Trabajo de Fin de Grado

Assessing TLR and PAR2 crosstalk in epithelial cells during COPD exacerbations

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1. ABSTRACT

Chronic obstructive pulmonary disease (COPD) is characterised by progressive deterioration of the airways, disrupting respiratory function as a result of exposure to tobacco smoke or airborne pollutants. In the course of the disease there are periods of exacerbations, episodes of worsening symptoms caused by bacterial or viral infections. Epithelial cells are essential for the maintenance of the protective barrier and for the development of the immune response against pathogens in the airways. These cells express toll-like receptors (TLR) and protease-activated receptors (PAR) which are involved in the pathogenesis of COPD. Characterising the expression of these receptors in respiratory epithelial cells and studying whether there is a crosstalk between them during exacerbations is essential for a complete understanding of the molecular mechanisms of the disease. Therefore, using epithelial cells of the A549 line, in this project, the expression of PAR2, TLR3 and TLR4 receptors in these cells was studied via immunofluorescence staining and different aspects of the activation of these receptors during exacerbation were evaluated by carrying out bioassays.

2. INTRODUCTION

2.1. Chronic Obstructive Pulmonary Disease (COPD)

COPD is characterised by the triggering of severe inflammatory processes in the airways leading to airway obstruction and the development of an uncontrolled chronic inflammatory response leading to the destruction of lung tissue. The main risk factor in the development of this disease is the prolonged exposure to substances such as tobacco smoke or air pollutants (Laniado-Laborín, 2009).

However, studies such as the one conducted by Yang, Jenkins and Salvi (2022) have been able to demonstrate that many cases of COPD are caused by risk factors other than tobacco smoke, such as poorly treated asthma or infectious diseases. More research on COPD in non-smokers is needed to fully understand the disease.

COPD is a heterogeneous disease that does not always present in the same way and depends on both environmental and genetic factors. The main genetic factor that plays a role in the development of the disease is alpha-1 antitrypsin (ATT) deficiency. ATT is a protease inhibitor essential for the control of inflammation as one of its main functions is to inactivate neutrophil elastase to prevent protease-mediated lung damage. ATT deficiency is an inherited condition that causes a proteolytic imbalance leading to lung damage and the development of COPD (Brode, Ling and Chapman, 2012).

2.1.1. Epidemiology

In 2019, the World Health Organization (WHO) determined that COPD is the third leading cause of death worldwide, which represents a major economic, health and social burden. The disease is a leading cause of mortality and morbidity in developed countries where social smoking is usual, but it is also becoming a problem in developing countries where air pollution levels are very high (Halpin, Vogelmeier and Agusti, 2021).

2.1.2. Symptoms and comorbidities

COPD presents with different types of characteristic symptoms such as dyspnoea, frequent coughing attacks, sputum production as well as general breathing difficulties, chest tightness and lung congestion. This set of symptoms compromises the quality of life of COPD patients and

complicates the performance of simple day-to-day activities. The types of symptoms and their frequency of occurrence depend on the stage of the disease, although many of them are common to all patients (Rodrigues *et al.*, 2021).

In addition to the symptoms previously mentioned, patients with COPD often have comorbidities that worsen their quality of life, the prognosis of the disease, the frequency of exacerbations and compromise patient survival (Smith and Wrobel, 2014). Vascular and cardiac diseases are the most common co-morbidities in COPD patients as it appears that the inflammation generated during COPD not only affects the pulmonary endothelium, but also affects the endothelium systematically generating major dysfunctions throughout the organism (Cavaillès *et al.*, 2013).

In addition, it has also been shown that common COPD comorbidities such as anxiety, depression, increased pulmonary stress and cardiovascular disease increase the risk of disease exacerbations (Smith and Wrobel, 2014). In this way, a loop is generated in which, while the pathophysiology of COPD predisposes patients to the development of comorbidities, these comorbidities in turn predispose patients to a higher frequency of exacerbations and thus a worsening of symptoms and disease prognosis.

2.1.3. Exacerbations

Exacerbations are episodes of worsening symptoms which have an adverse effect on COPD patients because with each exacerbation lung function declines irreversibly. Since one of the main pathological features of COPD is changes in the immune response, this also affects the defence against pathogens such as viruses and bacteria. This is why the main triggers of exacerbations are bacterial and viral infections, with viral infections being the most predominant cause (Ritchie and Wedzicha, 2020).

Several studies, such as the one conducted by Soler-Cataluña et al. (2005) have been able to demonstrate that the frequency of exacerbations is directly proportional to the risk of mortality in patients with COPD and that patients with more than 3 exacerbations per year are more likely to be hospitalised.

2.1.4. Pathophysiology

COPD is characterised by progressive airway limitation, hyperinflation, and the maintenance of chronic inflammatory processes in the airways. Thus, COPD triggers the destruction of the small airways and the development of chronic bronchitis and emphysema (**figure 1**).

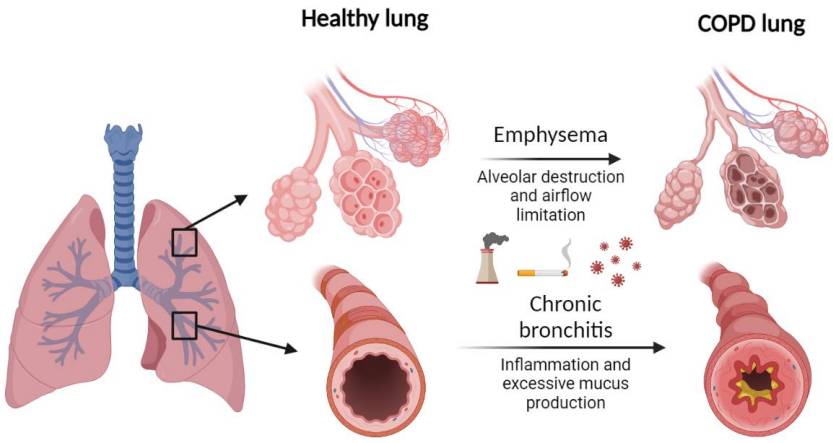

FIGURE 1. COPD PATHOPHYSIOLOGY IS CHARACTERIZED BY THE DEVELOPMENT OF ALVEOLAR EMPHYSEMA AND CHRONIC BRONCHITIS LEADING TO PROGRESSIVE AIRFLOW LIMITATIONS AND DESTRUCTION OF THE LUNG. ILLUSTRATED WITH BIORENDER.COM.

The subepithelial elastic fibres of the alveoli confer resistance to the muscular connective tissue which allows the lung tissue to deform and elastic retraction to take place without the need for energy input. Elastolysis is the process by which these elastic fibres are degraded, and this event is characteristic of COPD patients. In addition, the pathophysiology of COPD is also characterised by the development of emphysema, a term that refers to the gradual destruction of the pulmonary alveoli. Emphysema together with elastolysis results in reduced gas exchange, loss of elastic retractability, hyperinflation and limited expiratory flow (Rodrigues *et al.*, 2021).

The main pathophysiological features of COPD include the increased generation of reactive oxygen species (ROS), the development of major changes in different components of the immune response, and the imbalance between proteases and anti-proteases (Rodrigues *et al.*, 2021).

2.1.4.1. Oxidative stress

Oxidative stress occurs when the balance between oxidative and antioxidant species in the body is disrupted. The immune system can generate oxygen- and nitrogen-derived oxidative species which are essential in the development of the immune response against external pathogens and for certain signalling pathways but are also important in inflammatory reactions (Domej, Oettl and Renner, 2014).

Tobacco smoke is the main exogenous source of oxidative species introduced into the body. Thus, tobacco smoke-derived species enhance the development of oxidative stress and an imbalance between oxidative and antioxidant species (Domej, Oettl and Renner, 2014).

The organism has different antioxidant mechanisms that help to counteract the effect of oxidative species. These can be enzymatic mechanisms such as superoxide dismutase (SOD), catalase or glutathione peroxidase (Gpx) or non-enzymatic mechanisms such as glutathione (GSH), vitamins C and E, uric acid and albumin (Austin *et al.*, 2016).

While in healthy cells the antioxidant mechanisms can limit the production and action of ROS, if these cells are constantly exposed to pollutants or oxidative species such as those present in tobacco smoke, the production of reactive species becomes dysfunctional, and the redox imbalance is accentuated. This has been proven in studies such as the one conducted by

(Dietrich *et al.*, 2003) which shows that smokers have a significant decrease in the concentration of antioxidants such as vitamin C in their blood compared to non-smokers.

The increase in the amount of ROS involves damage to cellular components resulting in changes in cellular metabolism. In the case of airway tissues, when oxidative species from tobacco smoke meet epithelial cells, oxidation of membrane lipids in these cells can result in indirect or direct activation of innate sensing receptor called toll-like receptors (TLRs). Stimulation of these receptors leads to activation of the NF-κB (nuclear factor kappa B) mediated pathway which is responsible for the activation of the inflammatory response in COPD (Biabas *et al.*, 2016).

2.1.4.2. Immune cells and mediators

Exposure to the toxins present in tobacco smoke and to viral and bacterial components leads to the recruitment and mobilisation of inflammatory cells and the secretion of pro-inflammatory molecules in the airways, resulting in extensive damage to the tissues of the respiratory system. This damage is largely triggered by exposure of the airway epithelium to these toxins (Gao *et al.*, 2015).

EPITHELIAL CELLS IN COPD

Airway epithelial cells play a key role in maintaining the barrier against pathogens in the respiratory system and are responsible for maintaining normal respiratory function. In the respiratory epithelium there are different groups of cells: ciliated cells, goblet cells, club cells and basal cells (**figure 2**). Goblet and club cells create mucus to trap inhaled particles on the apical side of the epithelium and then the cilia from ciliated cells beat the trapped particles to transport them back to the mouth where they are swallowed or spit. Basal cells are part of the stem cell niche, and they can differentiate into other cell types. These cells have important functions in maintaining the integrity of respiratory tissue and participate in the immune response by recruiting other cells of the immune system through the production of protective mucus, cytokines, and other mediators such as TNF-α, IL-8, IL-6 or IL-1 (Calvén, Ax and Rådinger, 2020).

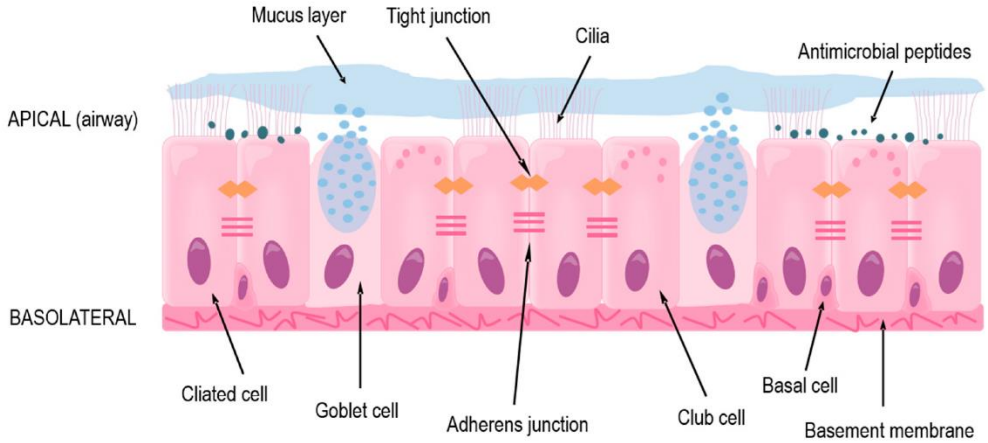

FIGURE 2. CELLULAR STRUCTURE OF THE AIRWAY EPITHELIUM (CALVÉN, AX AND RÅDINGER, 2020).

In response to oxidative stress caused by reactive substances from tobacco smoke and pollutants, signalling pathways such as MAPK (Mitogen-activated protein kinases) and NF-κB are activated in epithelial cells. These pathways result in increased secretion of mediators such as CXCL8 (C-X-C Motif Chemokine Ligand 8)/IL-8, TNF-α (Tumor necrosis factor alpha) or ICAM-1 (Intercellular adhesion molecule 1) that promote the influx and recruitment of inflammatory

cells such as neutrophils and thus enhance inflammatory processes in the airways (Gao *et al.*, 2015).

Reactive species from tobacco smoke and air pollutants cause significant alterations in the architecture of the respiratory epithelium as well as impairing its barrier function. The study by Kanazawa et al. (2014) shows that vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) levels are reduced in COPD patients. These two growth factors are essential for maintaining structural integrity and promoting cell survival, so decreased levels of them are associated with increased epithelial cell apoptosis.

Understanding the molecular mechanisms that occur in epithelial cells after exposure to reactive species is essential to fully understand the pathophysiology of COPD as these cells play a key role in airway protection.

2.1.4.3. Proteolytic imbalance

Proteolytic imbalance is also considered a characteristic pathophysiological feature of COPD. The main proteases associated with the development of COPD are serine proteases (such as trypsin, tryptase, metriptase or neutrophil elastase), cysteine proteases (such as caspases), aspartate proteases (such as cathepsins D and E) and metalloproteinases (MPPs). Many of these proteases are overexpressed in animal models, but also in clinical samples from COPD patients, thus confirming the role of proteolytic imbalance and protease accumulation in the pathophysiology of COPD (Dey *et al.*, 2018).

2.2. Protease-activated receptors (PARs)

The protease-activated receptor family consists of 4 different types of receptors -PAR1, PAR2, PAR3 and PAR4- which react and are activated by different types of proteases both exogenous and endogenous. The regulation of these receptors depends on the species and tissue, protease activity, dimerization and co-localisation with other receptors and other aspects. PAR receptors are composed of a domain consisting of 7 transmembrane helices linked to 3 intracellular loops (ICL1, ICL2, ICL3) and extracellular loops (ECL1, ECL2, ECL3) (Gieseler *et al.*, 2013).

As shown in **figure 3**, proteases, whether soluble or cell membrane-associated, can cleave peptides at the N-terminal end of PARs leading to the exposure of new N-terminal peptides that can serve as tethered activation ligands. In this way, these new ligands bind intramolecularly to the receptor itself, specifically to the extracellular loop ECL2. This binding leads to conformational changes in the receptor and its activation for signalling (Gieseler *et al.*, 2013).

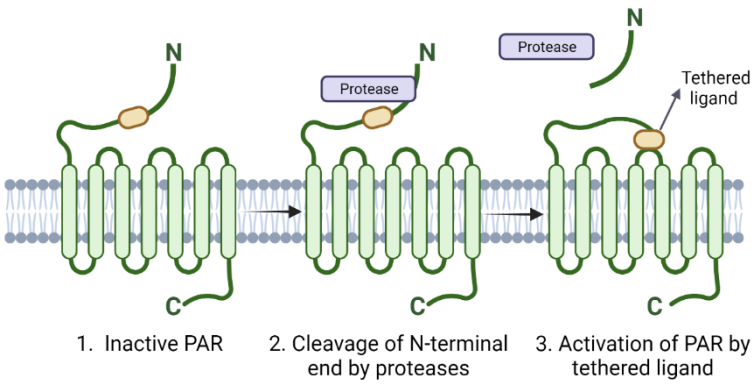

FIGURE 3. SCHEME OF THE ACTIVATION OF PAR RECEPTORS MEDIATED BY THE ACTION OF ENDOGENOUS AND EXOGENOUS PROTEASES. ILLUSTRATED WITH BIORENDER.COM

2.2.1. PAR2

PAR2 is mainly activated by serine proteases such as trypsin and tryptases, although many endogenous and exogenous proteases, as well as tethered ligand mimetic peptides like FLIGRL can activate the receptor. **Table 1** lists the different types of proteases that activate PAR2 and their cleavage sites (Heuberger and Schuepbach, 2019).

TABLE 1. LIST OF PROTEASES THAT CAN CLEAVE PAR2 AND THEIR CLEAVAGE SITES (HEUBERGER AND SCHUEPBACH, 2019).

	Protease	Major cleavage site	Additional cleavage sites
Mammalian proteases	Thrombin	R36S37	
	aPC	unknown	
	FXa	R36S37	
	Trypsin	R36S37	K34G35, K51G52, K72L73
	Tryptase	R36S37	
	Chymase	G35R36	L38I39, mouse
	Matriptase	R36S37	
	Cathepsin G	F65S66	F59S60, F64S65
	Cathepsin S	G40K41	E56P57, mouse
	Neutrophil elastase	A66S67, S67V68	V42D43,V48T49,V53T54,V58T59,T74T75,V76F77
	Proteinase-3	D62E63	V48T49,V55E56,T57V58 V61D62,K72L73,T74T75,T75V76,V76F77
	Plasmin	R36S37	K34G35
	Testisin	unknown	
	Kallikrein-4,	unknown	
	Kallikrein-5,-6,-14	R36S37	
	Calpain-2	unknown	
Non-mammalian proteases	Der-P1,-P2,-P3,-P9	unknown	
	Cockroach E1-E3	R36S37	
	Gingipain R	unknown	
	LepA	unknown	
	EPa	S37L38	S38L39, rat
	S.pneumoniae proteases	unknown	
	Thermolysin	unknown	
	Serralysin	unknown	
	P.acnes proteases	unknown	
	aPA	unknown	
	Bromelain	unknown	
	Ficin	unknown	
	Papain	unknown	
	penC	R36S37	

Proteases that cleave PAR2 at the arginine 36 residue, such as thrombin or trypsin, generate what is known as canonical activation, which would lead to the events explained above in which a ligand is generated that binds intramolecularly to the receptor itself, activates it and triggers the common signalling pathways. On the other hand, proteases that cut at sites other than this residue generate a non-canonical activation that results in the activation of other signalling pathways (Zhao *et al.*, 2014).

2.2.1.1. Canonical activation of PAR2

The canonical activation of PAR2 leads to the activation of different G protein-dependent and G protein-independent signalling pathways. PAR2 can bind to α-subunits of G proteins such as Gαq/11, Gαi, or Gα12/13 but can also induce G protein-independent pathways through its recruitment by β-arrestin (Rothmeier and Ruf, 2012).

Canonical activation of PAR2 results in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C (PLC) and further induces the Ca^{2+}/IP3/PKC signalling axis. This signalling pathway terminates in the phosphorylation of the IκB kinase complex (IKK) formed by IKKα and IKKβ resulting in nuclear translocation of NF-κB (Rothmeier and Ruf, 2012). NF-kB plays an essential role in carrying out transcriptional activation of cytokine genes, chemokines and cell adhesion molecules which can control the number and type of inflammatory cells infiltrating the airways (Schuliga, 2015). This highlights the role of PAR2 in inflammatory processes.

2.2.1.2. Desensitisation and termination of signalling

Since PAR2 is irreversibly activated by proteolysis and is not a constitutively internalised receptor, β-arrestin proteins interact with PAR2 on its C-terminal end to effectuate its internalisation and disrupt its signalling. When β-arrestins associate with PAR2, the MAPK signalling pathway is activated, resulting in down-regulation of G protein-associated receptor signalling pathways. Thus, when PAR2 is phosphorylated on its C-terminus, β-arrestin can bind to the receptor and promote the uncoupling of G-protein from the receptor and the internalisation of PAR2 by a clathrin-mediated mechanisms (Rothmeier and Ruf, 2012).

The PAR2-arrestin complex is then translocated to endosomes and due to its irreversible activation by proteolysis, the receptor usually terminates its signalling upon degradation. For this, the PAR2-arrestin complex dissociates and the receptor is ubiquitinated and degraded by the action of lysosomes. However, a portion of the internalised PAR2 receptors is not ubiquitinated and can circulate back to the cell surface (Rothmeier and Ruf, 2012).

2.2.1.3. PAR2 in inflammatory processes

The role of PAR2 in inflammatory processes and its involvement in the development of different diseases has been extensively demonstrated. Due to its localisation on the cell membrane and its activation by proteases, PAR2 enables cells to respond to changes in the proteolytic environment that occur during inflammation. Although this receptor is important for the initiation of the immune response and inflammation, its overstimulation can result in tissue damage (Sevigny *et al.*, 2011).

Many studies have referred to the role of PAR2 in inflammatory processes in different parts of the body such as the cardiovascular system where PAR2 has been found to play a key role in protective barrier activity and in the regulation of vasorelaxation. Due to its activation mediated by intestinal trypsins and tryptases, PAR2 is also directly involved in gastrointestinal inflammation. Furthermore, PAR2 overexpression has been demonstrated in atopic dermatitis and rheumatoid arthritis using animal models (Heuberger and Schuepbach, 2019).

2.2.1.4. PAR2 and COPD

The role of PAR2 in COPD is still under investigation. Expression of this receptor occurs in many airway cells such as lung epithelium and endothelium cells, respiratory smooth muscle cells, mast cells, macrophages, and alveolar neutrophils. This is why PAR2 is thought to play an important role in respiratory inflammatory diseases such as COPD. It has been shown that PAR2 activation in epithelial cells induces both the release of prostaglandin E2 (PGE2), which inhibits respiratory muscle contraction, and the secretion of the proinflammatory cytokines IL-6 and IL-8 (Knight *et al.*, 2001).

Previous studies such as the one conducted by Nystedt, Ramakrishnan and Sundelin (1996) have determined that PAR2 expression is up-regulated by different types of inflammatory stimuli such as TFN-α, interleukin 1α (IL-1α), IL-1β and lipopolysaccharide (LPS). These stimuli in turn induce the secretion of the macrophage chemoattractant CCL2/MCP1 and IL-8 and enhance the expression of extracellular adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 (vascular cell adhesion molecule 1), which facilitates leukocyte extravasation. Thus, PAR2 overexpression during inflammatory processes is critically related to the development of pathological mechanisms typical of vascular and respiratory inflammatory processes.

Previous research has shown that animal models of PAR2-deficient mice have delayed immune responses, which would highlight the importance of this receptor in the development of the

immune response. It has also been found that the epithelial expression of PAR2 in patients with asthma is quantitatively and qualitatively different from the expression of this receptor in people without asthma (Knight *et al.*, 2001). In addition, studies in animal models have shown that PAR2 activation in certain subpopulations of sensory neurons leads to an increase in TRPV1 (transient receptor potential vanilloid 1)-mediated cough, which again highlights the role of PAR2 activation in the development of COPD symptomatology (Gatti *et al.*, 2006).

Although numerous studies have highlighted the role of PAR2 in the development of inflammatory diseases such as COPD, there is growing evidence that the PAR2 receptor has a protective effect on the airways. Cocks et al. (1999) was able to demonstrate that activation of PAR2 by trypsin in the respiratory epithelium leads to airway relaxation and inhibition of bronchoconstriction. It is due to irreversible activation of the receptor and desensitisation of the receptor to inactivation mechanisms that the activated inflammatory responses persist over time and cause extensive damage.

2.3. Toll-Like Receptors (TLRs)

TLRs are essential in the initiation of the immune response as they belong to the family of pathogen recognition receptors (PRRs). These receptors can recognise pathogen-associated molecular patterns (PAMPs) such as LPS from Gram-negative bacteria, peptidoglycans from Gram-positive bacteria or viral double strand RNA (dsRNA) or cell damage-associated molecular patterns (DAMPs) such as heat-shock proteins or extracellular matrix components such as fibrinogen. Based on ligand recognition, TLRs generate a rapid response to the presence of pathogens in the organism or to the alteration of cellular homeostasis (El-Zayat, Sibaii and Mannaa, 2019).

TLR receptors are composed of 3 different domains: the extracellular leucine repeat-rich domain (LRRs) responsible for ligand recognition, the transmembrane domain, and the intracellular Toll/IL-1 receptor (TIR) domain that mediates the transmission of signals from the cell surface to adaptor proteins. TLRs can be located in endosomal membranes such as the endoplasmic reticulum membrane or the cell membrane and are expressed in most cells of the innate immune system (Sidletskaya, Vitkina and Denisenko, 2020).

Ligand binding to TLR can initiate two different types of signalling cascades: the MyD88-dependent pathway mediated by the MyD88/TIRAP adaptor protein complex, or the TRIF-dependent pathway mediated by the TRIF/TRAM adaptor protein complex. While the first pathway results in early activation of NF-κB for the synthesis of proinflammatory molecules, the second pathway leads to late activation of NF-κB and phosphorylation of the transcription factor IRF3 which regulates the expression of type 1 IFN genes important for antiviral immunity (Sidletskaya, Vitkina and Denisenko, 2020).

The respiratory epithelium, due to its constant exposure to airborne pathogens, functions as the first protective barrier against them. Thus, epithelial cells express TLR receptors on their surfaces to carry out antigen detection and initiate immune response and inflammation. Many studies have provided evidence for the contribution of dysregulated signalling of these receptors to the development of chronic inflammatory diseases such as COPD (El-Zayat, Sibaii and Mannaa, 2019).

2.3.1. TLR2, TLR4 and TLR3

Several TLRs are involved in the pathogenesis of COPD and among them the role of TLR2 and TLR4 can be highlighted. Thanks to the different research carried out on the subject, it has been

possible to determine that exposure to tobacco smoke generates an increase in the expression of the genes coding for these TLRs (Sidletskaya, Vitkina and Denisenko, 2020). It has also been concluded that TLRs play an essential role in the development of COPD exacerbations due to the fact that TLR2 and TLR4 are the main mediators of the immune response against microbial invasion of the airways (Zuo *et al.*, 2015).

While TLR2 activation results in activation of the MyD88-dependent pathway, TLR4 activation can result in activation of both the MyD88-dependent and TRIF-dependent pathways. This means that the activation of both receptors by components of tobacco smoke or by microbial components results in the engagement of the NF-κB pathway leading to the consequent synthesis of proinflammatory cytokines such as IL-6, IL-8 or IL-1β and chemokines that will aid the recruitment of monocytes and neutrophils into the lung. In turn, the recruited cells mediate the secretion of proteolytic enzymes that contribute to lung destruction and TGF-β1 that mediates airway remodelling (Sidletskaya, Vitkina and Denisenko, 2020).

As shown in **figure 4**, TLR4 activation is mediated by the CD14 molecule. CD14 has several roles in the activation and function of the TLR4 receptor when activated by LPS. On the one hand, CD14 is the molecule responsible for transporting extracellular LPS to the TLR4/MD2 complex on the cell membrane to mediate the initial activation of the receptor and activate the MyD88-dependent pathway that culminates in the rapid activation of the transcription factor NF-κB and the subsequent production of pro-inflammatory cytokines. This signalling pathway is followed by the TRIF-dependent pathway that occurs at the intracellular level and results in the upregulation of type 1 IFN gene expression and late activation of NF-κB. It is here that CD14 again plays a role as it is primarily responsible for mediating the internalisation of the TLR4 receptor so that this TRIF-dependent pathway can be activated and the TLR4 receptor is ultimately degraded (Ciesielska, Matyjek and Kwiatkowska, 2021).

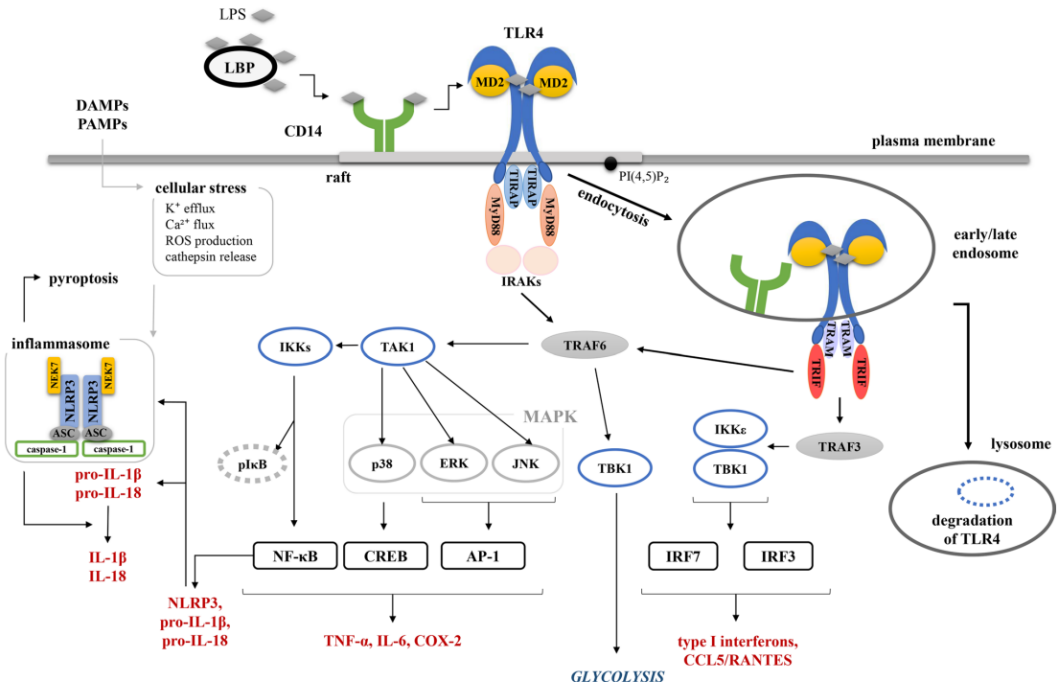

FIGURE 4. CD14 FIRST MEDIATES THE BINDING OF EXTRACELLULAR LIPOPOLYSACCHARIDE (LPS) TO THE TOLL-LIKE RECEPTOR 4 (TLR4) EXPRESSED ON THE CELL SURFACE LEADING TO ACTIVATION OF THE MY-D88-DEPENDENT PATHWAY CULMINATING IN EARLY ACTIVATION OF NUCLEAR FACTOR KB (NF-KB) AND RAPID

SECRETION OF PROINFLAMMATORY CYTOKINES SUCH AS IL-6 AND IL-8. AFTER THIS, CD14 MEDIATES THE INTERNALISATION OF TLR4 ACTIVATING THE TRIF-DEPENDENT PATHWAY CULMINATING IN THE ACTIVATION OF TYPE 1 INTERFERON (IFN) GENES AND THE LATE ACTIVATION OF NF-κB AND LATE SECRETION OF CYTOKINES. EXTRACTED FROM (CIESIELSKA, MATYJEK AND KWIATKOWSKA, 2021).

TLR2 is involved in the defence against pathogens entering the airways. Some studies have been able to determine that in COPD patients the antibacterial response is more attenuated than in patients with lung health in a way that demonstrates that there is a decrease in the synthesis of antimicrobial defence peptides due to truncated TLR2 signalling (Amatngalim *et al.*, 2017).

The role of TLR2 and TLR4 in the development of COPD exacerbations has been highlighted as these two receptors are the TLRs that are most responsive to bacterial and viral invasion of the airways. During exacerbations there is increased expression of TLR4 on bronchial epithelial cells resulting in increased inflammatory signalling and increased lung destruction (Pace *et al.*, 2013).

Regarding the involvement of TLR3 in the pathogenesis of COPD, the role of this receptor in airway inflammation has been discovered in recent years and it has been demonstrated that it is able to recognize viral dsRNA and that it is widely expressed in different respiratory cell types.

Studies such as that carried out by (Koarai *et al.*, 2010) have also been able to demonstrate using bronchial epithelial cells that oxidative stress generated by substances present in tobacco smoke enhances the immune response of airway epithelial cells through an increase in TLR3 expression, which in turn leads to an increase in nuclear translocation of NF-κB and greater inflammation.

2.4. Crosstalk between PAR2 and TLRs

It has been widely proven that PAR2 and TLR4 may have some interaction in the development of the immune response. In this sense, it has been demonstrated that the simultaneous activation of PAR2 by its corresponding activating peptide and TLR4 by LPS amplifies the activation of NF-κB resulting in an increase in the production of inflammatory cytokines by epithelial cells (Rallabhandi *et al.*, 2008).

The interaction of these two receptors has also been demonstrated in diseases such as colitis caused by *Citrobacter rodentium* in which inflammation is mediated by both receptors or the inflammatory response generated in response to *Aspergillus* infection where TLR4 mediates suppression of the PAR2 signalling pathway (Rallabhandi *et al.*, 2008).

On the other hand, TLR3 and PAR2 can interact and generate a synergy that leads to an increase in the inflammatory response. When these receptors are co-stimulated at the same time by their respective ligands, an increase in the production of proinflammatory mediators such as IL-8 is generated from an enhancement of NF-κB nuclear translocation (Nhu *et al.*, 2009).

Due to these previous demonstrations of the interaction between PAR2 and TLRs in other inflammatory diseases, the colocalization of both types of receptors on the epithelial cell membrane and the essential role of these two receptors in the development of the immune response and inflammation, it is essential to know whether these also interact in the pathophysiology of COPD.

3. HYPOTHESIS AND OBJECTIVES

The hypothesis on which this project is based is that a crosstalk might exist between the PAR2 receptor and the TLR receptors in the development of the inflammatory response in COPD.

The main objective of this work is to learn more about the role of PAR2 in the development of COPD inflammation by studying its expression in A549 cells (human epithelial cells from the lung) using immunofluorescence staining. Another objective is to study the expression of TLR receptors, in particular TLR2, TLR3 and TLR4, on these cells and to assess certain aspects of the possible interaction between PAR2 and TLR receptors during COPD exacerbations by carrying out different bioassays.

4. MATERIALS AND METHODS

4.1. Cell culture maintenance

A549 cells are a human type II epithelial cell line from a lung tumour, and they were sourced from the American Type Culture Collection (ATCC). These cells were frozen in aliquots at -70°C, so to establish the cell culture, they had to be progressively thawed and incubated at room temperature. Once thawed, the cells were washed with phosphate-buffered saline (PBS) and centrifuged at 1200 rpm for 6 minutes. Similar to other studies such as the one conducted by Schulz *et al.* (2002) where A549 cells were also used, the pellet of cells obtained was resuspended with RPMI medium supplemented with streptomycin/penicillin at 1% (v/v) and fetal calf serum (FCS) at 5% (v/v) and the cell suspension obtained was transferred to a 75 cm^3 culture flask to begin the cell acclimation process at 37°C and 5% CO_2.

After the acclimatisation period of approximately 5 days, it was possible to carry out a cell passage and use the cells to run different assays. For this, the medium was first removed and reserved, the monolayer of cells adhering to the culture flask floor was washed with PBS and a volume of about 2.5 mL of trypsin/EDTA was added and left to act for about 6 minutes at 37°C. After this, the previously reserved medium was added to inhibit the effect of trypsin and the cell suspension was collected. In case the cell concentration was needed, a cell count could be carried out from a ½ dilution of cell suspension with trypan blue using a Neubauer chamber or the TC20 automatic counter (Bio-Rad).

The suspension obtained was then centrifuged at 1200 rpm for 6 minutes to obtain a clean cell pellet. The pellet obtained was resuspended with fresh RPMI / 5% FCS medium. From this fresh cell suspension, the volume necessary to obtain the desired cell concentration was taken and supplemented with additional fresh medium.

4.2. Detection of PAR2, TLR3 and TLR4 using immunofluorescence

To carry out the immunofluorescence protocol, cells were seeded on round coverslips in wells of a 24-well plate. In each well, 1 mL of cell suspension was placed at a concentration of $5x10^4$ cell/mL, and the cells were left to grow for 24 hours. After the growth period cell fixation was carried out by incubating the cells for 10 minutes with Invitrogen's fixative solution made up of 4% formaldehyde prepared in PBS.

Detection of the PAR2 receptor in A549 cells was carried out using an anti-PAR2 (rabbit polyclonal; Alomone Labs) primary antibody and an anti-rabbit 568 (red) secondary antibody both at a concentration of 1/200. Cells were also incubated with Phalloidin 488 (green) at a concentration of 1/400 for F-actin staining.

For the detection of TLR3 and TLR4 the primary antibodies used were the anti-TLR3 and anti-TLR4 antibodies (mouse monoclonals; Novus Biologicals; both from a stock solution at 1mg/mL),

respectively, which were used at a concentration of 1/500 and 1/1000. The secondary antibody was a goat anti-mouse antibody 568 at a concentration of 1/200 and the slides were also incubated with Phalloidin 488 at a concentration of 1/400.

Detection of these receptors was performed using an immunofluorescence protocol similar to that previously described in studies such as the one performed by Ciencewicki et al. (2006). Briefly, the coverslips with the cells were first blocked and permeabilised for 15 minutes with 5% BSA solution prepared in PBS-Tx-100 (0.05%). After this, cells were left incubating with the primary antibody for 1 hour at room temperature inside a wet chamber and then they were washed 3 times with PBS-Tx-100 (0.05%). They were then incubated for another hour with the secondary antibody and the Phalloidin and washed again 3 times with PBS-Tx-100 (0.05%). The coverslips were finally washed with distilled water to remove any remaining PBS and they were mounted onto microscope slides using DAPI Fluoromont-G (SouthernBiotech).

Visualisation and imaging of the stained cells was performed using the fluorescence microscope Nikon Eclipse i80 and ImageJ software.

4.3. Cell Bioassays

4.3.1. Inhibition of PAR2 using AZ8838

For this assay, two 96-well plates were seeded at a concentration of $5x10^4$ cells/mL with a volume of 200 µL of cell suspension per well. The cells were grown at 37°C and 5% CO_2 for 24 hours.

The aim of this assay was to study the effect of PAR2 on the response of TLR4 activated by different concentrations of LPS (1000 ng/mL, 100 ng/mL, 10 ng/mL, 1 ng/mL, 0.1 ng/mL), a natural component of the cell wall of Gram-negative bacteria, to simulate an exacerbation episode. For this, PAR2 inhibition was carried out using the inhibitor AZ8838 (gift from Professor Robin Plevin, University of Strathclyde) at a final concentration of 30 µM (100 mM stock) to compare the TLR4-mediated response when PAR2 is inhibited and when it is active. Since AZ8838 is prepared in DMSO, a vehicle control was included in this assay to ensure that DMSO has no effect on the cellular response. For this a final concentration of DMSO of 0.06% was used.

As can be seen in **figure 5**, each different condition was carried out in triplicate to obtain more reliable results. For each of the treatments, a control without LPS was included.

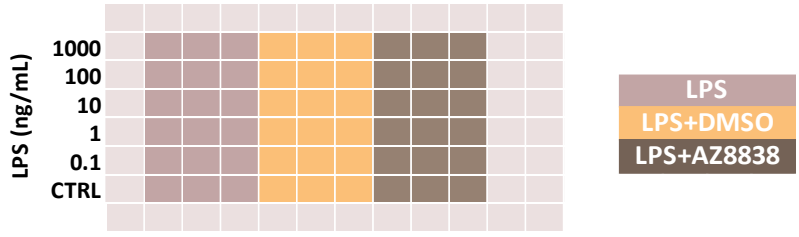

FIGURE 5. SCHEMATIC DIAGRAM OF THE 96-WELL PLATE SET-UP FOR THIS BIOASSAY.

The different LPS concentrations were all prepared with RPMI / 5% FCS. All concentrations (LPS, DMSO and AZ8838) were prepared at a working concentration of 2X. This is because after adding 100 µL of the LPS concentrations to the corresponding wells, 100 µL of RPM with no FCS, DMSO or AZ8838 were added to the wells, diluting the different concentrations and FCS by half to obtain 1X concentrations. As a result, all wells ended up with an FCS concentration of 2.5%.

After setting up the assay, one of the plates was left with the treatments for 24 hours and the other one was left with the treatments for 48 hours to compare the impact that treatment duration has on the cells.

4.3.2. Stimulation of TLR4 using CD14

For this assay, two 96-well plates were seeded at a concentration of $5x10^4$ cells/mL with a volume of 200 µL of cell suspension per well. The cells were grown at 37°C and 5% CO_2 for 24 hours.

CD14 is a molecule physiologically available to TLR4 and its presence stimulates and regulates TLR4 activity. This assay aims to determine the effect of this molecule on the TLR4 response to different concentrations of LPS (1000 ng/mL, 100 ng/mL, 10 ng/mL, 1 ng/mL, 0.1 ng/mL) in A549 cells. For this, two different working concentrations of CD14 (2 ng/mL and 8 ng/mL) were used to obtain two final concentrations of 1 ng/mL and 4 ng/mL.

As seen in **figure 6**, each different condition was carried out in triplicate to obtain more reliable results. For each of the treatments, a control without LPS was included.

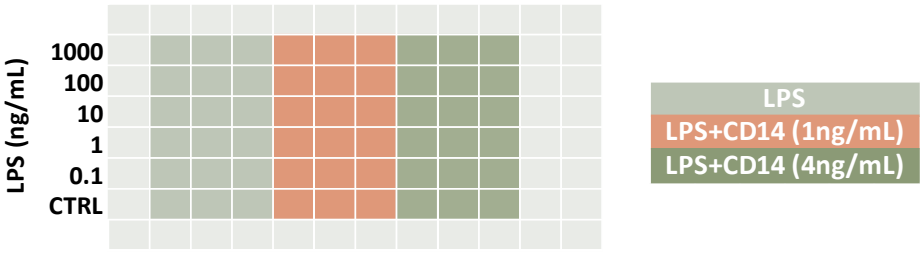

FIGURE 6. SCHEMATIC DIAGRAM OF THE 96-WELL PLATE SET-UP FOR THIS BIOASSAY.

The different LPS concentrations were all prepared with RPMI / 5% FCS. All concentrations (LPS and CD14) were prepared at a working concentration of 2X. This is because after adding 100 µL of the LPS concentrations to the corresponding wells, 100 µL of RPM with no FCS or the corresponding CD14 concentration were added to the wells, diluting the different concentrations and FCS by half to obtain 1X concentrations. As a result, all wells ended up with an FCS concentration of 2.5%.

After setting up the assay, one of the plates was left with the treatments for 24h and the other one was left with the treatments for 48h to compare the impact that treatment duration has on the cells.

4.3.3. TLR and PAR2 stimulation: LPS, Pam3SCK4, Poly(I:C) and FLIGRL

For this assay, two 96-well plates were seeded at a concentration of $5x10^4$ cells/mL with a volume of 200 µL of cell suspension per well. The cells were grown at 37°C and 5% CO_2 for 24 hours.

In this assay, the stimulation of TLR and PAR2 receptors was carried out in A549 cells to determine the effect of the activation of these receptors on the development of the inflammatory response. RPMI / 5% FCS was added to one plate while RPMI / 5% FCS with DMSO at a final concentration of 0.06% was added to the other plate to confirm that DMSO has no influence on the response of the cells to the different stimulations. **Table 2** shows the various concentrations of the different agonists used:

TABLE 2. LIST OF AGONISTS USED TO PERFORM THE TLR AND PAR2 STIMULATION BIOASSAYS WITH THE CONCENTRATIONS USED FOR EACH OF THEM AND THE ORIGINAL CONCENTRATION IN THE STOCK SOLUTION.

Name of agonist	Receptor	Nature	Concentrations used	Stock solution
LPS	TLR4	Natural component of the outer membrane of Gram-negative bacteria	1000, 100, 10, 1, 0.1 ng/mL	1mg/mL
Pam3CSK4	TLR2	Synthetic analogue of the active N-terminal portion of bacterial lipoprotein	10, 1, 0.1, 0.01, 0.001 ng/mL	1mg/mL
Poly(I:C)	TLR3	Synthetic dsRNA analogue	10, 1, 0.1, 0.01, 0.001 µg/mL	1mg/mL
FLIGRL	PAR2	Synthetic agonist peptide mimicking the tethered ligand of PAR2	100 and 10µM	10mM

Each different condition was carried out in triplicate to obtain more reliable results. For each of the stimulations, a control without agonist was included. The layout of both plates was the one shown in **figure 7**.

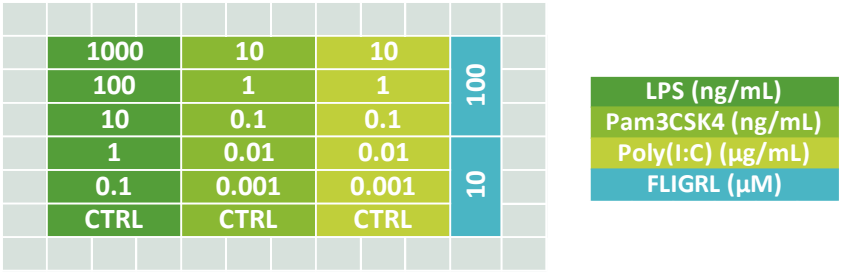

FIGURE 7. SCHEMATIC DIAGRAM OF THE 96-WELL PLATE SET-UP FOR THIS BIOASSAY.

All the agonists' concentrations were prepared with RPMI without FCS and were also prepared at a 2X concentration. This is because after adding 100 µL of the treatments to the corresponding wells, 100 µL of RPMI / 5% FCS or RPMI / 5% FCS + DMSO was added to the wells, diluting the agonists and FCS by half to obtain the desired concentrations. As a result, all wells ended up with an FCS concentration of 2.5%. After setting up the assay, the plates were left with the agonists for 24h.

4.4. MTT assay

On the cells used for the stimulation and inhibition assays, an MTT assay could be performed to determine the viability of these cells after subjecting them to the different treatment conditions. MTT reagent (Triazolyl Blue Tetrazolium Bromide) was prepared at 5 mg/mL in PBS and added at a 1/10 dilution (dilution made with RPMI without FCS) to the cells and left to incubate for 4 hours at 37°C.

In metabolically active cells the MTT reagent can cross the cell membrane due to its positive charge and lipophilic nature to be reduced to formazan blue crystals by the action of mitochondrial dehydrogenases. The medium with the MTT was carefully removed and the formazan crystals were solubilised using DMSO and the absorbance could be read at 570nm using a Multiskan Sky plate spectrophotometer (Thermo Scientific).

4.5. Determination of cytokines

Commercially available sandwich ELISA (Enzyme-linked immunosorbent assay; paired antibodies) was used to carry out the detection of different inflammatory cytokines of interest, including IL-6 and IL-8, in the supernatant of the cells used in the bioassays discussed previously.

The determination of cytokine concentration in the cell supernatant could be carried out by using a standard of known concentrations.

The following kits with their corresponding protocols were used to carry out this determination:

- Human IL-6 uncoated ELISA kit (Invitrogen)
- Human IL-8 uncoated ELISA kit (Invitrogen)

As shown in **figure 8**, the ELISA microplates were first coated with the capture antibody overnight. The next day, after a blocking step, standards were added to duplicate wells (IL-6: 0-200 pg/mL; IL-8: 0-250 pg/mL) and the cellular supernatant was added into the wells in a ½ dilution to bind to the capture antibody. Plates were incubated at room temperature for 2 hours after which plates were washed 3 times with wash buffer (PBS and 0.05% Tween 20). This was followed by the addition of the detection antibody conjugated with biotin and the incubation of the plates for 1 hour. Plates were washed 3 times with wash buffer followed by the addition of the enzyme HRP (horseradish peroxidase) conjugated with streptavidin. After 30 minutes of incubation, plates were washed 3 times and the substrate solution was added to the wells to generate a colorimetric reaction. This was stopped by the addition of 12.5% acid solution (H_2SO_4). Absorbance was read at 450nm using the Multiskan Sky plate spectrophotometer (Thermo Scientific).

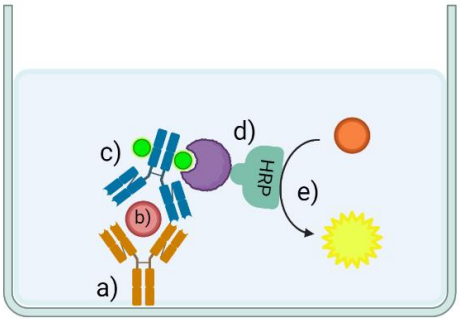

FIGURE 8. SCHEME OF A SANDWICH ELISA. A) CAPTURE ANTIBODY (AB). B) CYTOKINE BOUND TO CAPTURE AB. C) DETECTION AB CONJUGATED TO BIOTIN. D) HRP CONJUGATED TO STREPTAVIDIN. E) SUBSTRATE CONVERSION TO DETECTABLE SIGNAL. ILLUSTRATED WITH BIORENDER.COM.

4.6. Statistical analysis

The statistical analysis of the results was performed by applying the Student's T-test with two tails to find significant differences when comparing the means between the different groups of samples studied. To perform this statistical test, a significance level of 5% was used, the p-value was determined for each comparison of means and a significant difference was considered when $P \leq 0.05$.

5. RESULTS

5.1. Immunofluorescence staining of A549 cells

5.1.1. Detection of PAR2 in A549 cells

Detection of the PAR2 receptor in A549 cells was possible following the immunofluorescence protocol explained above. **Figures 9A** and **9D** are the images obtained by generating the

composite of the three fluorescence channels using ImageJ software. These images show the presence of the PAR2 receptor in the perinuclear areas and in the cell cytoplasm.

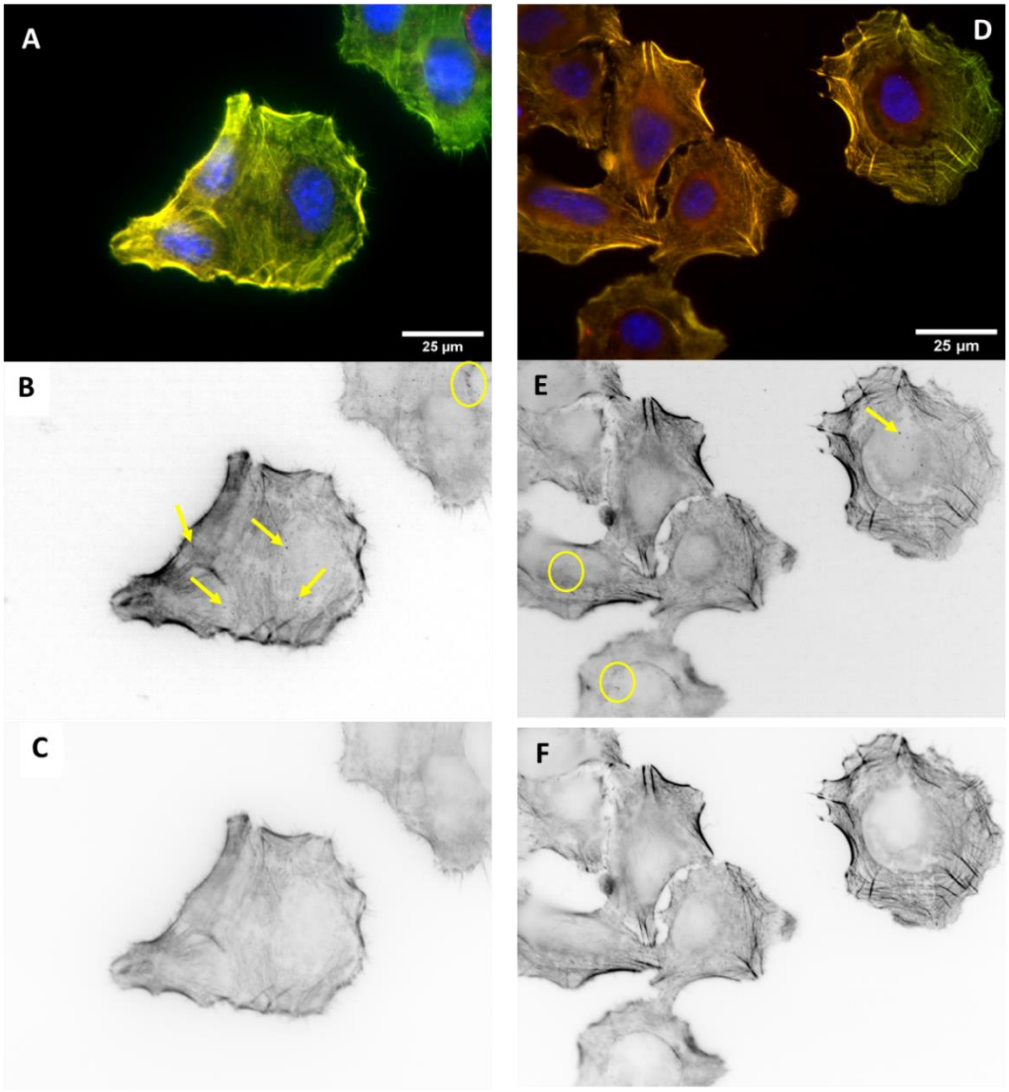

FIGURE 9. IMMUNOFLUORESCENCE STAINING OF A549 CELLS FOR THE DETECTION OF PAR2. A AND D ARE THE COMPOSITES OF THE THREE FLUORESCENCE CHANNELS: DAPI (CELL NUCLEUS IN BLUE), FITC (ACTIN IN GREEN) AND CY3 (TLR3 IN RED). IMAGES B AND E ARE THE GRAYSCALE INVERTED IMAGES OF THE DETECTION OF THE ANTI-RABBIT 568 SECONDARY ANTIBODY USED TO LOCALIZE PAR2 WHILE IMAGES C AND F ARE THE GRAYSCALE INVERTED IMAGE OF PHALLOIDIN 488. THESE IMAGES WERE OBTAINED AT 60X MAGNIFICATION.

Since there was too much overlap between the fluorescence of Phalloidin 488 and the fluorescence of the anti-rabbit 568 secondary antibody, images were also obtained inverted in greyscale for both fluorescence channels to better detect the PAR2 receptor. When comparing **figures 9B** and **9C**, in **image 9B**, which corresponds to the detection of the secondary antibody for PAR2, black dots corresponding to the detection of PAR2 are indicated with yellow arrows. Similarly, when comparing **figures 9E** and **9F**, these points of PAR2 detection can also be seen around the cell nucleus and in the cell cytoplasm in **figure 9E**.

5.1.2. Detection of TLR3 in A549 cells

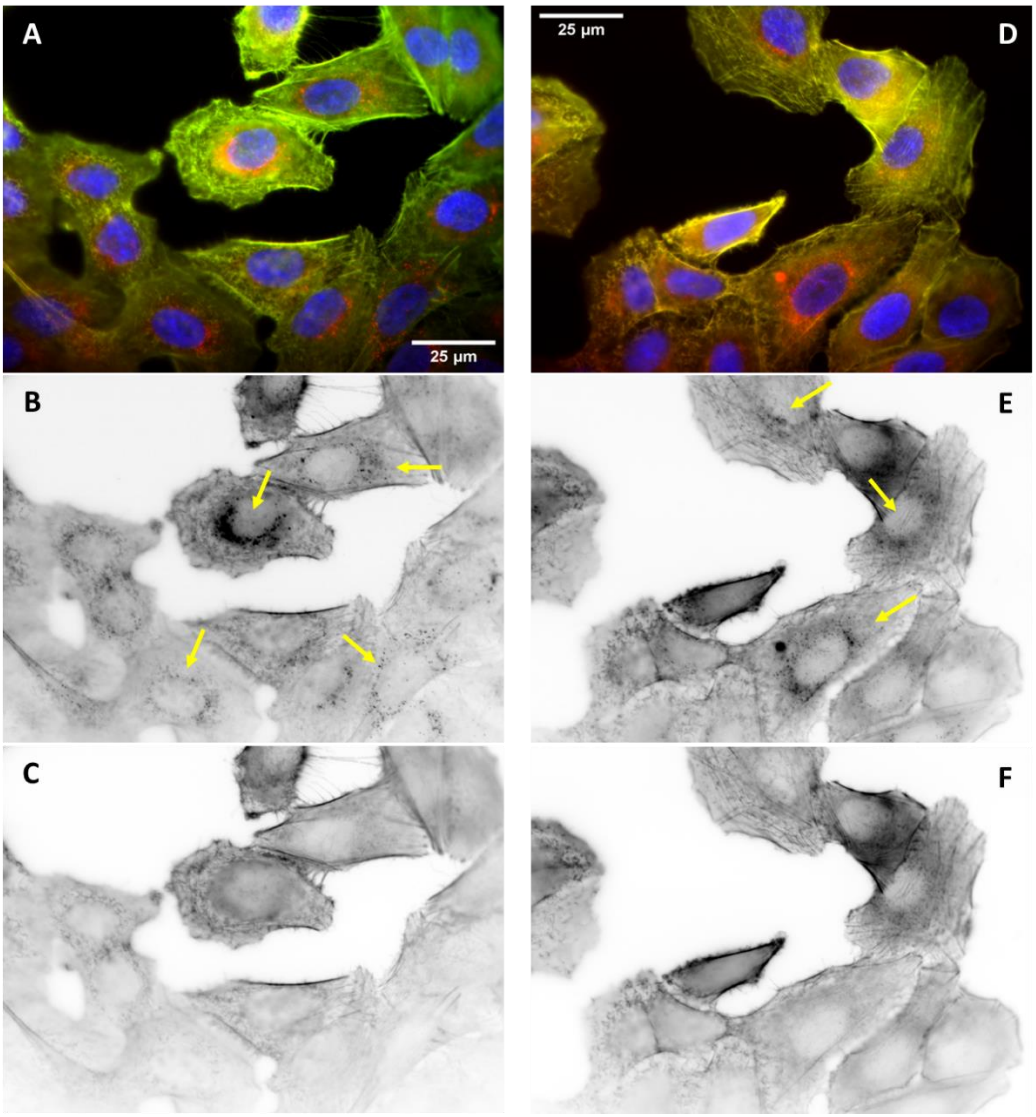

FIGURE 10. IMMUNOFLUORESCENCE STAINING OF A549 CELLS FOR THE DETECTION OF TLR3. TWO CONCENTRATIONS OF ANTI-TLR3 PRIMARY ANTIBODY WERE USED: A, B AND C CORRESPOND TO THE 1/500 CONCENTRATION WHILE D, E AND F CORRESPOND TO THE 1/1000 CONCENTRATION. A AND D ARE THE COMPOSITES OF THE THREE FLUORESCENCE CHANNELS: DAPI (CELL NUCLEUS IN BLUE), FITC (ACTIN IN GREEN) AND CY3 (TLR3 IN RED). IMAGES B AND E ARE THE GRAYSCALE INVERTED IMAGES OF THE DETECTION OF THE ANTI-MOUSE 568 SECONDARY ANTIBODY USED TO LOCALIZE TLR3 WHILE IMAGES C AND F ARE THE GRAYSCALE INVERTED IMAGE OF PHALLOIDIN 488. THESE IMAGES WERE OBTAINED AT 60X MAGNIFICATION.

As with the PAR2 detection images, detection of the TLR3 receptor in A549 cells was also possible thanks to the immunofluorescence protocol used. In this case, the detection of the receptor of interest can also be better appreciated by looking at the areas pointed by the yellow arrows in **images 10B** and **10E** and comparing them with the **images 10C** and **10F** corresponding to the detection of cellular actin.

As explained in the figure caption, the first column of **images 10A**, **10B** and **10C** is for TLR3 detection using a 1/500 concentration of primary antibody and the second column of **images 10D**, **10E** and **10F** is for receptor detection using a 1/1000 concentration of primary antibody. The use of two different concentrations was carried out according to the advice of the

manufacturer and was useful to determine the ideal concentration of antibody to use for better detection.

5.1.3. Detection of TLR4 in A549 cells

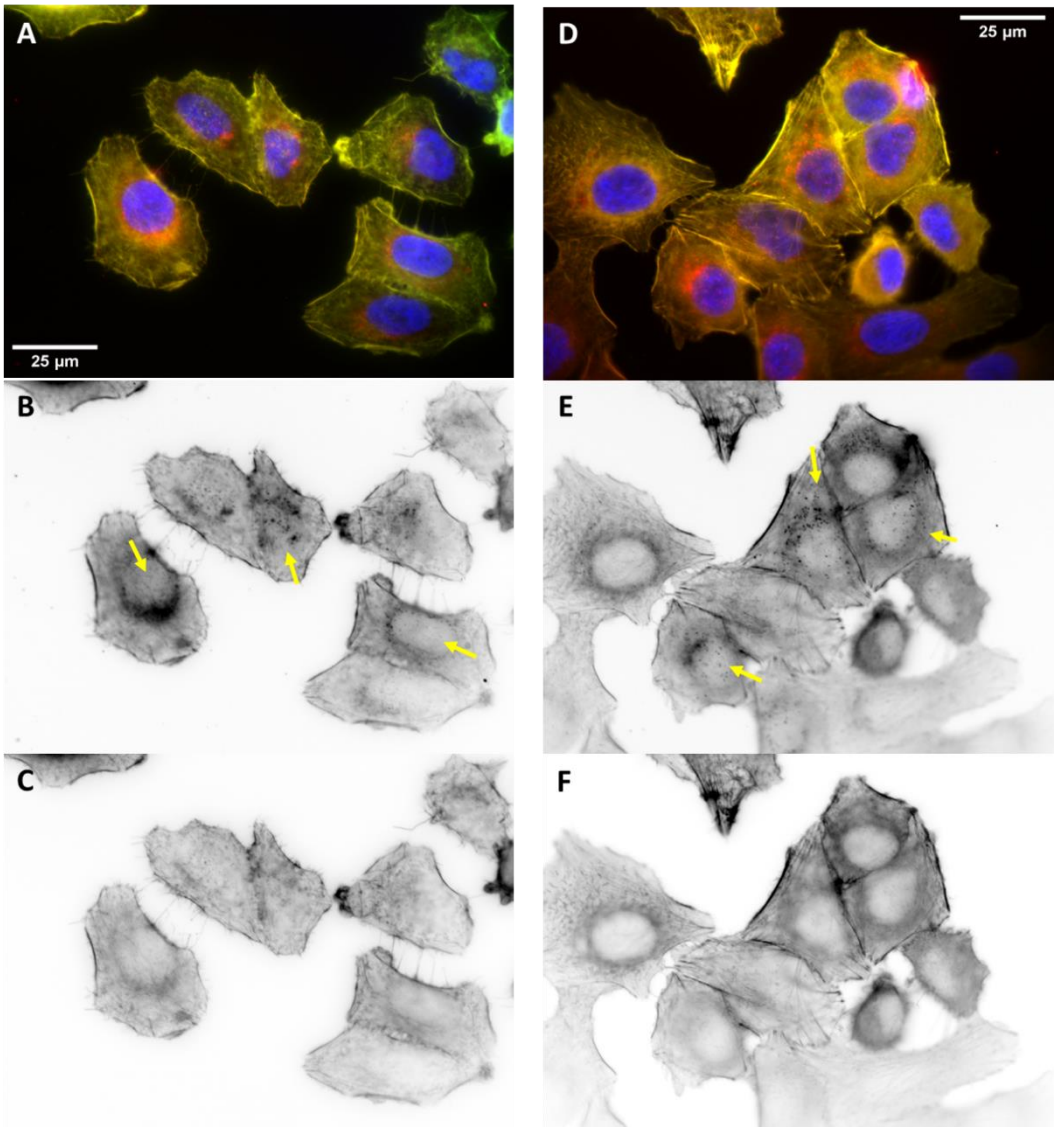

FIGURE 11. IMMUNOFLUORESCENCE STAINING OF A549 CELLS FOR THE DETECTION OF TLR4. TWO CONCENTRATIONS OF ANTI-TLR4 PRIMARY ANTIBODY WERE USED: A, B AND C CORRESPOND TO THE 1/500 CONCENTRATION WHILE D, E AND F CORRESPOND TO THE 1/1000 CONCENTRATION. A AND D ARE THE COMPOSITES OF THE THREE FLUORESCENCE CHANNELS: DAPI (CELL NUCLEUS IN BLUE), FITC (ACTIN IN GREEN) AND CY3 (TLR4 IN RED). IMAGES B AND E ARE THE GRAYSCALE INVERTED IMAGES OF THE DETECTION OF THE ANTI-MOUSE 568 SECONDARY ANTIBODY USED TO LOCALIZE TLR4 WHILE IMAGES C AND F ARE THE GRAYSCALE INVERTED IMAGE OF PHALLOIDIN 488. THESE IMAGES WERE OBTAINED AT 60X MAGNIFICATION.

Figure 11 shows images of TLR4 receptor detection in A549 cells after using the immunofluorescence protocol. As with PAR2 and TLR3, the presence of the receptor can be better appreciated by looking at the areas marked with arrows in **images 11B** and **11E**. Furthermore, as with TLR3 detection, two different concentrations of primary antibody were also used in this case, so the first column of images belongs to the images obtained after using the 1/500 concentration and the second column belongs to the 1/1000 concentration.

5.2. Analysis of cytokine release

After following the ELISA protocol explained in the methodology section and obtaining the equation of the standard line from the standards provided in the kits, this equation can be extrapolated to the absorbance values measured for each group of samples and a mean of the concentration of cytokines present in the cell supernatants can be obtained, together with the standard deviation for each group of samples, all this taking into account that the supernatant of the cells was added to the ELISA plates in a ½ dilution.

5.2.1. Role of PAR2 on TLR4-mediated inflammatory response: Inhibition assay with AZ8838

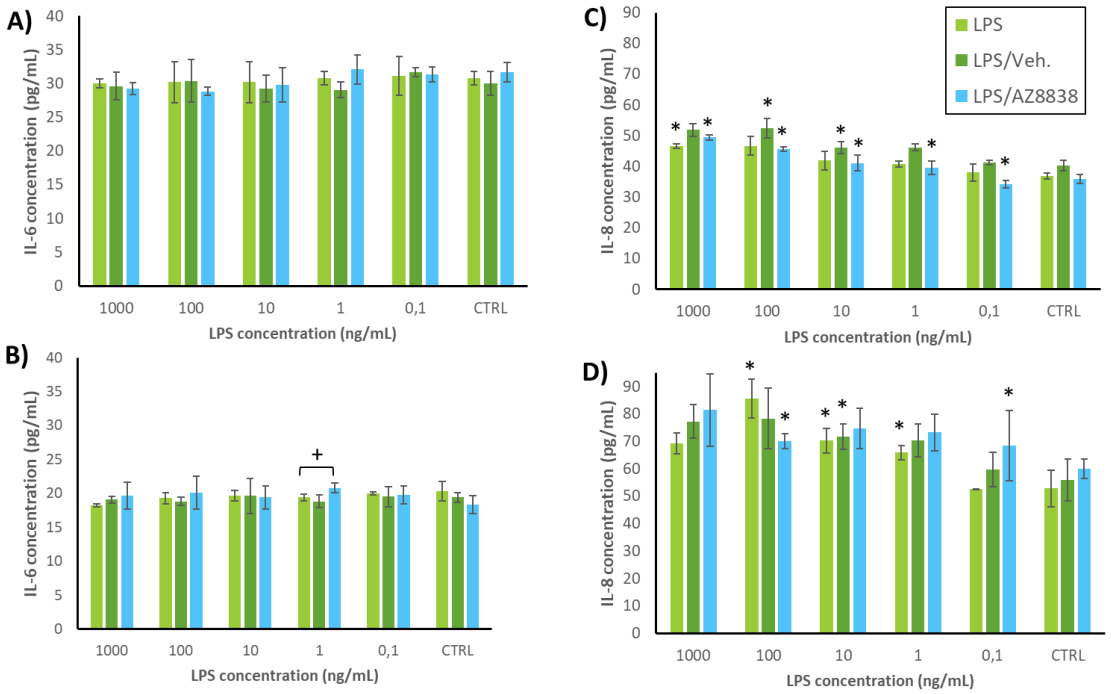

FIGURE 12. EFFECT OF PAR2 ON THE TLR4-MEDIATED RESPONSE IN A549 CELLS. IN THIS ASSAY, DIFFERENT CONCENTRATIONS OF LPS TO STIMULATE THE TLR4 RESPONSE, THE PAR2 INHIBITOR AZ8838 AND A VEHICLE (DMSO) CONTROL WERE USED. A) AND B) REPRESENT THE CONCENTRATION OF IL-6 IN THE CELL SUPERNATANT AFTER 24HR AND 48HR OF TREATMENT, RESPECTIVELY. C) AND D) REPRESENT THE CONCENTRATION OF IL-8 IN THE CELL SUPERNATANT AFTER 24HR AND 48HR, RESPECTIVELY. AFTER T-TEST ANALYSIS: *P ≤ 0.05 COMPARED TO THE CONTROL WITHOUT LPS, +P ≤ 0.05 COMPARING LPS TREATED CELLS WITH LPS/VEHICLE OR LPS/AZ8838 TREATED CELLS.

The aim of this assay was to study the effect of the PAR2 receptor on the TLR4-mediated inflammatory response in A549 cells. Different concentrations of LPS, a TLR4-activating molecule, were used to test the production of IL-6 and IL-8 in the absence and presence of AZ8838, a specific PAR2 inhibitor. For each concentration, a control without LPS was included.

When analysing **figure 12**, the first thing to note is that IL-6 secretion is much lower than IL-8. Furthermore, when comparing the concentration of IL-6 in the supernatant of cells treated for 24 hours (**figure 12A**) with those treated for 48 hours (**figure 12B**), a significant decrease of this cytokine can be seen. In contrast, in the case of IL-8 concentration, a significant increase can be seen from the 24-hour treatment (**figure 12C**) to the 48-hour treatment (**figure 12D**).

In the case of IL-6 no significant differences could be found between the different treatments and their respective controls while, as can be seen in the figure, in the case of IL-8 there are significant differences with respect to the respective controls in each condition. Also, no significant differences can be observed between the LPS treatment and the LPS+DMSO and LPS+AZ8838 treatments.

5.2.2. Stimulation of TLR4-mediated inflammatory response with CD14

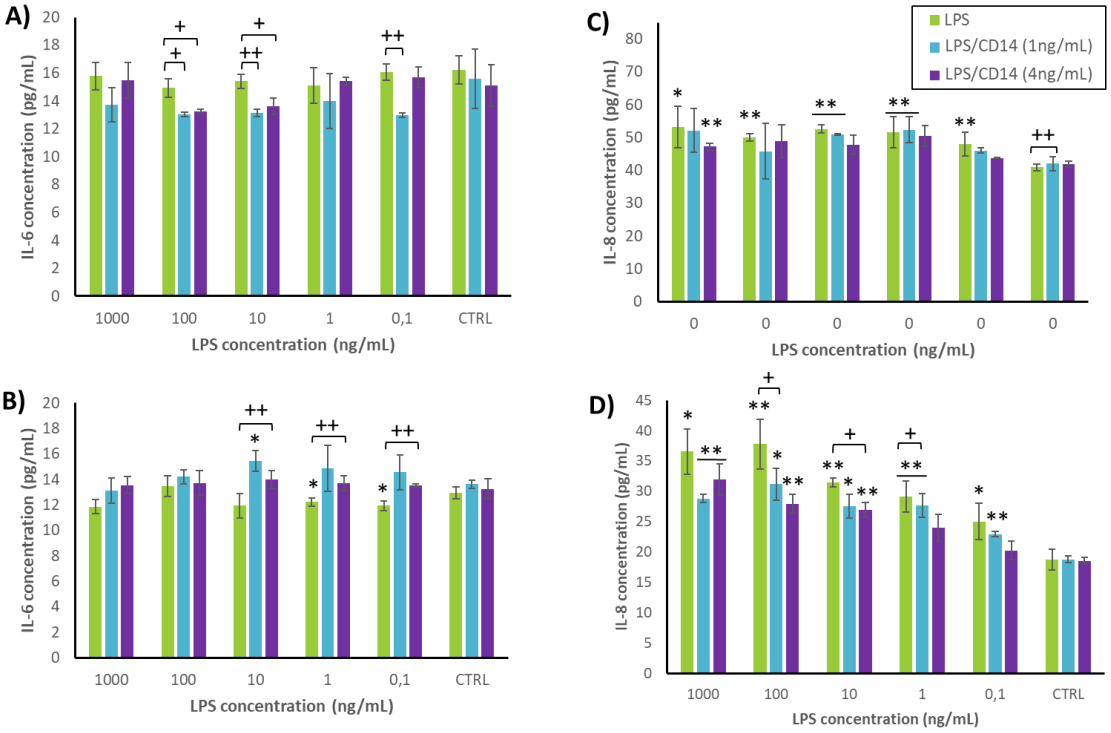

FIGURE 13. EFFECT OF CD14 ON THE TLR4-MEDIATED RESPONSE IN A549 CELLS. IN THIS ASSAY, DIFFERENT CONCENTRATIONS OF LPS TO STIMULATE THE TLR4 RESPONSE AND TWO DIFFERENT CONCENTRATIONS OF CD14 (1 NG/ML, 4 NG/ML) WERE USED. A) AND B) REPRESENT THE CONCENTRATION OF IL-6 IN THE CELL SUPERNATANT AFTER 24HR AND 48HR OF TREATMENT, RESPECTIVELY. C) AND D) REPRESENT THE CONCENTRATION OF IL-8 IN THE CELL SUPERNATANT AFTER 24HR AND 48HR, RESPECTIVELY. AFTER T-TEST ANALYSIS: *P ≤ 0.05, **P ≤ 0.01 COMPARED TO THE CONTROL WITHOUT LPS, +P ≤ 0.05, ++P ≤ 0.1 COMPARING LPS TREATED CELLS AND LPS/CD14 TREATED CELLS.

This assay was performed to determine the effect of CD14 on the TLR4-mediated inflammatory response in A549 cells. This molecule mediates TLR4 activation and internalisation.

With the shown results, it is confirmed again that IL-6 production by A549 cells is significantly lower than IL-8 production. In the case of IL-6 no large significant differences are observed between the different conditions and the respective controls without LPS. When analysing **figure 13A** of cells treated for 24 hours, it appears that there is a significant decrease between IL-6 production in cells treated with LPS alone and production in cells treated with CD14. In contrast, in **figure 13B** of cells treated for 48 hours, a significant increase in IL-8 production is observed in cells treated with a combination of 10, 1 and 0.1 ng/mL LPS and 4 ng/mL CD14.

Several significant differences can be seen regarding IL-8 concentration between the different conditions and their respective controls, which would confirm that the different conditions to which the cells are being subjected have an impact on IL-8 concentration. Particularly in **figure 13D** IL-8 production is dependent on the dose of LPS administered since the lower the LPS

concentration, the lower the production of this cytokine. Also in this figure, significant decreases can be seen between cells treated with LPS alone and cells treated with LPS and CD14.

5.2.3. Stimulation of TLRs and PAR2 using different agonists

As explained above in the methodology section, in this assay different specific agonists were used to stimulate TLR2, TLR3, TLR4 and PAR2 receptors to simulate an exacerbation episode and to see the effect of the stimulation of these receptors on the extracellular concentration of proinflammatory cytokines.

Another aim of this assay was to compare the treatment when applied with RPMI / 2.5% FCS medium to when applied with RPMI / 2.5% FCS supplemented with DMSO (0.06%). DMSO is the vehicle in which the PAR2 inhibitor AZ8838 is prepared, so it is important to determine whether DMSO interferes with cytokine production for future experiments aimed at inhibiting PAR2 action and stimulate the aforementioned TLRs with their agonists to assess TLR-PAR2 crosstalk.

5.2.3.1. Stimulation of TLR4 with agonist LPS

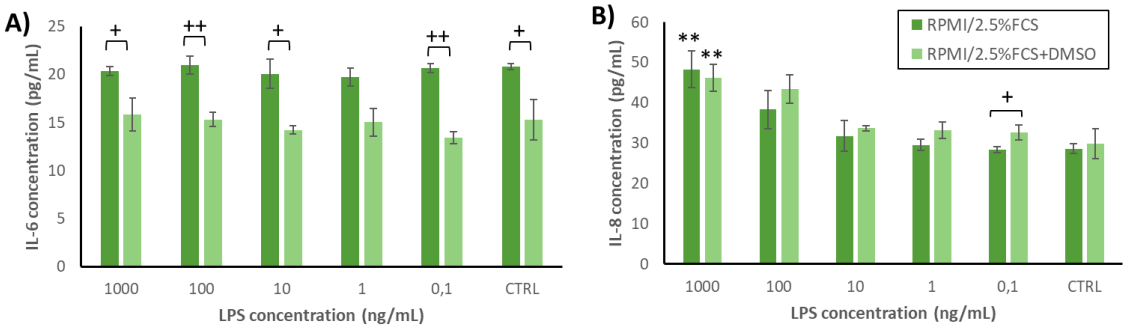

FIGURE 14. EFFECT OF DIFFERENT CONCENTRATIONS OF LPS ON IL-6 (FIGURE A) AND IL-8 (FIGURE B) PRODUCTION IN A549 CELLS. TWO DIFFERENT CONDITIONS, CELLS TREATED WITH LPS + RPMI / 2.5% FCS AND CELLS TREATED WITH LPS + RPMI / 2.5% FCS + DMSO (0.06%), WERE USED IN THIS ASSAY TO DETERMINE THE EFFECT OF DMSO ON CELL RESPONSE. AFTER T-TEST ANALYSIS: *P ≤ 0.05, **P ≤ 0.01 COMPARED TO THE CONTROL WITHOUT AGONIST, +P ≤ 0.05, ++P ≤ 0.1 COMPARING LPS + RPMI / 2.5% FCS TREATED CELLS AND LPS + RPMI / 2.5% FCS + DMSO (0.06%) TREATED CELLS.

The aim of this assay was to study the effect of LPS on IL-6 and IL-8 production in A549 cells. In this assay, LPS is used as a specific agonist for the TLR4 receptor and different concentrations are used to determine if there is a dose-dependent response from the cells.

Again, A549 cells seem to produce more IL-6 than IL-8. In the case of IL-6 (**figure 14A**) there are no major differences between the different conditions and their respective controls without agonist, but there are major differences between the two medium conditions used, with significant decreases of even P ≤ 0.01 when DMSO is present in the medium.

On the other hand, significant differences in IL-8 concentration from the control can be seen in **figure 14B** when using an LPS concentration of 1000 ng/mL. In addition, it again appears that IL-8 production is dependent on the dose of LPS administered since the lower the LPS concentration, the lower the production of this cytokine. No significant differences can be seen between the use of RPMI and RPMI with DMSO.

5.2.3.2. Stimulation of TLR2 with agonist Pam3CSK4

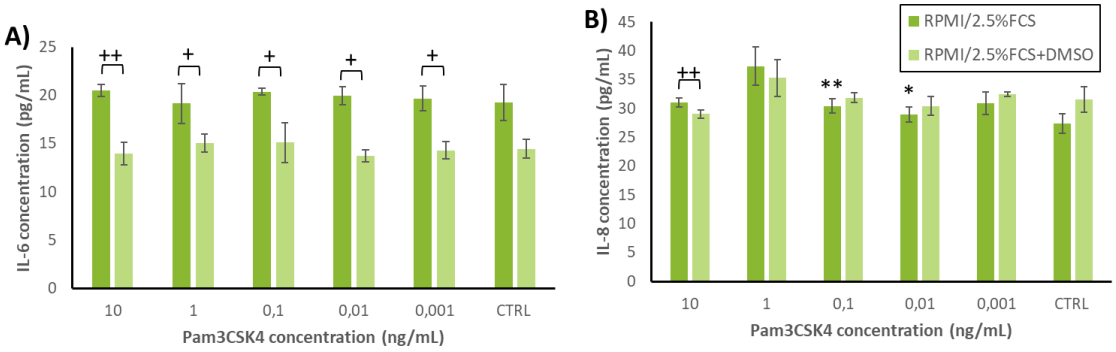

FIGURE 15. EFFECT OF DIFFERENT CONCENTRATIONS OF PAM3CSK4 ON IL-6 (FIGURE A) AND IL-8 (FIGURE B) PRODUCTION IN A549 CELLS. TWO DIFFERENT CONDITIONS, CELLS TREATED WITH PAM3CSK4 + RPMI / 2.5% FCS AND CELLS TREATED WITH PAM3CSK4 + RPMI / 2.5% FCS + DMSO (0.06%), WERE USED IN THIS ASSAY TO DETERMINE THE EFFECT OF DMSO ON CELL RESPONSE. AFTER T-TEST ANALYSIS: *P ≤ 0.05, **P ≤ 0.01 COMPARED TO THE CONTROL WITHOUT AGONIST, +P ≤ 0.05, ++P ≤ 0.1 COMPARING PAM3CSK4 + RPMI / 2.5% FCS TREATED CELLS AND PAM3CSK4 + RPMI / 2.5% FCS + DMSO (0.06%) TREATED CELLS.

This part of the assay aimed to determine the effect of the TLR2-specific agonist Pam3CSK4 on the response generated by A549 cells. Again, a series of serial dilutions were used to obtain different concentrations of the agonist and to study whether there is a dose-dependent response.

In the case of IL-6, in **figure 15A** no significant differences exist when comparing the different conditions with their corresponding controls without agonist, although, similarly to what was seen in the LPS stimulation, significant decreases in IL-6 levels can be seen when DMSO is present in the treatment. With respect to IL-8, although its production is higher than IL-6, no major differences are observed between the different conditions and their respective controls, nor between RPMI medium and RPMI medium supplemented with DMSO.

5.2.3.3. Stimulation of TLR3 with agonist Poly(I:C)

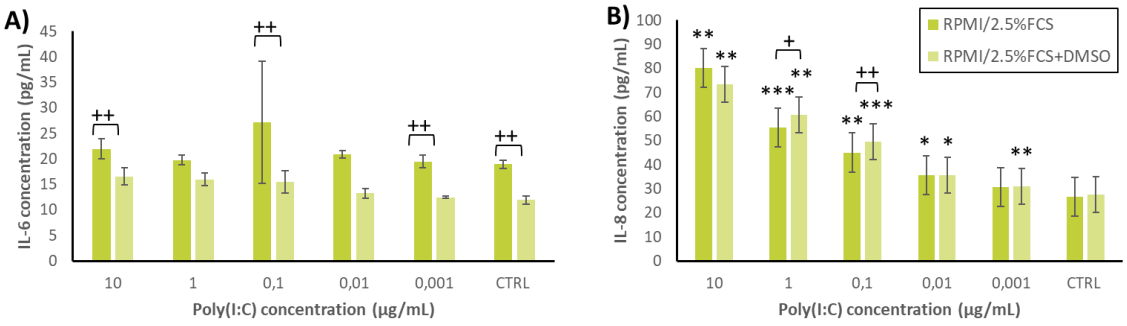

FIGURE 16. EFFECT OF DIFFERENT CONCENTRATIONS OF POLY(I:C) ON IL-6 (FIGURE A) AND IL-8 (FIGURE B) PRODUCTION IN A549 CELLS. TWO DIFFERENT CONDITIONS, CELLS TREATED WITH POLY(I:C) + RPMI / 2.5% FCS AND CELLS TREATED WITH POLY(I:C) + RPMI / 2.5% FCS + DMSO (0.06%), WERE USED IN THIS ASSAY TO DETERMINE THE EFFECT OF DMSO ON CELL RESPONSE. AFTER T-TEST ANALYSIS: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 COMPARED TO THE CONTROL WITHOUT AGONIST, +P ≤ 0.05, ++P ≤ 0.1 COMPARING POLY(I:C) + RPMI / 2.5% FCS TREATED CELLS AND POLY(I:C) + RPMI / 2.5% FCS + DMSO (0.06%) TREATED CELLS.

In this case, the agonist Poly(I:C) was used to carry out the specific stimulation of the TLR3 receptor on A549 cells. Again, in the case of IL-6, no significant differences are observed between extracellular concentration of this cytokine in the presence of the different concentrations of

the agonist and the control conditions of each condition. **Figure 16A** shows some significant decreases in IL-6 levels in the presence of DMSO.

When looking at **figure 16B** it can be seen that there are significant differences of even P ≤ 0.001 of the different conditions with respect to the controls without agonist. A good dose-dependent cellular response to the agonist administered can be observed since the lower the agonist concentration is, the lower the IL-8 production is. Again, no significant differences are observed when DMSO is present in the medium.

5.2.3.4. Stimulation of PAR2 with agonist FLIGRL

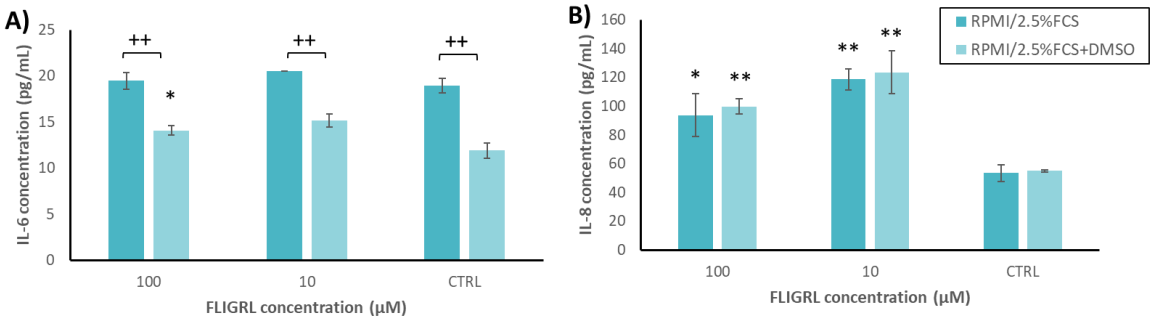

FIGURE 17. EFFECT OF DIFFERENT CONCENTRATIONS OF FLIGRL ON IL-6 (FIGURE A) AND IL-8 (FIGURE B) PRODUCTION IN A549 CELLS. TWO DIFFERENT CONDITIONS, CELLS TREATED WITH FLIGRL + RPMI / 2.5% FCS AND CELLS TREATED WITH FLIGRL + RPMI / 2.5% FCS + DMSO (0.06%), WERE USED IN THIS ASSAY TO DETERMINE THE EFFECT OF DMSO ON CELL RESPONSE. AFTER T-TEST ANALYSIS: *P ≤ 0.05, **P ≤ 0.01 COMPARED TO THE CONTROL WITHOUT AGONIST, +P ≤ 0.05, ++P ≤ 0.1 COMPARING FLIGRL + RPMI / 2.5% FCS TREATED CELLS AND FLIGRL + RPMI / 2.5% FCS + DMSO (0.06%) TREATED CELLS.

This part of the assay aimed to determine the effect of FLIGRL, a specific PAR2 agonist, on the production of the cytokines IL-6 and IL-8 in the A549 cells used. As observed in the rest of the stimulation assays with the other agonists, in the case of IL-6, there are no significant differences in the concentration of this cytokine when the agonist is present. Similarly, to all the other results from this assay, when looking at **figure 17A** a significant decrease in cytokine levels can be observed when DMSO is present.

In contrast, in the case of IL-8 (**figure 17B**), significant increases in IL-8 levels are seen when comparing the use of different concentrations of agonist with control conditions. Again, no significant influence of DMSO on the production of this cytokine is seen.

5.3. MTT assays results

To determine cell viability after each treatment, MTT assays were performed on the cells used in the experiments. After following the protocol of this assay, the optical density (OD) was measured at 570nm to get an idea of the viability of the cells, as a higher OD can be interpreted as a higher number of metabolically active cells.

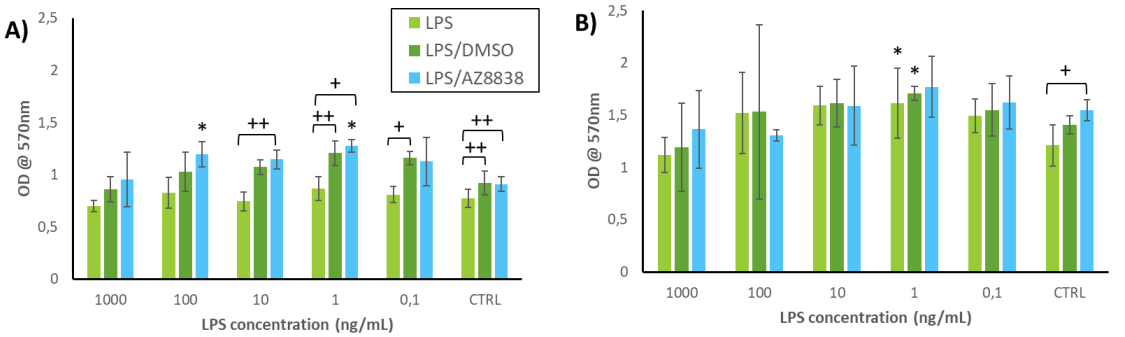

FIGURE 18. MTT ASSAY RESULTS FOR THE INHIBITION ASSAY WITH AZ8838. A) AND B) REPRESENT THE LEVELS OF OPTICAL DENSITY READ AT 570NM AFTER CARRYING OUT THE PROTOCOL FOR THE MTT ASSAY ON THE CELLS TREATED FOR 24HR AND 48HR, RESPECTIVELY. AFTER T-TEST ANALYSIS: *P ≤ 0.05, COMPARED TO THE CONTROL WITHOUT AGONIST, +P ≤ 0.05, COMPARING LPS TREATED CELLS WITH LPS/VEHICLE OR LPS/AZ8838 TREATED CELLS.

Thus, by comparing **figures 18A** and **18B** it can be seen that cell viability is higher in cells treated for 48hr than in cells that were treated for 24hr. This makes sense as the cells treated for 48hr had more time to grow which would explain the higher OD values.

Both figures show some significant differences between the OD of the treated cells and their respective controls, although this does not seem to be uniform and common in all cases. When comparing cells treated with LPS and cells treated with LPS and vehicle or AZ8838, especially in cells treated for 24hr (**figure 18A**), there are significant increases in OD in cells with LPS and vehicle or AZ8838 with respect to cells with only LPS.

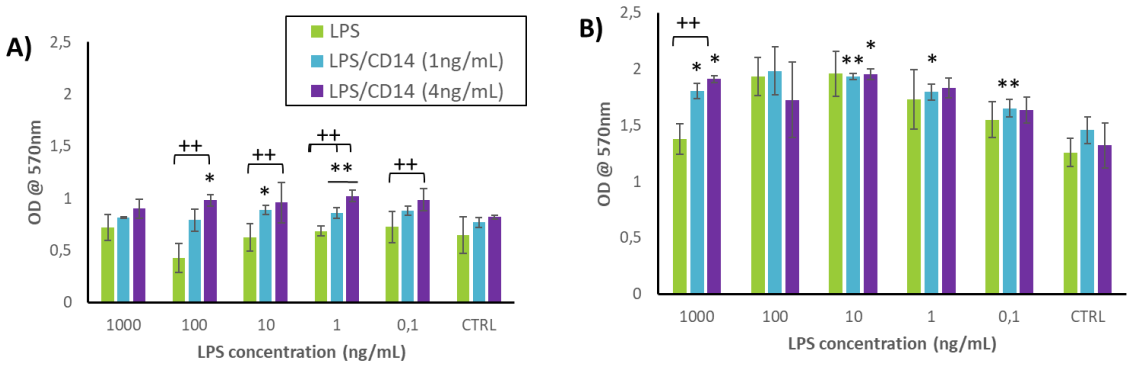

FIGURE 19. MTT ASSAY RESULTS FOR THE TLR4 STIMULATION ASSAY WITH CD14. A) AND B) REPRESENT THE LEVELS OF OPTICAL DENSITY READ AT 570NM AFTER CARRYING OUT THE PROTOCOL FOR THE MTT ASSAY ON THE CELLS TREATED FOR 24HR AND 48HR, RESPECTIVELY. AFTER T-TEST ANALYSIS: *P ≤ 0.05, **P ≤ 0.01 COMPARED TO THE CONTROL WITHOUT AGONIST, +P ≤ 0.05, ++P ≤ 0.1 COMPARING LPS TREATED CELLS AND LPS/CD14 TREATED CELLS.

In the case of **figure 19**, again the OD of cells treated for 48hr is higher than that of cells treated for 24hr. Again, some significant differences can be seen between the different conditions and their respective controls, suggesting that the treatments applied may have some influence on cell viability.

As in the previous case, when comparing cells treated with LPS and cells treated with LPS and CD14, especially in cells treated for 24hr (**figure 19A**), there are significant increases in the OD in cells with LPS and CD14 compared to cells with only LPS.

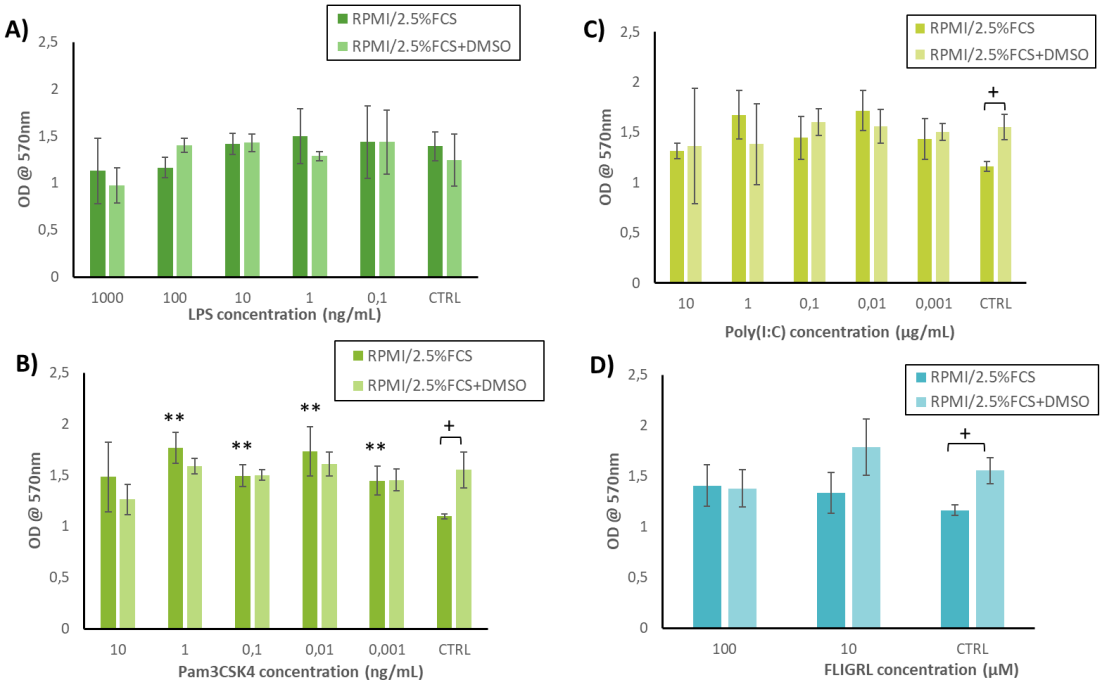

FIGURE 20. MTT ASSAY RESULTS FOR THE ASSAY WITH THE DIFFERENT AGONISTS: LPS (A), PAM3CSK4 (B), POLY(I:C) (C) AND FLIGRL (D). THE FIGURES REPRESENT THE LEVELS OF OPTICAL DENSITY READ AT 570NM AFTER CARRYING OUT THE PROTOCOL FOR THE MTT ASSAY ON THE TREATED CELLS. AFTER T-TEST ANALYSIS: *P ≤ 0.05, **P ≤ 0.01 COMPARED TO THE CONTROL WITHOUT AGONIST, +P ≤ 0.05 COMPARING AGONIST + RPMI / 2.5% FCS TREATED CELLS AND AGONIST + RPMI / 2.5% FCS + DMSO (0.06%) TREATED CELLS.

In this last case, it is only worth noting that in **figure 20B**, corresponding to the treatment of the cells with the agonist Pam3CSK4, significant differences can be seen between the different treatments with Pam3CSK4 and RPMI / 2.5% FCS and their respective controls, suggesting that the addition of this agonist can have an influence on cell viability. With the other agonists, there were no remarkable differences between the different groups.

6. DISCUSSION OF THE RESULTS

6.1. PAR2, TLR3 and TLR4 are mostly located in the cytoplasm of A549 cells.

The characterisation process of these cells includes immunofluorescence staining protocols for the detection of TLR3, TLR4 and PAR2. In the future, to analyse the level of expression of each receptor in A549 cells PCRs with specific primers or analysis of the fluorescence intensity could be carried out.

In the case of PAR2 fluorescence detection, detection spots can be observed at the perinuclear level but also at the cytoplasmic level. As explained in the introduction, PAR2 activity must be interrupted by a β-arrestin-mediated internalisation process as it is a receptor that is irreversibly activated by proteolysis. Thus, the detection points marked in **figures 9B** and **9E** may be endosomes of PAR2 receptor that has been internalised from the cell membrane to be ubiquitinated and degraded or recycled back to the cell surface (Rothmeier and Ruf, 2012).

For TLR3 and TLR4 detection, two different concentrations of anti-TLR3 and anti-TLR4 primary antibodies were used as this type of protocol had never been used in the laboratory with A549

cells. Although both concentrations gave good quality images as shown in **figures 10** and **11**, in future occasions it would be more convenient to use the 1/1000 concentration as when observing under the microscope the slides where the 1/500 concentration was used, there were regions of detection too saturated with antibody.

The specific location of the TLR3 receptor in respiratory epithelial cells is controversial and some studies have confirmed that its location is mainly in cytoplasmic vesicles and that this receptor has little or no presence in the membrane of A549 cells (Ciencewicki *et al.*, 2006). In the case of the TLR4 receptor, there is also some controversy about its localisation in A549 cells, as previous research has shown that this receptor is not expressed on the cell surface of A549 cells and that its localisation is mostly at the level of intracellular compartments (Guillott et al., 2004).

This is consistent with the results obtained and presented in **images B** and **E** in **figures 10** and **11** where it can be seen that the detection sites of TLR3 and TLR4 respectively are located in granules in the cytoplasm, i.e., in cytoplasmic vesicles. In contrast, there is not much detection of these receptors at the cell membrane level. However, it must be considered that the cells used have been previously permeabilised, so it could be expected that there would not be a high detection of the receptor at the membrane level. To determine the presence or absence of the receptor in the cell membrane, the immunofluorescence protocol should be performed with unpermeabilised cells. A western immunoblotting protocol of the membrane fraction of the cells could also be performed, as studies such as the one performed by MacRedmond et al. (2005) have been able to demonstrate the presence of TLR4 in the membrane of A549 cells using this technique.

6.2. Bioassays for the study of crosstalk between PAR2 and TLRs

To assess certain aspects of the TLR and PAR2 crosstalk on A549 cells different bioassays were carried out where the extracellular concentration of proinflammatory cytokines like IL-6 and IL-8 were then quantified. It is worth mentioning that when analysing from **figure 12** to **figure 17**, it is common to all that IL-8 levels are much higher than IL-6 levels confirming what other studies such as the one performed by Grandel et al. (2009) have previously demonstrated with respect to IL-6 production by A549 cells. It can be concluded that this cell type is not a good producer of the pro-inflammatory cytokine IL-6 as they produce it at a basal level and its production does not respond to the presence of stimulators.

In future experiments, in addition to performing ELISAs that allow us to determine the amount of cytokine secreted to the outside of the cell, the Western-Blot technique could also be used to determine the amount of protein that is accumulated intracellularly. Other pro-inflammatory cytokines such as IL-1 or TNF-α, the expression of which also depend on the activation of the transcription factor NF-κB, could also be studied, and the influence of PAR2 and TLR receptors and their crosstalk on the production and secretion of these cytokines could also be determined using ELISAs or Western-Blot (Rallabhandi *et al.*, 2008).

6.2.1. AZ8838, a specific inhibitor for PAR2, does not act as expected.

In the first bioassay, the aim was to study the influence of the PAR2 receptor on the response of A549 cells to TLR4 receptor stimulation using LPS. To study the effect of PAR2 on the production of inflammatory mediators derived from TLR4 activation, the PAR2-specific inhibitor AZ8838 was prepared with DMSO as a vehicle. First, the DMSO vehicle was found to have no influence on

cytokine production, which is confirmed by looking at **figure 12** and finding no significant differences between cytokine secretion with and without DMSO.

Previous studies have confirmed that there is a crosstalk between PAR2 and TLR4 that results in an enhancement of the NF-κB-mediated inflammatory response resulting in increased production of pro-inflammatory cytokines (Rallabhandi *et al.*, 2008). In this way, it would have been expected that the presence of the PAR2-specific inhibitor AZ8838 would have had a negative effect on cytokine secretion initiated by TLR4 activation by LPS. As seen in **figure 12**, this effect could not be seen as there are no significant differences on cytokine secretion between cells treated only with LPS and those treated with LPS and AZ8838, and it was concluded that the inhibitor used may not have worked as expected probably because the stock from which it is derived is too old and the inhibitor may has lost its properties.

To ensure that PAR2 activity is completely inhibited, other concentrations or a new stock of inhibitor could be used in future occasions. Other PAR2 inhibitors such as I-287 could also be used in following experiments to assure complete interruption of PAR2 proinflammatory signalling (Avet *et al.*, 2020). Another way to ensure PAR2 inhibition in epithelial cells like A549 cells would be by using PAR2 knock out cells that could be obtained following a gene-editing protocol using the CRISPR-Cas molecular tool, which allows the silencing of specific genes (Ran *et al.*, 2013).

To study TLR-PAR2 crosstalk, one could also co-stimulate PAR2 and TLR receptors by using their corresponding agonists simultaneously. Studies such as the one by Rallabhandi et al. (2008) have already carried out experiments of this type using an agonist peptide to stimulate PAR2 and LPS to stimulate TLR4 at the same time. In this way, by carrying out this co-stimulation, it would be possible to compare cytokine production and secretion when both PAR2 and the different types of TLRs are activated to production and secretion of cytokines when PAR2 is inhibited.

Regarding the MTT assay, it appears that in cells treated for 24hr (**figure 18A**) both the inhibitor AZ8838 and DMSO have some significant effect on cell viability and seem to increase it, confirming that these compounds are not toxic to A549 cells and do not affect negatively to cell viability. This effect is not seen with cells treated for 48 hours.

6.2.2. CD14-mediated stimulation leads to increased cytokine production over time.

The following bioassay aimed to study the effect of the CD14 molecule on the response of A549 cells to TLR4 receptor activation by LPS. As discussed in the introduction, TLR4 activation by agonists such as LPS results in the activation of two different but consecutive pathways: MyD88-dependent, which is from TLR4 expressed on the cell membrane, and then TRIF-dependent, which is from TLR4 expressed on endosomes. Thus, CD14 first mediates the binding of LPS to TLR4 to carry out the first signalling pathway, and then CD14 mediates the internalisation of TLR4 to enable the second pathway to occur (Ciesielska, Matyjek and Kwiatkowska, 2021).

When analysing the results of the CD14 assay (**figure 13**), IL-6 levels at 24hrs of treatment decrease when CD14 is present in the medium. When only LPS is used as treatment, it can be assumed that CD14, which is naturally present on the membrane of A549 cells as proved by previous studies (Barbosa *et al.*, 2007), mediates the binding of LPS to the TLR4 receptor and leads to activation of the My-D88-dependent pathway culminating in rapid activation of NF-κB and production of basal levels of cytokine without a dose-dependent response to LPS. In contrast, when CD14 is added to the medium, the excess CD14 and the lack of TLR4 on the

surface of A549 cells triggers increased TLR4 receptor internalisation and activation of the TRIF-dependent pathway which mediates a late rather than early NF-kB response and so it is observed that with CD14 present, IL-6 production at 24 hours falls in comparison to the treatment with only LPS and no CD14 (**figure 13A**). In contrast, at 48 hours (**figure 13B**) the production of IL-6 increases when CD14 is present compared to the LPS-only treatment because of the late activation of NF-κB by CD14-mediated internalisation.

With the cytokine IL-8 one would expect to see the same response to the presence of CD14, but as can be seen in the cells treated for 24 hours (**figure 13C**) there are no significant differences between treatment with LPS alone and treatment with LPS and CD14. As with IL-6, in cells treated for 48 hours, as seen in other studies such the one conducted by Schulz et al. (2002) one would expect to see an increase in IL-8 production after treatment with CD14 as this molecule is a natural stimulator of TLR4.

This lack of influence of CD14 on IL-8 production may be attributed to the fact that the concentration of CD14 used is not sufficient to exert an effect on IL-8 production. To verify all the above, a Western-Blot could be performed to detect proteins of these signalling pathways in their phosphorylated state and confirm that activation of these pathways is occurring as described above.

Regarding the MTT assay, it appears that in cells treated for 24hr (**figure 19A**) CD14 has some effect on cell viability and seems to increase it, confirming that this compound is not toxic to A549 cells and does not affect negatively to cell viability. This effect is not seen with cells treated for 48 hours.

6.2.3. PAR2, TLR3 and TLR4 on A549 cells are stimulated by their respective agonists in a dose-dependent manner.

The last bioassay was based on the study of the effect of different agonists on IL-6 and IL-8 levels and it was determined whether DMSO, vehicle of the PAR2 inhibitor AZ8838, influences this response for future experiments where this inhibitor is to be combined with the agonists studied. It was concluded that DMSO influences IL-6 production as can be seen in **figures 14A**, **15A**, **16A** and **17A**, but not on IL-8.

From the use of LPS as a TLR4 agonist it can be seen that A549 cells have a concentration-dependent response to LPS present in the medium as the higher the concentration of LPS the higher the IL-8 production. This effect is not seen with IL-6. In the case of the use of the TLR2 agonist Pam3CSK4, it was found that no dose-dependent response appears to be seen for either IL-6 or IL-8 production. The clearest example of agonist influence is seen in **figure 16B** where the agonist Poly(I:C) was used to stimulate TLR3 activation and where a clear Poly(I:C) dose-dependent response can be seen as the higher the agonist concentration, the higher the IL-8 production. This effect is not noticeable with IL-6. Finally, the PAR2 agonist FLIGRL was used, and it was determined that IL-8 production levels are maximal when using a concentration of 10μM of this agonist.

On future occasions, it would also be interesting to determine whether these agonists generate an increase in the expression of the receptors by carrying out an analysis and quantification of the mRNA levels by qPCR and it would also be useful to confirm the expression of TLR2 in A549 cells using immunofluorescence or other techniques like flow cytometry.

Regarding the MTT assay, it appears that the used agonists do not have significant effect on cell viability or proliferation (**figure 20**).

7. CONCLUSION

One of the main objectives of this project was to characterise the presence of PAR2, TLR3 and TLR4 receptors in A549 cells. Thanks to the results obtained by the immunofluorescence staining, it was possible to conclude that these cells express these receptors, and that this expression is mainly at the level of cytoplasmic vesicles. In future occasions, detection of TLR2 could also be carried out using a similar immunofluorescence protocol.

Thanks to the bioassays carried out, it was also possible to determine that the cell line used is a poor producer of the pro-inflammatory cytokine IL-6 and that the production of this cytokine does not respond to the presence of different agonists that activate inflammation-mediating receptors. In contrast, IL-8 production by these cells is much more efficient and responds to increasing doses of agonist stimulators of the receptors studied, especially in the case of PAR2, TLR3 and TLR4 stimulation, which will be useful in the future for the design of new experiments focused on the study of crosstalk between PAR2 and TLRs.

To study the crosstalk between PAR2 and TLR4, an inhibition assay was carried out with AZ8838, a PAR2 inhibitor, using LPS as an activator of TLR4. The expected results of this assay were not as expected, as no influence of PAR2 could be seen, leading to the conclusion that the inhibitor is not acting as expected, probably because the stock from which it is derived is too old. When TLR4 was stimulated with CD14, it was found that the stimulation was efficient after 48 hours with respect to IL-6 production but not IL-8. To determine whether IL-8 production is stimulated by the presence of CD14 in the medium, this bioassay would have to be repeated with other concentrations of CD14 and should also include western-blot results of proteins of the signalling pathways involved, as well as a study of changes in the expression of the receptors of interest after CD14 stimulation.

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Thanks also to Dr Jamie Whitelaw for providing me with the protocol and some of the reactives necessary to carry out the immunofluorescence staining, for teaching me how to use the Nikon i80 fluorescence microscope and for giving me guidance to obtain images with the best possible quality.

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9. BIBLIOGRAPHY

Amatngalim, G.D. *et al.* (2017) 'Antibacterial Defense of Human Airway Epithelial Cells from Chronic Obstructive Pulmonary Disease Patients Induced by Acute Exposure to Nontypeable Haemophilus influenzae: Modulation by Cigarette Smoke', *Journal of innate immunity*, 9(4), pp. 359–374. Available at: https://doi.org/10.1159/000455193.

Austin, V. *et al.* (2016) 'COPD and stroke: are systemic inflammation and oxidative stress the missing links?', *Clinical Science*, 130, pp. 1039–1050. Available at: https://doi.org/10.1042/CS20160043.

Avet, C. *et al.* (2020) 'The PAR2 inhibitor I-287 selectively targets Gαq and Gα12/13 signaling and has anti-inflammatory effects', *Communications Biology 2020 3:1*, 3(1), pp. 1–13. Available at: https://doi.org/10.1038/s42003-020-01453-8.

Barbosa, F.M. *et al.* (2007) 'Binding of glucuronoxylomannan to the CD14 receptor in human A549 alveolar cells induces interleukin-8 production', *Clinical and Vaccine Immunology*, 14(1), pp. 94–98. Available at: https://doi.org/10.1128/CVI.00296-06.

Biabas, A.J. *et al.* (2016) 'The Role of Mitochondria and Oxidative/Antioxidative Imbalance in Pathobiology of Chronic Obstructive Pulmonary Disease'. Available at: https://doi.org/10.1155/2016/7808576.

Brode, S.K., Ling, S.C. and Chapman, K.R. (2012) 'Alpha-1 antitrypsin deficiency: a commonly overlooked cause of lung disease', *CMAJ : Canadian Medical Association Journal*, 184(12), p. 1365. Available at: https://doi.org/10.1503/CMAJ.111749.

Calvén, J., Ax, E. and Rådinger, M. (2020) 'The Airway Epithelium—A Central Player in Asthma Pathogenesis', *International Journal of Molecular Sciences 2020, Vol. 21, Page 8907*, 21(23), p. 8907. Available at: https://doi.org/10.3390/IJMS21238907.

Cavaillès, A. *et al.* (2013) 'Comorbidities of COPD', *European Respiratory Review*, 22(130), pp. 454–475. Available at: https://doi.org/10.1183/09059180.00008612.

Ciencewicki, J. *et al.* (2006) 'Diesel exhaust enhances virus- and poly(I:C)-induced Toll-like receptor 3 expression and signaling in respiratory epithelial cells', *American journal of physiology. Lung cellular and molecular physiology*, 290(6). Available at: https://doi.org/10.1152/AJPLUNG.00318.2005.

Ciesielska, A., Matyjek, M. and Kwiatkowska, K. (2021) 'TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling', *Cellular and molecular life sciences : CMLS*, 78(4), pp. 1233–1261. Available at: https://doi.org/10.1007/S00018-020-03656-Y.

Cocks, T.M. *et al.* (1999) 'A protective role for protease-activated receptors in the airways', *Nature*, 398(6723), pp. 156–160. Available at: https://doi.org/10.1038/18223.

Dey, T. *et al.* (2018) 'Proteases and Their Inhibitors in Chronic Obstructive Pulmonary Disease', *Journal of Clinical Medicine*, 7(9). Available at: https://doi.org/10.3390/JCM7090244.

Dietrich, M. *et al.* (2003) 'Smoking and exposure to environmental tobacco smoke decrease some plasma antioxidants and increase gamma-tocopherol in vivo after adjustment for dietary antioxidant intakes', *The American journal of clinical nutrition*, 77(1), pp. 160–166. Available at: https://doi.org/10.1093/AJCN/77.1.160.

Domej, W., Oettl, K. and Renner, W. (2014) 'Oxidative stress and free radicals in COPD – implications and relevance for treatment', *International Journal of Chronic Obstructive Pulmonary Disease*, 9, p. 1207. Available at: https://doi.org/10.2147/COPD.S51226.

El-Zayat, S.R., Sibaii, H. and Mannaa, F.A. (2019) 'Toll-like receptors activation, signaling, and targeting: an overview', *Bulletin of the National Research Centre 2019 43:1*, 43(1), pp. 1–12. Available at: https://doi.org/10.1186/S42269-019-0227-2.

Gao, W. *et al.* (2015) 'Bronchial epithelial cells: The key effector cells in the pathogenesis of chronic obstructive pulmonary disease?', *Respirology*, 20(5), pp. 722–729. Available at: https://doi.org/10.1111/RESP.12542.

Gatti, R. *et al.* (2006) 'Protease-activated receptor-2 activation exaggerates TRPV1-mediated cough in guinea pigs', *Journal of Applied Physiology*, 101(2), pp. 506–511. Available at: https://doi.org/10.1152/JAPPLPHYSIOL.01558.2005.

Gieseler, F. *et al.* (2013) 'Proteinase-activated receptors (PARs) – focus on receptor-receptor-interactions and their physiological and pathophysiological impact', *Cell Communication and Signaling : CCS*, 11(1), p. 86. Available at: https://doi.org/10.1186/1478-811X-11-86.

Grandel, U. *et al.* (2009) 'Amplification of lipopolysaccharide-induced cytokine synthesis in non-small cell lung cancer/neutrophil cocultures', *Molecular cancer research : MCR*, 7(10), pp. 1729–1735. Available at: https://doi.org/10.1158/1541-7786.MCR-09-0048.

Guillott, L. *et al.* (2004) 'Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4', *The Journal of biological chemistry*, 279(4), pp. 2712–2718. Available at: https://doi.org/10.1074/JBC.M305790200.

Halpin, D.M.G., Vogelmeier, C.F. and Agusti, A. (2021) 'COVID-19 and COPD: Lessons beyond the pandemic', *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 321(5), pp. L978–L982. Available at: https://doi.org/10.1152/AJPLUNG.00400.2021.

Heuberger, D.M. and Schuepbach, R.A. (2019) 'Protease-activated receptors (PARs): Mechanisms of action and potential therapeutic modulators in PAR-driven inflammatory diseases', *Thrombosis Journal*, 17(1), pp. 1–24. Available at: https://doi.org/10.1186/S12959-019-0194-8/TABLES/7.

Kanazawa, H. *et al.* (2014) 'Simultaneous assessment of hepatocyte growth factor and vascular endothelial growth factor in epithelial lining fluid from patients with COPD', *Chest*, 146(5), pp. 1159–1165. Available at: https://doi.org/10.1378/CHEST.14-0373.

Knight, D.A. *et al.* (2001) 'Protease-activated receptors in human airways: upregulation of PAR-2 in respiratory epithelium from patients with asthma', *The Journal of allergy and clinical immunology*, 108(5), pp. 797–803. Available at: https://doi.org/10.1067/MAI.2001.119025.

Koarai, A. *et al.* (2010) 'Oxidative stress enhances toll-like receptor 3 response to double-stranded RNA in airway epithelial cells', *American journal of respiratory cell and molecular biology*, 42(6), pp. 651–660. Available at: https://doi.org/10.1165/RCMB.2008-0345OC.

Laniado-Laborín, R. (2009) 'Smoking and Chronic Obstructive Pulmonary Disease (COPD). Parallel Epidemics of the 21 st Century', *Int. J. Environ. Res. Public Health*, 6, pp. 209–224. Available at: https://doi.org/10.3390/ijerph6010209.

MacRedmond, R.E. *et al.* (2005) 'Respiratory epithelial cells require Toll-like receptor 4 for induction of human beta-defensin 2 by lipopolysaccharide', *Respiratory research*, 6(1). Available at: https://doi.org/10.1186/1465-9921-6-116.

Nhu, Q.M. *et al.* (2009) 'Novel signaling interactions between proteinase-activated receptor 2 and Toll-like receptors in vitro and in vivo', *Mucosal Immunology*, 3. Available at: https://doi.org/10.1038/mi.2009.120.

Nystedt, S., Ramakrishnan, V. and Sundelin, J. (1996) 'The proteinase-activated receptor 2 is induced by inflammatory mediators in human endothelial cells. Comparison with the thrombin receptor', *The Journal of biological chemistry*, 271(25), pp. 14910–14915. Available at: https://doi.org/10.1074/JBC.271.25.14910.

Pace, E. *et al.* (2013) 'TLR4 up-regulation and reduced Foxp3 expression in mechanically ventilated smokers with obstructive chronic bronchitis', *COPD*, 10(2), pp. 147–155. Available at: https://doi.org/10.3109/15412555.2012.730565.

Rallabhandi, P. *et al.* (2008) 'Analysis of Proteinase-activated Receptor 2 and TLR4 Signal Transduction: A NOVEL PARADIGM FOR RECEPTOR COOPERATIVITY*S', *The Journal of Biological Chemistry*, 283(36), p. 24314. Available at: https://doi.org/10.1074/JBC.M804800200.

Ran, F.A. *et al.* (2013) 'Genome engineering using the CRISPR-Cas9 system', *Nature Protocols 2013 8:11*, 8(11), pp. 2281–2308. Available at: https://doi.org/10.1038/nprot.2013.143.

Ritchie, A.I. and Wedzicha, J.A. (2020) 'Definition, Causes, Pathogenesis, and Consequences of Chronic Obstructive Pulmonary Disease Exacerbations', *Clinics in Chest Medicine*, 41(3), p. 421. Available at: https://doi.org/10.1016/J.CCM.2020.06.007.

Rodrigues, S. de O. *et al.* (2021) 'Mechanisms, Pathophysiology and Currently Proposed Treatments of Chronic Obstructive Pulmonary Disease', *Pharmaceuticals*, 14(10). Available at: https://doi.org/10.3390/PH14100979.

Rothmeier, A.S. and Ruf, W. (2012) 'Protease-activated receptor 2 signaling in inflammation', *Seminars in immunopathology*, 34(1), pp. 133–149. Available at: https://doi.org/10.1007/S00281-011-0289-1.

Schuliga, M. (2015) 'NF-kappaB Signaling in Chronic Inflammatory Airway Disease', *Biomolecules 2015, Vol. 5, Pages 1266-1283*, 5(3), pp. 1266–1283. Available at: https://doi.org/10.3390/BIOM5031266.

Schulz, C. *et al.* (2002) 'Differences in LPS-induced activation of bronchial epithelial cells (BEAS-2B) and type II-like pneumocytes (A-549)', *Scandinavian journal of immunology*, 56(3), pp. 294–302. Available at: https://doi.org/10.1046/J.1365-3083.2002.01137.X.

Sevigny, L.M. *et al.* (2011) 'Interdicting protease-activated receptor-2-driven inflammation with cell-penetrating pepducins', *Proceedings of the National Academy of Sciences of the United States of America*, 108(20), pp. 8491–8496. Available at: https://doi.org/10.1073/PNAS.1017091108/-/DCSUPPLEMENTAL/PNAS.201017091SI.PDF.

Sidletskaya, K., Vitkina, T. and Denisenko, Y. (2020) 'The Role of Toll-Like Receptors 2 and 4 in the Pathogenesis of Chronic Obstructive Pulmonary Disease', *International Journal of Chronic Obstructive Pulmonary Disease*, 15, p. 1481. Available at: https://doi.org/10.2147/COPD.S249131.

Smith, M.C. and Wrobel, J.P. (2014) 'Epidemiology and clinical impact of major comorbidities in patients with COPD', *International Journal of COPD*, 9, pp. 871–888. Available at: https://doi.org/10.2147/COPD.S49621.

Soler-Cataluña, J.J. *et al.* (2005) 'Severe acute exacerbations and mortality in patients with chronic obstructive pulmonary disease', *Thorax*, 60(11), pp. 925–931. Available at: https://doi.org/10.1136/THX.2005.040527.

Yang, I.A., Jenkins, C.R. and Salvi, S.S. (2022) 'Chronic obstructive pulmonary disease in never-smokers: risk factors, pathogenesis, and implications for prevention and treatment', *The Lancet. Respiratory medicine*, 10(5), pp. 497–511. Available at: https://doi.org/10.1016/S2213-2600(21)00506-3.

Zhao, P. *et al.* (2014) 'Biased signaling of protease-activated receptors'. Available at: https://doi.org/10.3389/fendo.2014.00067.

Zuo, L. *et al.* (2015) 'Molecular Regulation of Toll-like Receptors in Asthma and COPD', *Frontiers in Physiology | www.frontiersin.org*, 6, p. 312. Available at: https://doi.org/10.3389/fphys.2015.00312.