimulation with LPS. Several concentrations were tested: 100, 10, 1, 0.1, 0.01 ng/mL and non-stimulated cells. In order to get these concentrations, a 200 ng/mL solution was prepared in complete medium using the LPS stock and serial dilutions subsequently were performed. The old medium was exchanged with fresh medium containing LPS. Moreover, a cell row was left without LPS stimulation as a control. LPS concentrations were added as is shown in the following diagram (Figure 9).

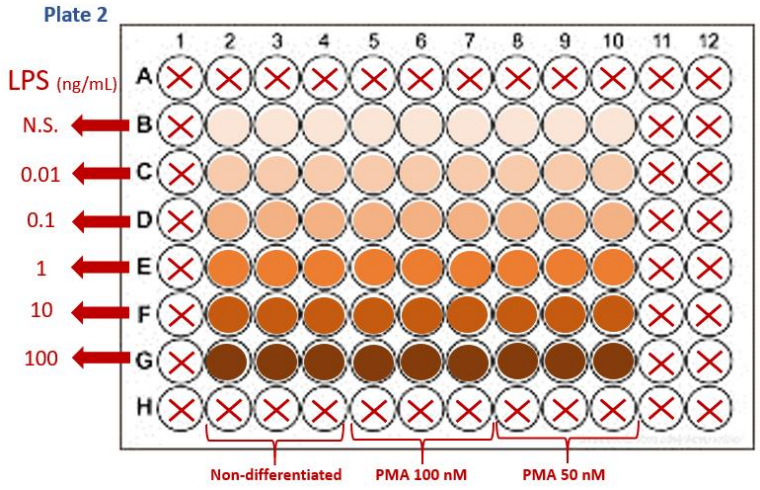


***Figure 9.*** Plate 2. Diagram of the plate used for the stimulation, which is carried out on the same plate in which the cell differentiation was performed. A different concentration of LPS is added in each row.

After 24 hours of incubation with LPS, the supernatants were collected and placed in another plate to freeze at -20 ºC until they could be used for posterior studies.

## PMA differentiation and LPS stimulation with the inhibitor

After the results obtained above, the ideal concentration of PMA and LPS were selected to carry out the study with several PAR2 activators and the PAR2 inhibitor tested. Therefore, 50 nM of PMA and 100 ng/mL of LPS were used in this case.

First, cells were differentiated with PMA 50 nM for three days long. After this time, the PMA was replaced with fresh medium and the cells were incubated overnight.

AZ8838[6] (100 mM stock prepared in DMSO) was used as an inhibitor of PAR2 and tested over the following concentrations: 30 µM, 3 µM, 0.3 µM, 0.03 µM and 0.003 µM. In order to be able to obtain these concentrations, the diluent used was RPMI/10% FCS with dimethyl sulfoxide (6µL of DMSO in 10 mL of medium).

The cells were incubated at 37ºC and 5% $CO_2$ for one hour with the inhibitor. After this time, the cells were stimulated with different molecules: LPS (100 ng/mL), FLIGRLO-$NH_2$ (100 µM) and SLIGKV-$NH_2$ (100 µM). In addition, one group of cells was not stimulated, and different controls were tested as it is shown in Table 2. Diagrams of the plates that were used in this part of the study are shown in Figure 10 and 11.

**Table 2.** Controls used in the study of the THP-1 cells with a PAR2 inhibitor.

| | |
|---|---|
| **DMSO + MEDIUM** | DMSO medium alone |
| | DMSO medium + LPS |
| | DMSO medium + FLIGRL |
| | DMSO medium + FLIGRL RP |
| | DMSO medium + SLIGKV-$NH_2$ |
| | DMSO medium + SLIGKV-$NH_2$ RP |
| **NORMAL MEDIUM** | NORMAL MEDIUM ALONE |
| | NORMAL MEDIUM + LPS |
| | NORMAL MEDIUM + FLIGRL |
| | NORMAL MEDIUM + FLIGRL RP |

*RP = reverse peptide

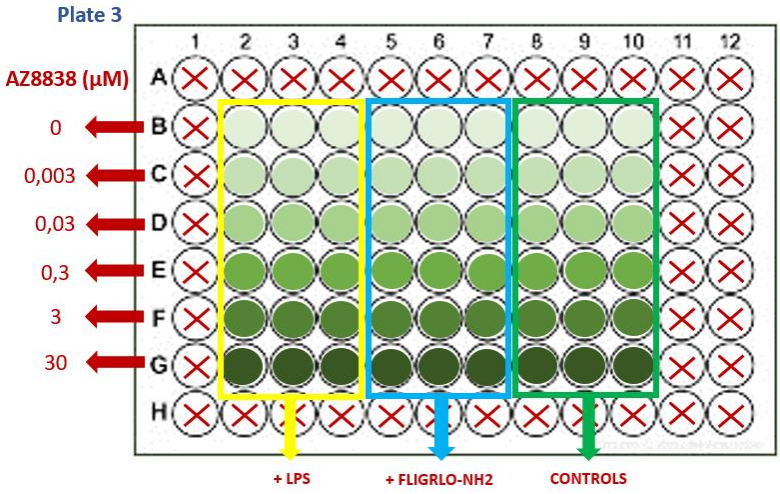


***Figure 10.*** Plate 3. Diagram of a plate used for the study in response to PAR2 inhibition. Different concentrations of AZ8838 were used. There are three different groups of cells: LPS stimulation, FLIGRL stimulation and controls.

[6] AZ8838 was given by Professor Robin Plevin from the University of Strathclyde, Glasgow.

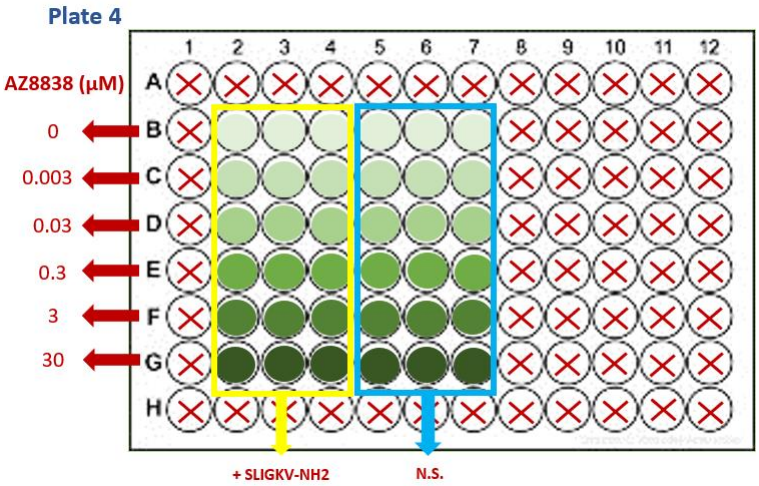


***Figure 11.*** Plate 4. Diagram of a plate used for the study in response to PAR2 inhibition. Different concentrations of AZ8838 were in each row of cells. One group of cells was stimulated with SLIGKV-$NH_2$ and the other cells were left without any stimulation.

After one day of incubation (24 hours), the supernatants were collected stored at -20ºC until they could be used for ELISA. Remaining attached cells were used to carry out an MTT assay.

## Determination of morphological characteristics

In order to study the changes in cell morphology, a microscope was used. Images were collected using a camera attached to the microscope and modified with ImageJ.

## Detection of cytokines and quantification

The detection and quantification of cytokines were done thanks to ELISA (Enzyme-linked immunosorbent assay). This technique detects the presence of a ligand (cytokines in this case) using two different antibodies. Three different pro-inflammatory cytokines were measured in the THP-1 supernatants without inhibition: IL-6, IL-8 and TNFα. On the other hand, only IL-6 was measured in the supernatants after PAR2 inhibition.

In order to perform this technique, several commercial human kits were used: Human IL-6 ELISA Kit, Human IL-8 ELISA Kit and Human TNFα ELISA Kit. Although all the kits work in the same way, they are using different capture antibodies which are specific for each cytokine.

First, ELISA plates were coated with the capture antibody (1st antibody) diluted in Coating Buffer overnight (4ºC). The day after, plates were blocked with ELISA diluent and incubated at room temperature for 1 hour.

In order to quantify the cytokines, a Standard Curve, also known as a Calibration Curve, was done for each cytokine. Serial dilutions were done from the stock reagent to create a Standard Curve with 8 different concentrations (in duplicate). The comprising range and the concentrations may vary between cytokines and are shown in the next table:

**Table 3.** The concentration of each well comprised the Standard Curve.

| | IL-6 (pg/mL) | IL-8 (pg/mL) | TNFα (pg/mL) |
|---|---|---|---|
| 1 | 0 | 0 | 0 |
| 2 | 3.13 | 3.9 | 7.82 |
| 3 | 6.25 | 7.82 | 15.63 |
| 4 | 12.5 | 15.63 | 31.25 |
| 5 | 25 | 31.25 | 62.5 |
| 6 | 50 | 62.5 | 125 |
| 7 | 100 | 125 | 250 |
| 8 | 200 | 250 | 500 |

To continue the experiment, in the next step, the solution with the antigen (sample or standard) was added which binds to the first antibody. The supernatants had been diluted with the dilution buffer to be placed inside the standard curve range (1:2 or 1:4). In this step, two columns were used for the Standard Curve and some wells for the blank (Figure 12, 13 and 14).

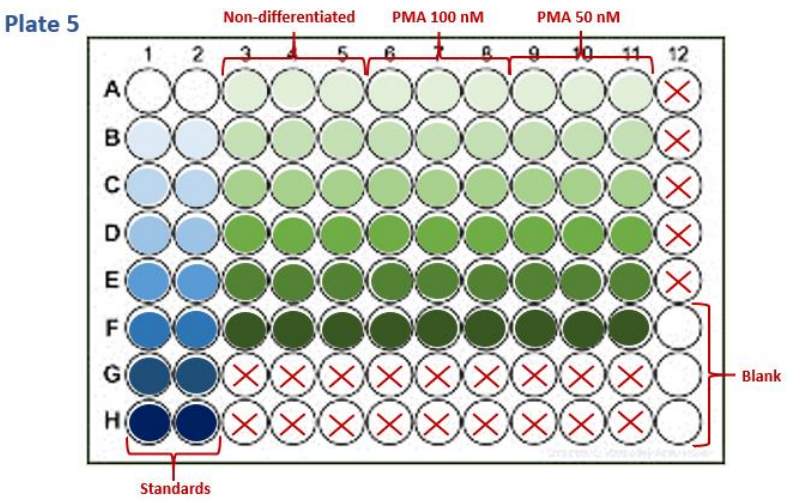


***Figure 12***. Plate 5 (supernatants from Plate 2). Diagram of the disposition of the plate used to determine the cytokines without PAR2 inhibition.

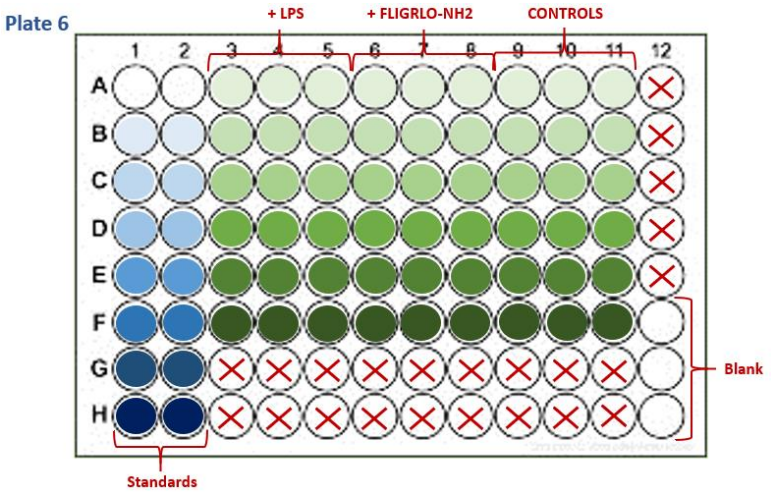


***Figure 13.*** Plate 6 (supernatants from plate 3). Diagram of the disposition of a plate used to determine the cytokines in the presence of a PAR2 inhibitor.

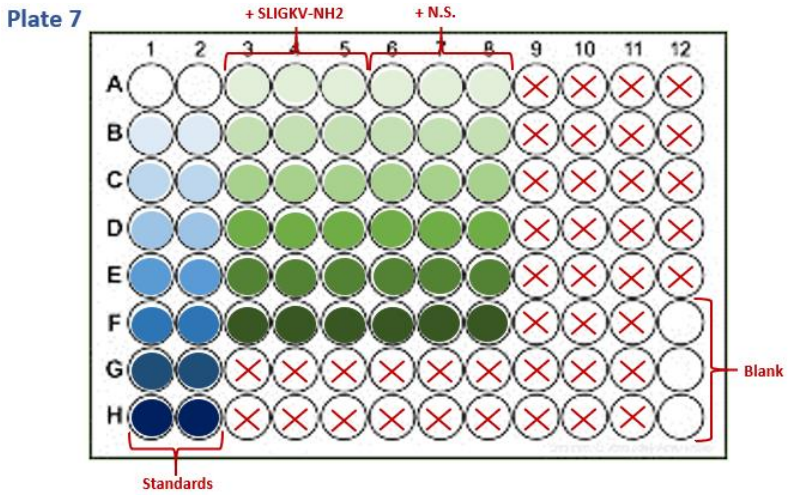


***Figure 14.*** Plate 7 (supernatants from plate 4). Diagram of the disposition of a plate used to determine the cytokines in the presence of the PAR2 inhibitor.

After 2 hours of incubation at room temperature, the detection antibody was added (2º antibody) to all the wells and the plate was incubated for 1 hour. The secondary antibody binds to the antigen and, as it is a biotin-conjugated antibody, can be detected with the Streptavidin-HRP enzyme (Streptavidin-Horseradish peroxidase enzyme). After the enzyme addition, a last incubation period of 30 minutes was needed.

TMB solution (3,3',5,5'-Tetramethylbenzidine) was used as Streptavidin-HRP substrate which generates a blue colour that can be read at 570 nm with spectrophotometry. The last step consisted of addition of Stop Solution (an acid solution as $H_2SO_4$). Therefore, the blue colour changed to yellow which was read at 450 nm in a plate reader. Finally, all the plates were read at the two wavelengths and the software (BioTek Gen5) subtracts the values of 570 nm from those of 450 nm to give the concentration of the cytokine in each well.

During the ELISA protocol, the plates were washed at least 3 times between each step with ELISA Wash Buffer which contained phosphate-buffered saline (PBS) and Tween 20 at 0.01%.

## Cell viability

MTT assay was performed in order to measure the viability of the cells. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is converted into formazan only for the viable cells and this allows to study the cellular viability. This assay was performed in the remaining cells of the plate after the supernatants were collected. Cell viability was only studied in the plate used to carry out the study in front of the inhibitors.

In order to perform this technique, 10 mg of MTT were diluted in 2 mL of PBS (5 mg/ml solution) and 20 µL added to each well which contained 200 µL of fresh medium. The plates were incubated at 37ºC and 5% CO2 for 3.5 – 4 hours. After this incubation, medium was removed leaving behind the blue crystals. DMSO (100 ml per well) was then used to dilute the crystals. Once the crystals had dissolved, the plate was read on the BioTek plate reader using 595 nm wavelength and optical density (O.D.) values collected.

## Graphics and statistics

Graphics were generated using GraphPad Prism 8. All graphics show the standard deviation directly generated with the same software.

Statistical analysis was done using Excel (Microsoft Office) and GraphPad InStat 3. Excel was used in order to perform unpaired T-test analysis to get the p-value in front of two different groups of samples. On the other hand, GraphPad InStat 3 was used for one-way ANOVA (Analysis of variance) and post hoc test (Bonferroni Procedure) to find significant differences between more than two groups of samples.

During the whole project, it was considered that the samples followed a normal distribution. There were significant differences when the p-value was under 0.05. In addition, a p-value under 0.01 was considered as very significant and under 0.001 very highly significant.

# Results

## Cell growth

Plate 1 was used to study cell growth with two different concentrations: $5x10^4$ and $1x10^5$ (cells/mL). Images taken from both concentrations after 48 hours of the plate inoculation are shown below:

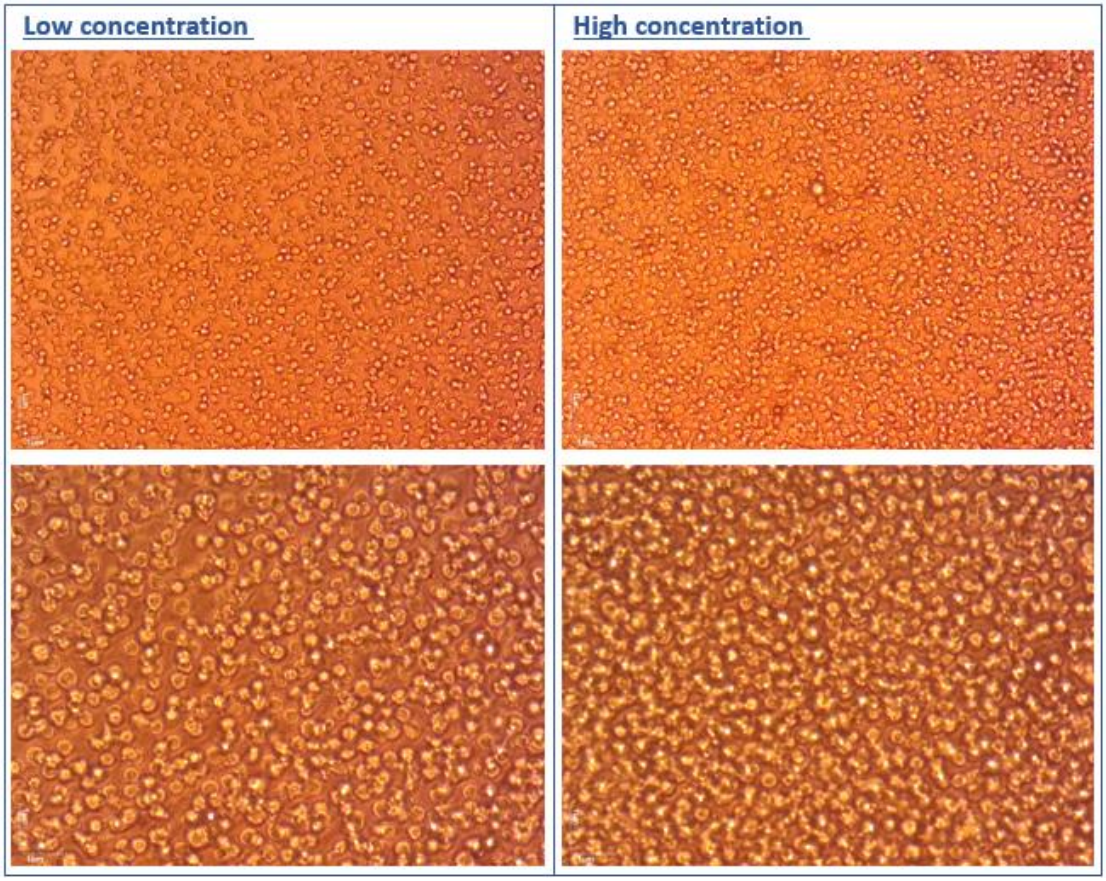


***Figure 15.*** Images of the THP-1 cells after 48 hours of culture. The left panels show the cells of the lowest initial concentration ($5x10^4$) while the panels on the right the highest initial concentration ($1x10^5$). 10x microscope objective was used in the upper panels and 20x was used in the ones below.

The cell counting for 10 days was used to generate the growth curve that is shown in Figure 16. The graphic only includes the viable cells.

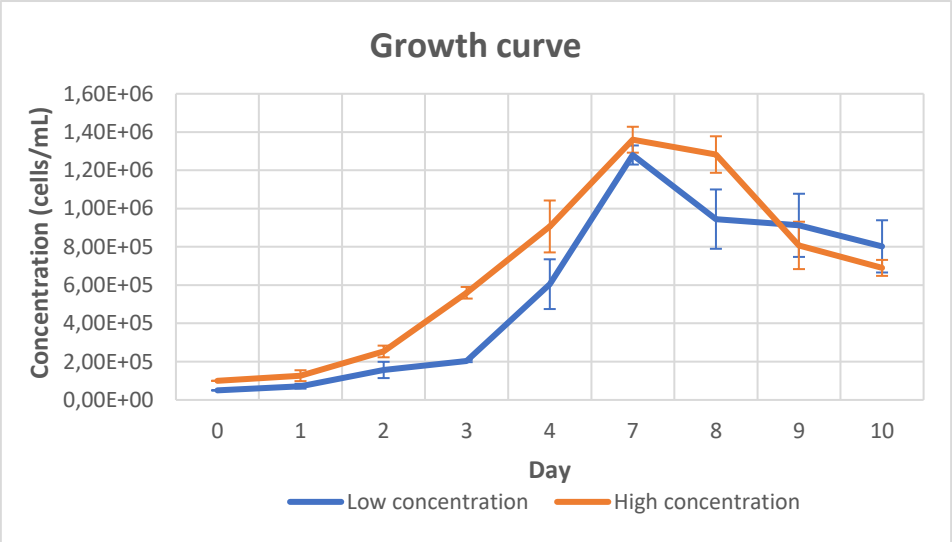


***Figure 16.*** Growth curve of THP-1 cells from two different initial concentrations: low concentration ($5x10^4$ cells/mL) and high concentration ($1x10^5$ cells/mL). Y-axis represents the concentration (cells/mL) while X-axis the days.

Both initial concentrations show a similar cellular development along the days. The highest concentration occurs on the 7th day after the inoculation which is $1.28x10^6$ cells/mL for the lowest initial concentration and $1.36x10^6$ for the highest.

## Morphological changes in response to PMA

Several pictures were taken during the experiment in order to check the cell differentiation using PMA. Figure 17 shows the morphological changes of the cells.

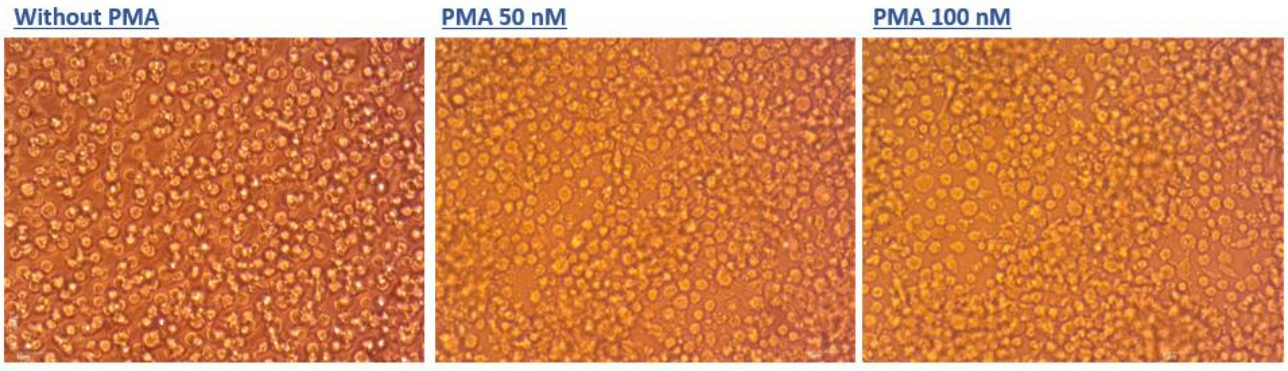


***Figure 17.*** Images of the cells without PMA and in response to 50 and 100 nM of PMA. Pictures were taken using the 20x objective of the microscope.

## Determination of cytokines

### Cytokines analysis without PAR2 inhibition

Results obtained of the cells differentiated with PMA and stimulated with LPS without any inhibition of the protein PAR2 are shown in this section. The inflammatory cytokines IL-6, IL-8 and TNFα have been analysed.

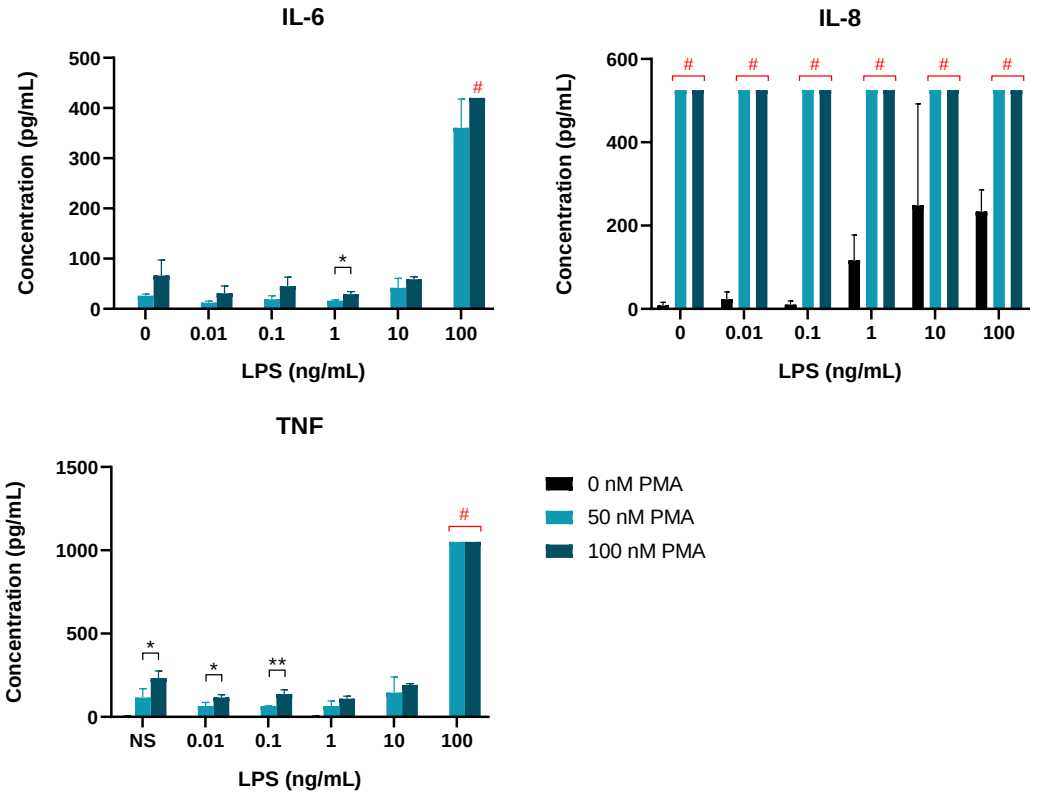


***Figure 18.*** Cytokine release by THP-1 cells differentiated with PMA (0, 50 and 100 nM) in response to several LPS over a range of concentrations. Three different cytokines are presented: IL-6, IL-8 and TNFα. Only significant differences between PMA 50 and 100 nM are marked in the graphic. Y-axis represents the cytokine concentration (pg/mL) while X-axis the LPS dose (ng/mL) in each case. *p-value < 0.05; **p-value < 0.01; #: results above the range.

Regarding the results, non-differentiated cells (0 nM PMA) do not release IL-6 and IL-8 at any LPS concentration. On the other hand, the highest concentration measured is produced in of 100 ng/mL of LPS for IL-6 and TNFα (p-value < 0.001; Bonferroni test). In the case of IL-8, the results obtained for cells cultured with PMA are above the range of the ELISA in response to all LPS doses. In addition, cells without PMA do not show

significant differences in response to LPS doses according to one-way ANOVA and Bonferroni post hoc test (p-value > 0.05).

Focusing on PMA different concentrations tested, in the case of IL-6, cells with PMA (50 or 100 nM) release higher concentrations of the cytokine in response to all LPS doses than non-differentiated cells (p-value < 0.05; Bonferroni). Furthermore, both PMA concentrations do not show significant differences except in response to 1 ng/mL of LPS (p-value = 0.0129; t-test). Nevertheless, THP-1 cells differentiated with 100 nM of PMA in response to 100 ng/mL of LPS express a number of cytokines above the range measured.

For TNFα, THP-1 cells with PMA 100 nM always released significantly higher cytokine than the cells without PMA (p-value < 0.001; multiple t-test). In the same way, cells with 50 nM, more TNFα was released compared to the non-differentiated cells (p-value < 0.05; multiple t-test) except in response to 10 ng/mL of LPS (p-value = 0.0556; t-test). Finally, comparing both PMA concentrations, significant differences between the first three LPS doses were observed (p-value < 0.05; multiple t-test), therefore, in front of 0, 0.01 and 0.1 ng/mL of LPS. On the other hand, for 1 and 10 ng/mL there were no differences (p-value = 0.0835 and 0.446, respectively). The results obtained in response to the highest LPS doses were above the range.

For IL-8, the cells that were differentiated with PMA and activated with LPS show a release of cytokines above the range of the ELISA. Furthermore, non-differentiated cells seem to release lower amounts of the cytokine. Comparison between the results from the cells without PMA and the highest concentration studied (525 pg/mL of IL-8) has been done. Multiple t-test analysis found highly significant differences in response to all LPS doses (p-value < 0.001) except with 10 ng/mL of LPS (p-value = 0.121).

Comparison between the three cytokines analysed in response to two different LPS doses (0 and 10 ng/mL) is shown in Figure 19. Results obtained of the cells in front of the highest LPS dose has not been used because some levels of cytokines were above the range studied. Nevertheless, in the next graphic, IL-8 secretion is still outside the interval.

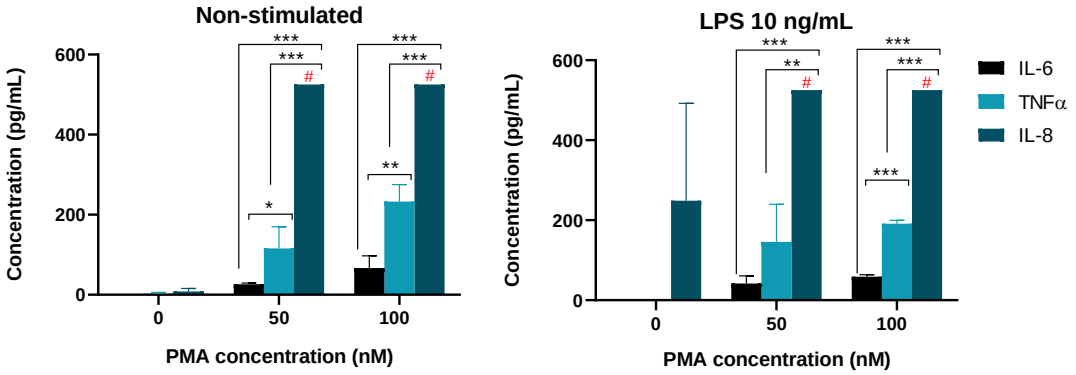


***Figure 19.*** Results of the three studied cytokines (IL-6, IL-8 and TNFα) in response to 0 and 10 ng/mL of LPS. Three different PMA concentrations tested are shown (0, 50 and 100 nM). Y-axis represents the cytokine concentration (pg/mL) while X-axis the PMA used (nM). *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001; #: results above the range.

According to the results presented in Figure 17, cells without PMA do not show differences in the cytokines analysed. On the other hand, IL-8 is always highest than the other two cytokines with a very significant difference (p-value < 0.01; multiple t-tests). It is important to mention that in order to compare these cytokines, the maximum concentration of the IL-8 interval was used (525 pg/mL) due to the results being outside the range of the standard curve. When IL-8 was compared with IL-6, the p-value was always under 0.001. IL-8 shows a highly significant difference (p-value < 0.001; multiple t-tests) when compared with TNFα in almost all cases except in response to 10 ng/mL of LPS and 50 nM of PMA, which was less significant (p-value = 0.00223; t-test).

Finally, regarding the other two cytokines, TNFα and IL-6, several different results were observed. On one hand, non-stimulated cells show a low significant difference between these two cytokines in front of PMA 50 nM (p-value = 0.0450; t-test) and a very significant difference in front of PMA 100 nM (p-value = 0.00536; t-test). On the other hand, THP-1 cells in response to 10 ng/mL of LPS release higher amounts of TNFα with a very highly significance in PMA 100 nM (p-value = 0.00000002; t-test) while there is no significant difference between both cytokines in PMA 50 nM (p-value = 0.135; t-test).

## Cytokines analysis in response to PAR2 inhibition

In order to study the effect of the inhibitor AZ8838, only IL-6 was determined using ELISA assay. All the cells were differentiated using 50 nM of PMA and stimulated with different molecules: LPS (100 ng/mL), FLIGRLO-$NH_2$ (100 µM) and SLIGKV-$NH_2$ (100 µM).

The inhibitor tested was diluted in medium with DMSO (as described in methods), therefore, RPMI 1640 Medium without DMSO is compared with RPMI 1640 Medium with DMSO in order to know the effect of this reagent. The results are represented in Figure 20 and they do not show significant differences between pairs according to t-test results (p-value > 0.05).

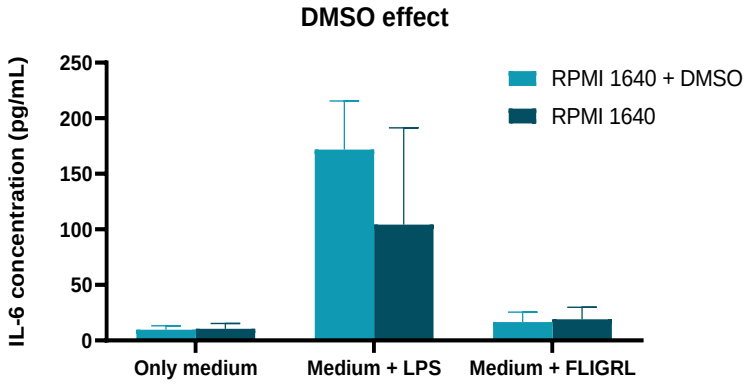


***Figure 20.*** Cytokine levels released using RPMI 1640 Medium with and without DMSO. The comparison has been done in medium alone, in medium plus LPS and in medium plus FLIGRLO-$NH_2$. Y-axis represents the concentration of IL-6 (pg/mL) while X-axis the different conditions tested.

Results for several inhibitor concentrations (0, 0.003, 0.03, 0.3, 3 and 30 µM) in response to the three stimulations studied are shown in Figure 21. Additionally, one group of cells without any stimulation is also shown.

According to the results presented in the previous graphics and the ANOVA test, there are no differences between the different inhibitor concentrations in any of the experiments set up. Furthermore, there are no differences in the non-stimulated cells either (p-value > 0.05; Bonferroni).

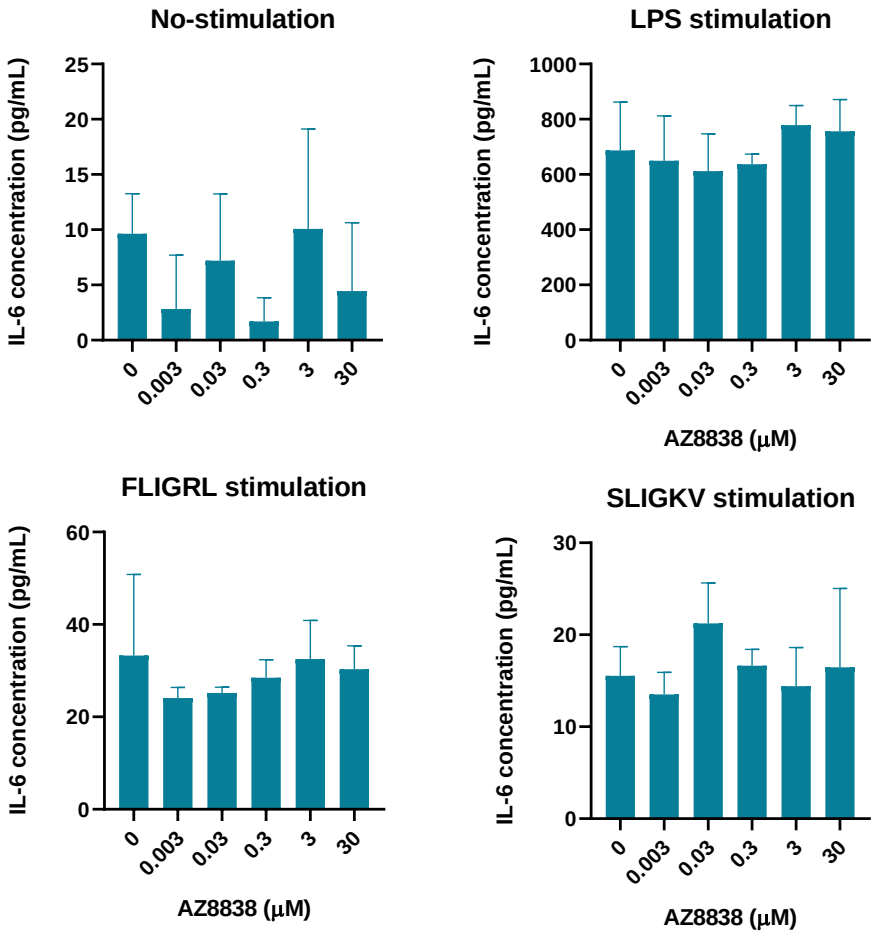


***Figure 21.*** IL-6 levels released by the cells with different inhibitor concentrations in response to three stimulation molecules: LPS, FLIGRLO-$NH_2$ and SLIGKV-$NH_2$. Also, the first panel shows the cells without any stimulation. Y-axis represents the IL-6 levels (pg/mL) and X-axis represents the inhibitor concentrations tested (μM).

The cytokine release in response to the different stimulations has been compared and is shown in Figure 22. In this case, inhibitor concentration has not been considered and all the samples for each stimulation have been used to calculate the average. In the first graphic, the results of the non-stimulated cells and the three activators used are presented: LPS, FLIGRLO-$NH_2$ and SLIGKV-$NH_2$. However, in the second graphic, LPS results have been removed in order to compare the effect of only the two PAR2-AP.

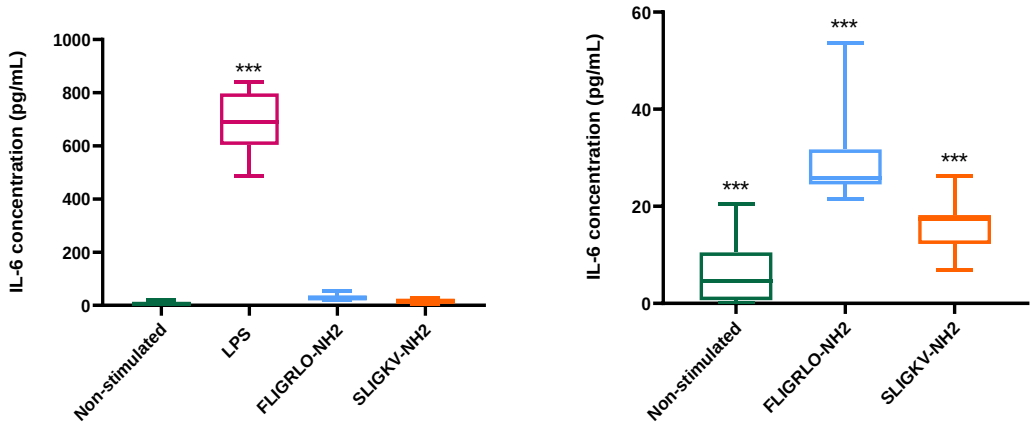


***Figure 22.*** IL-6 cytokine released by cells in response to different stimulations: non-stimulated, LPS (100 ng/mL), FLIGRLO-$NH_2$ (100 µM) and SLIGKV-$NH_2$ (100 µM). Y-axis represents the cytokine concentration (pg/mL) while X-axis the molecules studied. ***p-value < 0.001

According to one-way ANOVA test performed using the four groups of cells, cells stimulated with LPS express higher amounts of IL-6 (p-value < 0.001; Bonferroni). On the other hand, when the other three groups are compared without the LPS stimulation group, all groups show very highly significant differences between the others (p-value < 0.001; multiple t-tests).

The last comparison is done between FLIGRLO-$NH_2$ and SLIGKV-$NH_2$ and their respective reverse peptides and it is shown below:

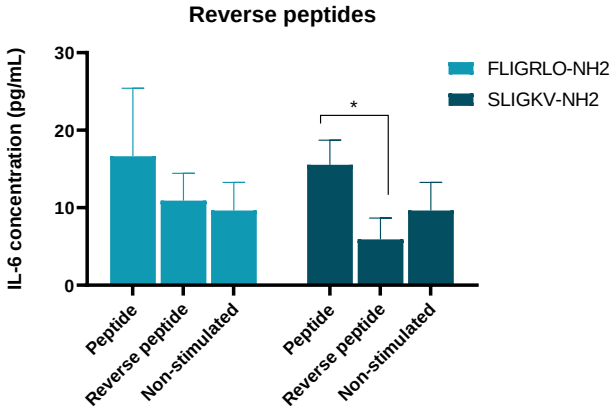


***Figure 23.*** Cytokine released by the cells stimulated with the FLIGRL and SLIGKV-$NH_2$ reverse peptides and the normal peptides. Y-axis shows the concentration of IL-6 (pg/mL) while X-axis the activator tested in each case. *p-value < 0.05

There are no significant differences between the FLIGRLO-$NH_2$ results, nevertheless, cells stimulated with SLIGKV-$NH_2$ release higher numbers of IL-6 than its reverse peptide (p-value = 0.0168; t-test).

## Cell viability

MTT assay was performed in the remaining cells attached to the bottom of the wells used to analyse the inhibitory treatment. Figure 24 shows the results obtained in response to the different activator molecules: non-stimulation, LPS, FLIGRLO-$NH_2$ and SLIGKV-$NH_2$. There are no significant differences between the groups stimulated with these molecules according to one-way ANOVA test.

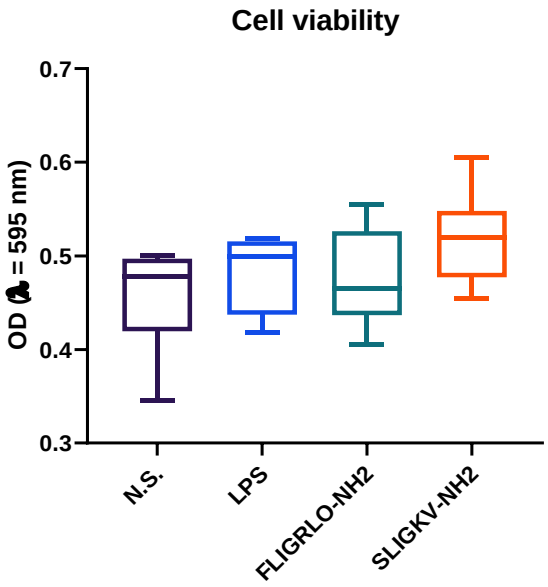


***Figure 24.*** Optical density measured at 595 nm of wavelength. Four different groups are represented in the graph: non-stimulated cells, LPS-stimulated cells, FLIGRLO-stimulated cells and SLIGKV-stimulated cells. Y-axis represents optical density and the different molecules are shown in the X-axis.

Figure 25 represents the cell viability in response to the different doses of inhibitor tested in this study: 0, 0.003, 0.03, 3 and 30 µM. All groups stimulated with different molecules are represented: non-stimulated cells, FLIGRLO-stimulated, LPS-stimulated and SLIGKV-stimulated. As in the previous case, no significant differences can be observed according to ANOVA test.

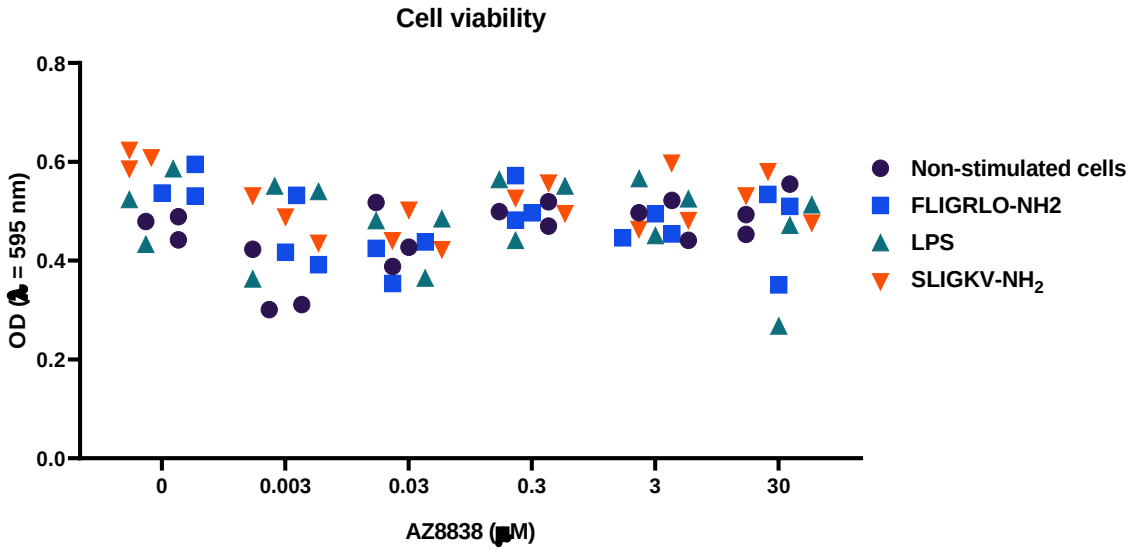


***Figure 25.*** Optical density at 595 nm of wavelength. The four groups in front of the different AZ8838 doses are presented (0, 0.003, 0.03, 0.3, 3 and 30 µM). Y-axis shows the optical density while X-axis represents the inhibitor concentrations (µM).

# Discussion

## Cell growth

According to Figure 15, differences in the number of cells between both initial concentrations cannot be seen with the 10x objective. However, using the 20x objective, a greater number of cells in the highest concentration is observed.

Observing the growth curve (Figure 16), both initial concentrations show a similar pattern of growth, being the highest concentration in the seventh day after the plate cultured. Furthermore, cells are still viable when the concentration is above $1x10^6$ cells/mL and the viability decreases because of the nutrient exhaustion in the medium. In fact, the initial difference between both cellular concentrations was $5x10^4$ cells/mL and, in the peak of growth, the difference is about $8x10^4$ cells/mL. These results suggest that both concentrations allow the proper development of the cells showing the highest cellular amount in the seventh day and they are viable in high concentrations.

In order to perform the posterior studies, a final concentration of $5x10^5$ cells/mL was used to obtain high levels of cytokines. In fact, the concentration used by Lung *et al.* (2016) for the PMA-induced maturation of THP-1 cells was $2x10^5$ cells/mL, which is quite close to our choice.

## Morphological changes

According to the images, the monocytes are well differentiated into macrophages using both concentrations of PMA (50 and 100 nM). The cells without PMA show a round shape, common in monocytes. On the other hand, PMA treatment induced a change in the morphology and the cells became more resembling “fried eggs”, which is a typical shape of M1-like macrophages. Moreover, the cells look more attached ones with the others and to the bottom of the well. In fact, Gatto *et al.* (2017) reported that the PMA-induced differentiation of THP-1 cells induces cell adhesion, irregular nucleus and nonuniform cell shape, as can be observed in Figure 17.

Additionally, both concentrations do not seem to show differences. Therefore, 50 and 100 nM of PMA have proved to be able to differentiate THP-1 cells in a proper way.

## PMA and LPS best concentrations

As it has been explained above, although THP-1 differentiation using PMA is very common, there is no established protocol for this procedure. Furthermore, Lund *et al.* (2016) reported that the concentration and the recovery period significantly influence the functionality of the macrophages.

According to the results presented in Figure 18 and 19, both PMA concentrations tested increase the cytokine secretion. It is important to mention that the cells without PMA do not release IL-6 and TNFα although express IL-8, suggesting that IL-8 might have a basal expression in monocytes and this is incremented depending the LPS concentration. Generally speaking, 100 nM of PMA produce a higher secretion of pro-inflammatory cytokines. These results agree with Lund *et al.* (2016), who said that more elevated concentrations of PMA induce higher amounts of this type of cytokines.

Regarding LPS dose, IL-6 and TNFα show higher levels in response to 100 ng/mL with a very significant difference compared with the other LPS stimulations. However, PMA-induced differentiated cells express high amounts of IL-8 independently of the LPS dose. It cannot be discussed the highest release in the case of IL-8 because the results are all above the range. However, to summarize, TNFα and IL-6 may be LPS dose-dependent in PMA-induced macrophages although IL-8 might not be.

Observing the results, concentrations allowing the proper differentiation of the THP-1 cells with PMA but maintaining the results inside the cytokine range studied were chosen. Therefore, 50 nM of PMA and 100 ng/mL of LPS was used for posterior studies. In addition, 24 hours of recovery after PMA addition was performed as it was recommended by Lund *et al.* (2016). Moreover, the dilution of the supernatants in ELISA assay was incremented (x2) in order to avoid results above the range when LPS was added as a macrophage activator in posterior studies.

## Cytokine profile in response to PMA differentiation

According to the graphics in Figure 19, THP-1 cells differentiated using PMA develop an M1-like phenotype as has been reported before (Lund *et al.,* 2016). This type of macrophages is able to secrete pro-inflammatory cytokines in response to LPS and they

have been associated with RA (Kennedy *et al.*, 2011). Therefore, it is logical to think that the attenuation or reversal of M1 phenotype may be a powerful therapy for this disease.

The three cytokines studied have been detected in PMA-induced cells (IL-6, IL-8 and TNFα). Nevertheless, it is interesting to mention that the concentration varies between them. IL-8 shows the highest concentration released with very significant difference compared to the other cytokines. In the second place, TNFα is found, showing a very low secretion compared to IL-8. Finally, IL-6 seems to be the cytokine less expressed in these macrophages.

Summarising the cytokine profile shown in PMA-induced macrophages, these cells may promote inflammatory responses releasing high amounts of IL-8 and lower levels of TNFα and IL-6.

### Different stimulations with SLIGKV-$NH_2$, FLIGRLO-$NH_2$ and LPS.

LPS stimulation induces the highest release of cytokines with a high significant difference compared to the other molecules tested as it is shown in Figure 22. LPS is a TLR4 agonist, a receptor involved in the defense against pathogens inducing activation of various cells stimulating inflammation. It is also important to remember the synergistic response existing between TLR4 and PAR2 (Bucci *et al.*, 2012; Rallabhandi *et al*, 2008). On the other hand, FLIGRLO-$NH_2$ and SLIGKV-$NH_2$, only activate PAR2. For that, cytokine secretion is higher in response to LPS because TLR pathway signalling is activated.

Talking about PAR2-APs, FLIGRLO-$NH_2$ and SLIGKV-$NH_2$, both increase the IL-6 release by macrophages compared to non-stimulated cells. FLIGRLO-$NH_2$ shows a higher cytokine secretion than SLIGKV-$NH_2$ using the same stimulator concentration (100 μM). These results suggest that FLIGRLO-$NH_2$ is a better PAR2 activator in the case of PMA-induced THP-1 macrophages and agree with the ones presented by McGuire *et al.* (2004). In McGuire study, FLIGRLO-$NH_2$ was described as the most potent and selective activator of PAR2.

Summarising, in all cases, when PAR2 is activated, a pro-inflammatory response by the macrophages is produced. Therefore, the results agree with Steven *et al.* (2013) who

reported that PAR2 is directly related to inflammatory immune responses because its activation enhances the pro-inflammatory profile of M1 macrophages.

Reverse sequences of the synthetic peptides have been also analysed as a negative control. In this case, the data suggest that none of the reverse peptides induce cytokine release as it was expected (Figure 23).

## PAR2 inhibition

AZ8838 was the inhibitor used in the study. It was diluted using DMSO, however, DMSO did not affect the functionality of the macrophages (Figure 20).

AZ8838 has been described as a possible PAR2 inhibitor but its effects have not been studied yet (Cheng *et al.*, 2017). According to the data obtained in this project and shown in Figure 21, AZ8838 do not inhibit PAR2 in any case. For that, regarding these results, it cannot be assumed that AZ8838 could be a potential treatment for RA. Nevertheless, for the association that has been seen between PAR2 and RA (Crilly *et al.* 2012), its inhibition is promising, and more research is needed to find a potent inhibitor.

These results could be consequence of the AZ8838 concentrations used that maybe were not enough. Future studies should test a panel of higher concentrations. On the other hand, stimulator concentrations can also affect this study. First, LPS activates TLR4 and may induce cytokine release even in the presence of PAR2 inhibitor. However, the pro-inflammatory cytokine release should be lower with an inhibitor according to their synergistic response (Rallabhandi *et al.,* 2008). Moreover, the inhibitor competes with the PAR2-AP to bind to the receptor and high concentrations of SLIGKV-$NH_2$ and FLIGRLO-$NH_2$ could affect the data. In order to study the effects of this inhibitor, I would propose to use a panel of lower concentrations for the activators.

## Cell viability after PAR2 inhibition

According to the results presented in Figure 24, there are no differences in the viability of the cells when PAR2 has been activated with different molecules (LPS, SLIGKV-$NH_2$ and FLIGRLO-$NH_2$). In addition, the inhibition of PAR2 using AZ8838 does not show an effect in the cell viability in any of the inhibitor concentrations tested (Figure 25).

## Limitations

Apart from the specific observations that have been described before, some general limitations need to be considered. First of all, the number of replications for each group of samples were low. In addition, IL-6 was the cytokine measured in the PAR2 inhibition, however, according to the results presented before, TNFα could be a better molecule to study. Therefore, I suggest that posterior studies determine both molecules, even other pro-inflammatory cytokines that can be associated with the disease such as IL-12.

Finally, this project was performed using the cell line THP-1 differentiated with PMA and other conditions applied may show different results. Moreover, the data obtained *in vitro* cannot be directly extrapolated *in vivo* due to the inflammatory mechanisms are more complex in a full organism.

According to this, the data presented in this project could be taken as a basis for future more specific and refined studies.

# Conclusion

Most of the treatments for RA are based in attenuating the inflammation produced by inhibiting the main inflammatory pathways. However, these therapies have important side-effects and do not work in all the patients. Therefore, more research is needed in order to understand the inflammatory network participating in the disease to develop targeted drugs. Since the ratio of M1/M2 seems to be increased in diseases such as RA, the modulation of this ratio could be an interesting line of study. Additionally, PAR2 inhibition could be a possible target in these cells.

According to the present study, PMA-induced THP-1 cells present features close to the M1 macrophages. Regarding the role of PAR2 in this subtype, its activation induces the release of pro-inflammatory cytokines associated with RA. Therefore, PAR2 may be involved in RA inflammatory immune response and be a potent target for treatments. Nevertheless, AZ8838 did not inhibit PAR2 at the concentrations studied, suggesting more research is needed around this competitive inhibitor.

In regard to the experimental conditions, cellular growth could be induced in concentrations above 1x106 cells/mL, with growth peaking around day 7 of culture. Furthermore, 100 nM of PMA and 100 ng/mL of LPS induced the highest release of IL-6 and TNFα while IL-8 gave a dose-independent response. Nevertheless, in this study, 50 nM of PMA and 100 ng/mL of LPS were the concentrations chosen in order to maintain the cytokine levels inside the range studied.

According to the cytokine profile of M1-like macrophages, they may drive the inflammation by secreting high amounts of IL-8 and less TNFα and IL-6. Additionally, LPS presents the higher ability to stimulate the macrophages. On the other hand, the PAR2-AP FLIGRLO-NH2 was shown to be a better activator of PAR2 than SLIGKV-NH2. However, in all cases, pro-inflammatory cytokines were released.

According to the morphological characteristics, both PMA concentrations tested (50 and 100 nM) allowed the differentiation of monocytes into M1-like macrophages. Finally, cell viability was not affected by the type of stimulator or the presence of the inhibitor AZ8838 at the concentrations studied.

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# Self-evaluation

I have had the opportunity to do this dissertation from the external internship performed at the University of the West of Scotland, in the United Kingdom. I decided to do the placement abroad because I thought it would help me to develop in a completely different environment and could learn from the research in another country. In addition, I have always been interested in immunology and this was presented as a good opportunity to know more about this field.

The stay, along with the writing of the dissertation, has allowed me to know more about a research group in Scotland. In addition, I have improved my skills in laboratory work, for example, in cell cultures. Furthermore, I also had the opportunity to participate in Journal Clubs and laboratory meetings, oral presentations and science fairs. All these events have helped me to improve my English skills and learn to develop myself in the scientific world.

For the simple fact of living for a time in another country, I have also been able to meet people from different parts of the world and learn more about British and Scottish culture.

Regarding the subject of the work itself, I think that research in the improvement of treatments for autoimmune diseases (such as RA) is necessary since, for now, neither the origin nor the definitive cure of these diseases are known. Finally, I am very fortunate to have been able to study a disease that touches me so closely.