ACADEMY OF ATHENS

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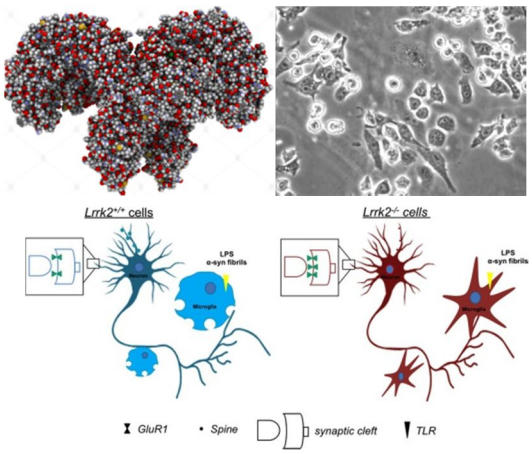
National and Kapodistrian UNIVERSITY OF ATHENS

Master of Science School of Medicine

in Molecular Biomedicine

The role of LRRK2 signaling in resident and infiltrating immune cells in different models of familial and sporadic Parkinson's disease.



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Περίληψη

Η νόσος του Πάρκινσον, είναι μια επιθετική κινητική διαταραχή, που προσβάλλει περίπου το 1.8% του παγκόσμιου πληθυσμού άνω των 65 και το 2.6% των ατόμων άνω των 85 ετών. Παρόλο που η νόσος προκαλεί σημαντική ψυχολογική, κοινωνική και οικονομική επιβάρυνση, μέχρι στιγμής δεν υπάρχει κάποια θεραπευτική αγωγή, η οποία να επιβραδύνει την εξέλιξη της ασθένειας. Αν και η αιτιολογία για την ανάπτυξη της ασθένειας δεν είναι σαφής, υπάρχουν γενετικοί παράγοντες που ωθούν στη ανάπτυξη της ασθένειας. Η έρευνά μας, επικεντρώνεται στους μηχανισμούς νευροεκφύλισης που σχετίζονται με το πιο συχνά μεταλλαγμένο γονίδιο που κωδικοποιεί την πρωτεϊνική κινάση LRRK2. Σε προηγούμενες μελέτες, έχουν χαρακτηριστεί πολλαπλοί μηχανισμοί μέσω των οποίων, μεταλλαγμένες μορφές της LRRK2, μπορούν να οδηγήσουν σε χαρακτηριστικό για Πάρκινσον, νευροεκφυλισμό. Μέσω της δράσης της στη ρύθμιση της φλεγμονής και της σηματοδότησης στο ανοσοποιητικό σύστημα, η LRRK2 ίσως να παίζει καθοριστικό ρόλο στη νόσο του Πάρκινσον, συμπεριλαμβανομένων σπανιότερων περιπτώσεων, που συσχετίζονται με μεταλλάξεις στο γονίδιο της α-συνουκλεΐνης. Η γενετική ή φαρμακολογική καταστολή της ενεργότητας κινάσης της LRRK2, καταστέλλει την ενεργοποίηση των μικρογλοιακών κυττάρων μετά από έκθεση σε αγωνιστές για το μονοπάτι των TLRs, όπως ο λιποπολυσακχαρίτης και τα ινίδια α-συνουκλεΐνης. Μέχρι σήμερα, δεν είναι ξεκάθαρο αν η φλεγμονή των νεύρων, χαρακτηριστικό της παθολογίας του Πάρκινσον, έχει προωθητικό ή προστατευτικό ρόλο στην απώλεια των νευρώνων, καθώς επίσης και ο ρόλος της LRRK2 στη σηματοδότηση του στο ανοσοποιητικό σύστημα , μέσα σε αυτό το πλαίσιο. Οι Rab GTPases, όπως η Rab10, που αποτελούν ενδογενή υποστρώματα φωσφορυλίωσης για τη LRRK2 κινάση, εμπλέκονται στους μηχανισμούς εξωκυττάρωσης των νευρώνων και άλλων κυτταρικών τύπων. Έχει δειχθεί πως η Rab10, προάγει τη σηματοδότηση του TLR4 μονοπατιού, ελέγχοντας τη μεταφορά του υποδοχέα στην πλασματική μεμβράνη. Στη συγκεκριμένη μελέτη, ελέγχουμε μέσω Δοκιμής ανοσοαποτύπωσης και κυτταρομετρίας ροής, αν η ρύθμιση της μεταφοράς των TLR4 και TLR2 από τη Rab10 σε RAW264.7 κύτταρα, εξαρτάται από τη LRRK2. Καθώς η φωσφορυλίωση της Rab10 μέσω της LRRK2 καταστέλλει τη λειτουργία της, αναμένουμε πως εμποδίζοντας αυτή τη φωσφορυλίωση στο μοντέλο μας, αποτρέπουμε και την ρύθμιση της επαγόμενης από λιποπολυσακχαρίτη και ινίδια α-συνουκλεΐνης μεταφοράς των TLRs από τη Rab10. Καταστέλλοντας την ενεργότητα κινάσης της LRRK2, εμποδίζεται η απώλεια των TLRs στη μεμβράνη, καταστέλλοντας αποτελεσματικά την ανακύκλωσή της, αν και δεν είναι σαφές εάν αυτή η ρύθμιση είναι άμεση, ή εάν συμμετέχουν επίσης άλλες κινάσες ή Rab GTPases.

Abstract

Parkinson's disease (PD) is a devastating, movement disorder that affects approximately 1.8 % people over the age of 65 and 2.6 % in people over the age of 85 of the worldwide population. While PD creates a substantial psychological, social and economic burden, there is not yet a biological treatment in which the progression of PD is stopped. Although, the etiology of Parkinson's disease is not clearly known, there are genetic factors that stimulate the disease development. Our work is focused on the mechanisms of neurodegeneration associated with the most commonly mutated gene: leucine-rich repeat kinase 2 (LRRK2). Previous studies have identified multiple mechanisms by which mutant forms of LRRK2 can lead to neurodegeneration typical of PD; however, there is evidence that normal, wild type, LRRK2 also plays a role in PD pathogenesis linked to other forms of the disease, as well as idiopathic PD. Through its action in regulating inflammation and immune system signaling, LRRK2 may play a vital role in PD in general, including rare cases associated with mutations in the alpha-synuclein gene. LRRK2 inhibition, either genetically or by pharmacological inhibition of its kinase activity, suppresses microglial activation following exposure to TLR4-pathway agonists such as LPS and fibrillar alpha-synuclein. Until now, it is not clearly known if neuroinflammation, a characteristic feature of Parkinson's disease pathology has a promoting or a protecting role in neuronal loss, as well as, the role of LRRK2 in immune signaling in this context. Endogenous phosphorylation substrates for the LRRK2 kinase, Rab GTPases, as Rab10 are implicated in exocytic mechanisms in neurons and other cell types. It has been shown that Rab10 facilitates Toll-like receptor-4 (TLR4) signaling by controlling its trafficking onto the plasma membrane. In this study, using Western immunoblotting and flow cytometry we test if the regulation of TLR4 and TLR2 trafficking by Rab10 is dependent on LRRK2 in RAW264.7 cells. As, the phosphorylation of Rab10 by LRRK2 inhibits its function, we predict that blocking this phosphorylation in our model would prevent the regulation of LPS- or PFF-induced TLR trafficking by Rab10. LRRK2 kinase inhibition blocks the loss of TLR at the membrane, effectively suppressing its recycling, although it is not clear if this is a direct regulation, or if kinases or Rab GTPases participate in this regulation.

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a. Introduction

1. Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative movement disorder, characterized by the loss of dopaminergic neurons in the substantia nigra (SN), which results in progressive motor system malfunction. This progressive movement disorder is the second most common neurodegenerative disease, which affects 1.8 % of population over the age of 65 and 2.6 % over the age of 85. The disease was first described by Dr. James Parkinson, in 1817. He gave a detailed characterization of the disease in his essay on the Shaking Palsy describing the resting tremor and a distinctive form of progressive motor disability of the disease, reporting six cases of paralysis agitans (Parkinson, J. 1817 published in J. Neuropsychiatry Clin. Neurosci. 2002).

What causes PD remains unknown, but almost all cases involve the formation of intraneuronal protein aggregates called Lewy bodies (LBs), primarily composed of the presynaptic protein, a-synuclein (SNCA gene) (Deng, Z., et. al. 2017). The cell bodies of nigrostriatal neurons are in the SNpc, and they project to the putamen. The loss of these neurons, which normally contain conspicuous amounts of neuromelanin, produces the classic gross neuropathological finding of SNpc depigmentation. The earliest documented pathological changes in PD brain have been observed in the medulla oblongata/pontine tegmentum and olfactory bulb.

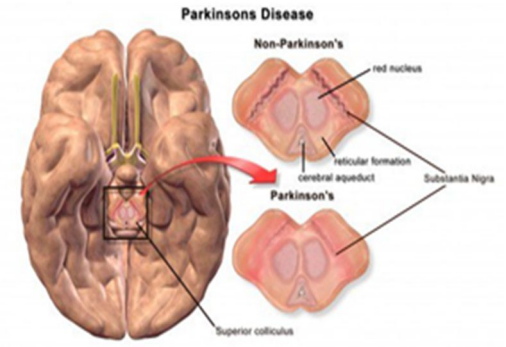

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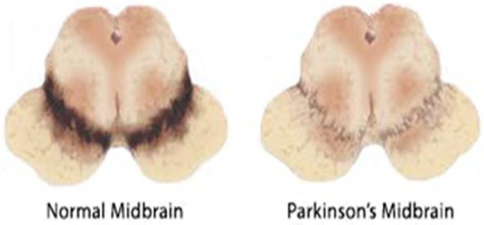
Figure 1. Comparison of PD patient's SN with non PD brain.



maplecarephysiotherapy.com

Figure 2. Normal and PD midbrain.

Despite the fact that PD is primarily a movement disorder, studies in animal models as well as in humans, have shown that certain cognitive functions may also be affected (Adeosun, S. O., et al, 2017). In addition, it is known that the non-motor symptoms of PD, including those cognitive impairments, are very common and frequently appear earlier than the motor symptoms (Chaudhuri and Odin, 2010; Magen, et. al. 2012; Mochizuki-Kawai, Mochizuki, and Kawamura, 2010; Paisan-Ruiz, Jain, Evans, et. al. 2004). Close to this, it has been suggested that three of the six proposed stages of Parkinson's pathology take place before motor symptoms become evident and a clinical diagnosis is feasible (Braak H, et. al. 2013).

Furthermore, non-linear loss of serotonergic terminals have been observed, which is associated with non-motor symptoms such as fatigue, apathy, as well as visual halluciations according to PET data (Politis M, et. al. 2010; Pagano G, et. al. 2017). As the clinical diagnosis and treatment response of PD are heterogeneous, the etiology is affected by various factors and the management of this disease is complex and challenging (Lesage S, Brice A., 2009).

As it is referred above, the etiology of Parkinson's disease is not clearly known. However, there are genetic factors that also contribute to the disease. The mutations that predispose to PD are called, monogenic, Mendelian or causative and are equivalent to 10% of PD cases (Paisán-Ruíz C,et. al. 2004). These mutations are rarer than risk factors, and current evidence suggests that PD pathogenesis is an association between environmental factors, aging and genetic susceptibility (Abeliovich A, et. al. 2000; Gao, H. M. & Hong, J. S. 2011).

1.2 Genes related to Parkinson's disease

The most convincing evidence that suggested the contribution of genetics came with the discovery of monogenic forms of Parkinson's disease. The first gene identified that is causative for PD was *SNCA* that encodes the protein a-synuclein (Polymeropoulos MH, et. al. 1997). In the matter of dominantly inherited PD, most common are the mutations in *LRRK2* gene (Corti O, et. al. 2011). The greatest genetic risk factor is mutations in *GBA*, which encodes β-glucocerebrosidase (Gaucher syndrome). In addition, other genes and mutations have been implicated in PD, such as DJ-1 and ATP13A2 which cause autosomal-recessive PD (Klein, C. & Westenberger, A., 2012).

Other gene mutations are correlated to monogenic forms of Parkinson's that can be shown on Table 1 together with the above genes.

	Protein	Pathogenic mutation(s)
Autosomal dominant		
SNCA	α-synuclein	Missense mutations (Ala18Thr, Ala29Ser, Ala30Pro, Glu46Lys, His50Gln, Gly51Asp, Ala53Glu, Ala53Thr); multiplications (duplications, triplications)
LRRK2	Leucine-rich repeat kinase 2	Missense mutations (Ile1371Val, Asn1437His, Arg1441Cys, Arg1441Gly, Arg1441His, Tyr1699Cys, Gly2019Ser [most common], Ile2020Thr)
VPS35	Vacuolar protein sorting 35	Missense mutation (Asp620Asn)
EIF4G1	Eukaryotic translation initiation factor 4-γ 1	Missense mutations (Arg1205His, Ala502Val)
DNAJC13	Receptor-mediated endocytosis 8 (REM-8)	Missense mutation (Asn855Ser)
CHCHD2	Coiled-coil-helix-coiled-coil-helix domain containing 2	Missense mutations (Thr61Ile, Arg145Gln); splice-site alteration
Autosomal recessive		
Parkin	Parkin	Exon rearrangements, including exon deletions or multiplications (most common); missense mutations, nonsense mutations, small deletions or insertions; splice-site alterations
PINK1	PTEN-induced putative kinase 1	Missense or nonsense mutations (most common); exon rearrangements, including exon deletions or duplications
DJ-1	DJ-1	Missense mutations or exon rearrangements (most common); splice-site alterations

Table 1: **Monogenic forms of Parkinson's disease, by gene**

Lorraine V Kalia, Anthony E Lang,

Lancet, 2015

Table 1. Genes correlated to monogenic forms of PD.

1.2 Other risk factors

For such a complicated disease as Parkinson's, there are various risk factors. Ethnicity has been identified as a risk factor based on different studies, and indicates that there is a trend towards higher prevalence in European, North American, and South American population compared with African, Asian, and Arabic countries (Kalia & Lang, 2015). Excluding ethnicity, gender is a risk factor too; with males comprising the majority of patients with PD. According to different epidemiological studies, the established male-to-female ratio is 3:2 showing that men are marginally more susceptible to the disorder than women (De Lau LML, Breteler MMB., 2006).

Age is the greatest established risk factor for the development of Parkinson's disease. The high incidence of the disease increases with aging starting at the threshold of 50 years of age (Driver, J. A. et. al. 2012). The prevalence and incidence increase with age and has a peak after 80 years (Driver JA, et. al. 2009; Pringsheim T., et. al. 2014). Considering the fact that the life expectancy has increased worldwide as well as the population itself, the number of PD patients is expected to increase more than 50% in the next ten years, resulting in significant public health considerations (Dorsey ER, 2005).

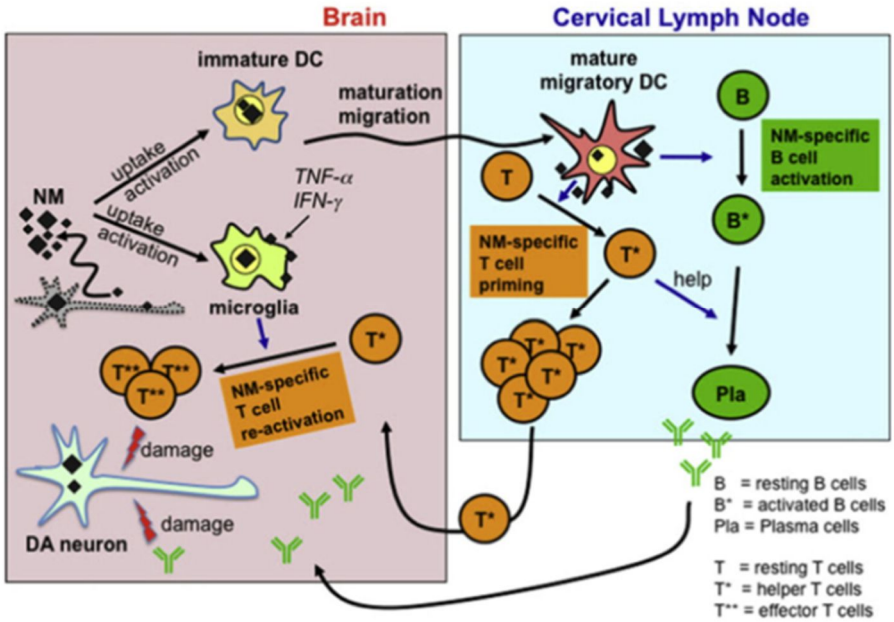
Environmental factors are also shown to be risk factors for the development of PD. The environmental risk factors with increased risk are the pesticide exposure, prior head injury, rural living, beta-blocker use (a competitive antagonist that blocks the receptor sites for the endogenous catecholamines epinephrine (adrenaline) and norepinephrine (noradrenaline) on adrenergic beta receptors , and is used in order to manage abnormal heart rhythms, of the sympathetic nervous system), and well water drinking. On the other hand, tobacco smoking, coffee drinking, NSAID use (Nonsteroidal anti-inflammatory drugs that reduce pain, decrease fever, prevent blood clots) , calcium channel blocker use (to decrease blood pressure in patients with hypertension) and moderate alcohol consumption constitutes the decreased environmental risk factors (Kalia & Lang, 2015).

1.3 Parkinson's disease and the immune system.

In addition to the genetic and environmental considerations, Parkinson's disease pathogenesis has been connected to immunologic factors. Recent studies indicate a significant role of the immune system in PD pathogenesis, through inflammation or autoimmune response (Armando De Virgilio, et. al. 2016). It is not well known if the immune activation is the cause or a response to the neuronal loss, which is observed in the SNpc region (Hawkes CH, et. al. 2010).

Several studies have revealed significantly increased levels of innate immune components such as IL-1, IL-2, IL-6 and TNF-a in the SN and cerebrospinal fluid (CSF) and γ/δ + T cells in the peripheral blood and CSF. Related to this, Benkler et al. suggested an autoimmune mechanism playing a role in PD pathogenesis (Liu B, Gao HM, Hong JS. 2003; Benkler M, et. al., 2012; Sulzer et al., 2017).

In this context, post mortem analyses from PD patients reveal IgG binding to dopaminergic neurons (Orr CF, et. al. 2005). The pigment neuromelanin that accumulates in dopaminergic neurons (catecholamine metabolism by-product), according to Oberlander et. al. triggers the functional dendritic cells (DC) maturation in vitro, which leads to a proliferative T cell response (Oberlander U, et. al. 2011). In the context of autoimmunity, Chen et. al. observed a marked loss of dopaminergic neurons (DN) in the SN of rats after transferring of plasma antibodies from PD patients (Chen S, Le WD, 1998).



Armando De Virgilio, et. al. Autoimmunity reviews, 2016.

Figure 3. Parkinson's disease pathogenesis and the immune system.

One suggested mechanism by which autoimmunity leads to dopaminergic cell loss is the activation of surrounding microglia, which take the role of macrophage cells in the CNS, and are the main glial cells in the inflammatory response (Le WD, et. al. 1999; reviewed by Armando De Virgilio, et. al. 2016). He Y, et. al. observed that IgG derived from PD patients can induce an Fcγ receptor pathway and as a result microglial activation (He Y, 2002). This suggests that there are peripheral inflammatory mediators which are elevated in PD, and which can trigger an activation response in other cells.

The activated innate immune system induces on a second time an adaptive immune response, with specific, targeted, and highly potent against the antigens presented in the inflamed region. The adaptive immune cells could promote both anti-inflammatory and pro-inflammatory reactions in response to specific antigens. In this context, they modulate certain environmental exposures and neuronal dysfunction (Shlomchik MJ, et al. 2012; Salmond RJ, et al. 2011). Although, adaptive immune responses are limited in the brain, through them neurons release proteins like a-synuclein to the surrounding neuroenvironment. There the modified proteins find their way to the peripheral lymphatic system, where microglia and macrophages infiltrate sites of neuronal injury and death initiatining proinflammatory responses (Reynolds, et. al. 2010; reviewed by R. Lee Mosley, et. al. 2016). Studies in the peripheral blood of PD patients, gave light in the role of adaptive immunity, in this specific neurodegenerative disease. Examination of the composition of T-cell subsets in the peripheral blood of PD patients showed decreased overall numbers of lymphocytes without a change in frequency, and reduced T helper cells (Stevens CH, et al. 2012). However recent studies suggest that the immune activation may be the cause of neuronal loss and not a response.

In peripheral macrophages, monocytic cells and central microglia express high levels of LRRK2, while altered activation of these cells have been observed in LRRK2 KO animals, which suggest a functional role of the protein in the innate immune system and its inhibition as a possible anti-inflammatory treatment for Parkinson's (Moehle MS., et. al. 2012; Thevenet J., et. al. 2011).

1.4 Current treatments

Currently, the available therapies for PD treat only the symptoms of the disease. Disease modifying drugs for slowing or stopping the neurodegeneration are not established yet, however there are some available treatments for the motor systems by stimulating dopamine receptors or regulate the dopamine concentrations. Such drugs are levodopa, dopamine agonists, monoamine oxidase type B inhibitors and amantadine (Fox SH., et. al. 2011; Connolly B., et. al. 2014). In addition, as the link between PD and the immune system has been strengthened, early immunomodulation may be a key treatment. An antibiotic, minocycline effectively crosses the blood brain barrier (BBB) and shows potent anti-inflammation in PD models, as well as LRRK2 inhibition as discussed above may have possible anti-inflammatory effects (Ton TG., et. al. 2006).

2. Leucine-rich repeat kinase 2

2.1 Domains, structure and physiological function of LRRK2

In 2004, two independent research groups identified LRRK2 as a causative gene in the PARK8 locus for autosomal dominant familial PD in multiple ethic families (Paisan-Ruiz C., et. al. 2004; Zimprich A, et. al, 2004). LRRK2 also known as dardarin, DRDN, PARK8, RIPK7 and ROCO2, translates to an unusual big sized protein of 2.527 amino acids with a molecular mass of 286.103 Da and is a multi-domain serine/threonine kinase.

The LRRK2 gene spans 145 kb and contains 51 exons (Mata et al., 2006). This gene is a member of the leucine-rich repeat kinase family and encodes a protein with an ankryin repeat region, a leucine-rich repeat (LRR) domain (N-terminal), a kinase domain (Ser/Thr), a GTPase domain,and a WD40 domain in the C-terminal (Figure 4). The protein is present largely in the cytoplasm as a monomeric protein but also associates with the mitochondrial outer membrane and cell membranes in a smaller dimeric population with higher kinase activity (Berger, Z., et. al. 2012; Ito, G. and Iwatsubo, T., 2012; Webber, P.J., et. al. 2011; Greggio, E., et. al. 2008).

The kinase activity of this enzyme is closely related to RIPKs, a family that consists of seven members and has a role in many different cellular pathways, including the recognition of cellular injury, pathogen and stress associated signaling (reviewed by Zhang D., et. al. 2010).

Rideout HJ., and Stefanis L., Neurochem Res 2014

Figure 4 . The LRRK2 domains.

LRRK2 expression is widely distributed in many mammalian cell types. The highest levels of expression have been observed in the kidney and lung, and in activated cells of the innate immune system, especially in macrophages (Moehle, M.S., et. al. 2012). In neurons the expression of endogenous LRRK2 is low and in the brain is restricted to medium spiny neurons in the striatum, in the cortex and hippocampus (Mandemakers, W., et. al. 2012).

Experimental approaches demonstrated that LRRK2 is localized in the cytoplasm of neurons and dendritic processes (Biskup, S., et. al. 2006). Thus LRRK2 is associated with structures in the vesicles and the membrane, as well as, the microtubules, mitochondria and other organelles binding to the membrane (Hatano, T., et. al. 2007). In 2006, Biskup et. al. studied the LRRK2 intracellular localization using different antibodies in rat primary cortical neurons and in rodent brains. LRRK2 found to co-localise to the Golgi apparatus and Golgi-associated vesicles, to the endoplasmic reticulum, lysosomes, mitochondria and in vesicles. Co-localisation was also evident between LRRK2 and microtubules in rodent brain slices (Biskup, S., et. al. 2006).

LRRK2 has also been linked with lysosomal degradation, mitochondrial function and interaction with alpha-synuclein (Esteves, a. R., et. al. 2014). According to Orenstein et al LRRK2, except from G2019S mutant, can be degraded in lysosomes by chaperone-mediated autophagy (CMA). Testing, CMA lysosomal markers in neuronal cultures and brains of LRRK2 transgenic mice, iPSC-derived dopaminergic neurons, and brains of mutant LRRK2 PD patients, they found that the CMA lysosomal binding substrates for wt and mutant LRRK2 interferes with the organization of the CMA complex, resulting in defective CMA (Orenstein et al 2013). In 2013, Manzoni et al. using primary fibroblasts from individuals carrying pathogenic mutations in the three central domains of LRRK2 detected for alterations in the autophagy/lysosomal pathway. In addition they assessed that inhibition of LRRK2 kinase activity stimulates macroautophagy in the absence of any alteration in the translational targets of mTORC1 signaling pathway (Manzoni et al. 2013). Studies in LRRK2 knock-out mice revealed that the protein is involved in protein homeostasis of the cell. Specifically, in the kidney of those mice, ubiquitinated proteins and aggregates of a-synuclein were detected, as well as altered levels of autophagy markers, such as p62 and LC3-II, which suggested a role in the autophagic pathway (Tong Y., et. al. 2010; Herzig MC, et. al. 2011; Tong Y., et. al. 2012).

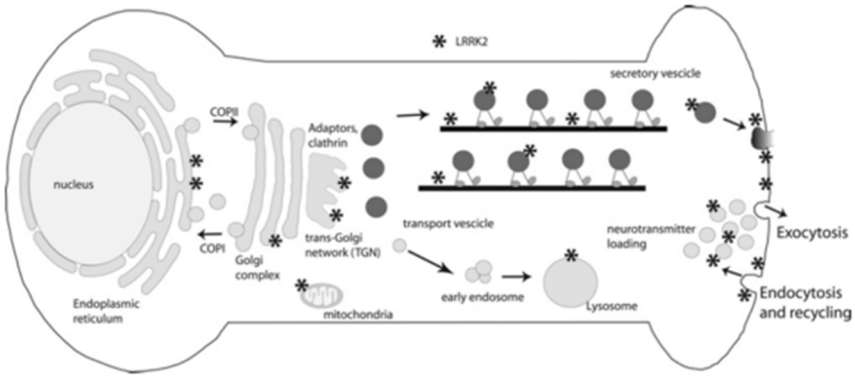
2.2 LRRK2 in membrane trafficking

It is known that LRRK2 associates with different vesicle types and intraluminal vesicles within MVBs or multivesicular bodies (Biskup, S., et. al. 2006). Moreover, Fraser et al. through purification of exosomes, showed that LRRK2 is secreted from intraluminal vesicles from MVBs in a variety of cells such as in kindney, brain and cells of the immune system (Fraser et. al.2013).

In 2011, Picoli et al. observed that LRRK2 silencing alters synaptic transmission, showing greater EPSC (excitatory postsynaptic current) generation over baseline after a presynaptic trigger, a decrease in docked vesicles and an increase in vesicle recycling. The same group tested LRRK2 implications in membrane trafficking. Although LRRK2 interacts with proteins involved in endocytosis and exocytosis of synaptic vesicles, by the WD domain, overexpression of LRRK2 was toxic (Picoli et. al. 2011). On the other hand, studies in other model organisms, like *Caenorhabditis elegans* reveals that a LRRK2 homologue, LRK-1 is important for axonal-dendritic polarity and synaptic vesicle localization (Sakaguchi-Nakashima A., et. al. 2007).

The same year, two different groups, using either overexpression or knockdown of endogenous LRRK2 in primary neuronal cell lines, revealed a role for LRRK2 in the control of synaptic vesicle endocytosis, which at the molecular level is related to its interaction with Rab5 (Shin N., et. al. 2008), highlighting its activity in retrograde vesicle trafficking from endosomes to the trans-Golgi.

LRRK2 interacts with 14-3-3 proteins, which modulate cell localization of their binding partners (Dzamko N., et. al. 2010). The interaction with 14-3-3 has been proposed to regulate LRRK2 function within the endocytic pathway, and its release in exosomes ((Fraser et. al.2013). And it has been shown by many groups that loss of 14-3-3 can lead to the re-distribution of LRRK2 into cytoplasmic filamentous structures that partially co-localize with microtubules (Kett et al., 2012). In figure 5 the LRRK2 localization and the cellular districts that affect the membrane trafficking are shown.



Giovanna Sanna et al., Biochemical Society Transactions (2012)

Figure 5. LRRK2 in membrane trafficking

2.3 LRRK2 and the immune system

Expression of LRRK2 is robust within the immune system, especially in T cells, and many subtypes of monocytes, where the highest expression is observed (Gardet et al., 2010; Hakimi et al., 2011; Thévenet et al., 2011; Schapansky et al., 2014). Recent genomic studies have revealed that LRRK2 is implicated in autoimmune disorders such as Crohn's disease and colitis (Cardoso et al., 2011; Umeno et al., 2011). The high expression of LRRK2 in monocytes, which is even higher than in neurons, implicates a role of LRRK2 in the innate immune system and the first line of defense against infection (Schapansky et al., 2014; Gardet et al., 2010).

Although it was suggested that cytokine production is regulated by LRRK2, studies in Bone marrow-derived macrophages from LRRK2 KO animals showed controversial results. One study from Liu et. al. revealed increased IL-12p40 and IL-6 levels, when in two other studies no significant differences were observed between KO and WT LRRK2 animals (Liu et al., 2011; Hakimi et al., 2011; Dzamko et al., 2012). It is possible that differences in the methodology between these studies can account for the divergent findings. Furthermore, studies in microglial and macrophage cells of transgenic mice expressing disease-linked mutations in LRRK2 featured similar results. While microglial cultures from R1441G KI mice showed a significantly increased cytokine expression compared to cultures derived from WT mice, in macrophages from R1441C KI mice the levels of IL-6 secretion were similar to WT cells (Gillardon et al., 2012; Hakimi et al., 2011; reviewed in Longitudinal S., et. al. 2015), suggesting here that the mechanism of cytokine release may be affected.

Monocyte activation by interferon gamma significantly increases LRRK2 expression, while LPS-induced activation through stimulation of the Toll-like receptor 4 (TLR4) boosts its expression and results in LRRK2 phosphorylation at Ser935 from minutes to hours ((Moehle et al., 2012; Dzamko et al., 2012; Schapansky et al., 2014; reviewed in Longitudinal S., et. al. 2015).

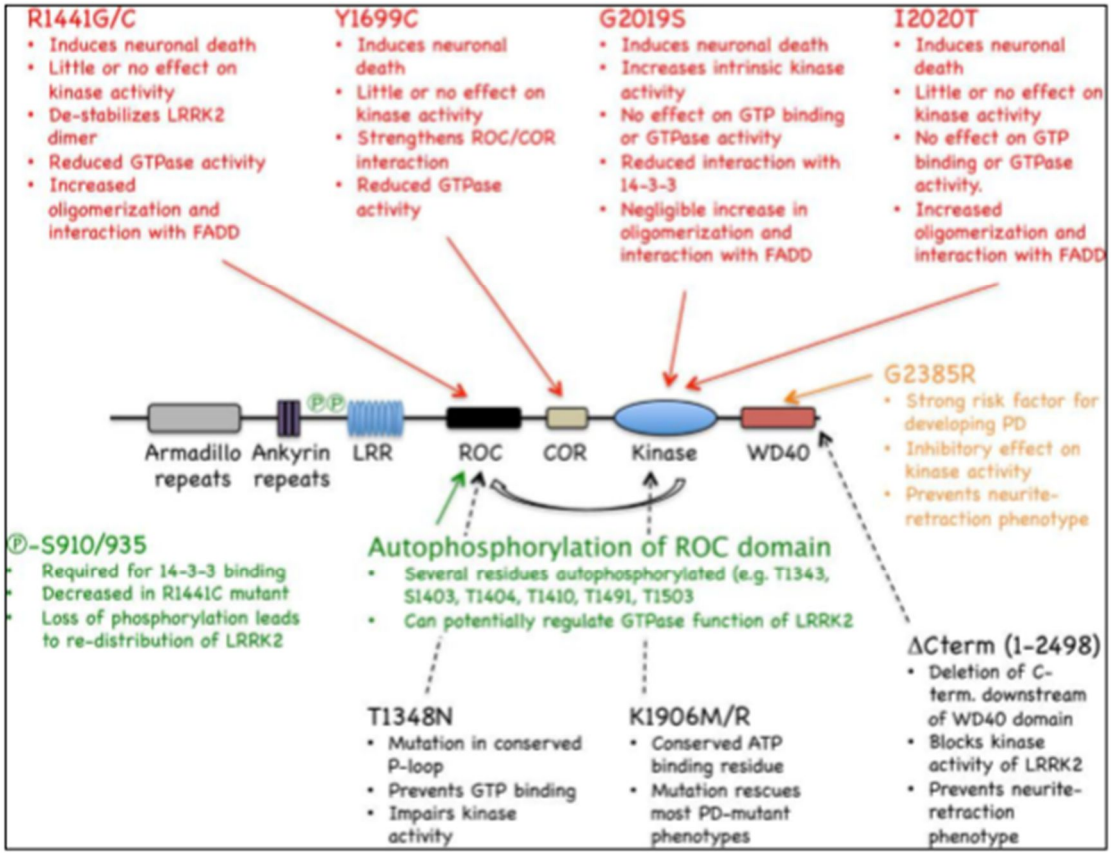
2.4 Mutations and PD pathogenesis

As it has already been mentioned, LRRK2 missense mutations are the most common known genetic cause of PD. They are inherited in an autosomal dominant fashion and the carriers are mostly heterozygous. Variants in LRRK2 are considered generally as pathogenic or risk factors, however some protective haplotypes have also been described. Pathogenic mutations from a genetic view, are those that clearly co-segregate with the occurrence of the disease (Greggio, E. & Cookson, M. R. 2009). Additionally, several loss of function mutations (e.g. early stop codons) have also been identified, but their prevalence is not different from healthy control populations.

Many mutations spanning the length of LRRK2 have been associated with PD, although only mutations in the enzymatic GTPase and kinase domains segregate with familial disease making evident that the enzymatic activities are important to disease development. Pathogenic variants include the missense mutations R1441C/G/H/S, Y1699C, G2019S, and I2020T; whereas the increased risk factors include R1628P, G2385R as (Dachsel and Farrer, 2010; Giasson and Van Deerlin, 2008).

Disease symptoms in LRRK2 mutation cariers are nearly indistinguishable from idiopathic PD (iPD). Neuropathologically, however, there is heterogeneity between the different mutations, and between carriers and iPD patients (Giasson et al., 2006; Ross et al., 2006; Taylor et al., 2006; Kett and Dauer, 2012). Some cases have both neuronal loss and Lewy body pathology, whereas some have neuronal loss without Lewy bodies. The reason for these differences is unclear.

Most of the familial disease-associated mutations tend to cluster within the central ROC COR-kinase region of LRRK2. This indicates that the GTPase and the kinase domain, as well as their regulation are of a high importance. R1441G, R1441C and R1441H mutations may reduce the GTPase activity and potentially alter GTP binding to the Roc domain (Liao et al., 2014). The above mutations together with Y1699C and I2020T have been reported to increase kinase activity in some of the studies as the methodology depending on the phospho-substrate that is assessed (Ray et al., 2014). In addition G2019S and I2020T are characterized by increase in the phosphorylation states (Martin et al., 2014). Figure 6 represents the most known LRRK2 mutations as well as the predicted functions of LRRK2 domains.



Rideout et al.Neurochemical Research, 2014.

Figure 6. The LRRK2 mutations within the LRRK2 domains.

3. *Rab GTPases*

The discovery of Rab GTPases came in the 1980s by Dieter Gallwitz in yeast, characterizing a GTP-binding protein, on the Golgi, named Ypt1. This protein was related to Ras protein family but was not identical to *RAS1* or *RAS2* genes so it was considered having a different role (Schmitt et al., 1986; Segev et al., 1988). Earlier in this decade, Peter Novick described the SEC genes responsible for secretion in yeast and focused on the delivery to the cell surface (Novick et al., 1980).

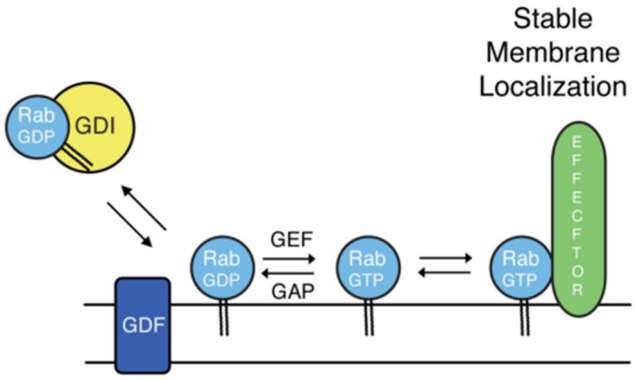
The first evidence that membrane trafficking might require a Ras-like GTPase, was with the sequencing of *SEC4* gene, which identified a Ras-related protein (Salminen et al., 1987) present on secretory vesicles (Goud et al., 1988). Almost ten years later, it was shown that this protein recruits the Exocyst tether to aid fusion of the delivery vesicles (Walch-Solimena et al., 1997; Guo et al., 1999). Zerial and colleagues showed that a Rab protein (Rab5) played a key role in membrane traffic, as Sec4, and using exclusively Rab5-binding proteins they characterized its function in endosome fusion and endocytosis (Gorvel et al., 1991)

Studying the mammalian homologues and cloning the yeast gene relatives Tavitian named these proteins Ras-like proteins from rat brain or Rabs (Touchot et al., 1987; Chavrier et al., 1990a). Rabs took their names (numbered) randomly in an order of their related yeast homologues (Rab1 was the homologue of Ypt1). Colicelli found that in humans at least 63 Rabs are known, while yeast possesses 11 (Colicelli, 2004). The number of Rabs is even larger in some protozoans (Saito-Nakano et al., 2005).

Rabs are comprised of a GTP-binding and hydrolysis domain, linked to an unstructured and hypervaliable C-terminal domain prenylated on one or two cysteine residues permitting their tight membrane association (Itzen A, Goody RS, 2011). Through prenylome-wide analysis revealed that at least 42 different Rabs can be found in a single cell, however some Rabs are expressed in a tissue-specific manner (Nguyen et al., 2009).

In 1990 Sakaki et al., discovered a cytosolic protein in rat brain with that could inhibit a small GTPase (smg p25) of releasing GDP. The GTPase identified later as Rab3A and the cytosolic protein named GDP-dissociation inhibitor (GDI) (Sasaki et al., 1990). Two GDIs have been identified in human and one in yeast that have the ability to bind in all Rab GTPases with a preference in GDP-bound states. Active Rabs carry GTP when inactive Rabs carry GDP. The GTP cycle that Rab proteins follow is explained in Figure 7. There, guanine nucleotide exchange factors (GEFs) activate Rabs and GDP is released. GEFs are major factors of Rabs' membrane targeting specificity (Blümer et al., 2013). On the other hand, GTPase activation proteins or GAPs inactivate Rabs (Itzen A, Goody RS, 2011; Barr F, Lambright DG, 2010). In addition it has been shown that Rab proteins are delivered onto membranes

in their GDP-bound forms and at least in vitro they converted into their GTP-bound forms after ~5 min (Soldati et al., 1994; Ullrich et al., 1994).



Suzanne R. Pfeffer, Curr Opin Cell Biol (2013).

Figure 7. Prenylated Rab GTPases are delivered to membranes by GDI.

Several studies showed that effector binding stabilizes Rabs on membrane surfaces, which is GTP-dependent (Aivazian et al., 2006). Almost 30 specific effector proteins have been recognized (Christoforidis et al., 1999). Through this regulation, each Rab could be the substrate for a next one, creating Rab signaling cascades that control the membrane trafficking (Vitale et al., 1998; de Renzis et al., 2002).

3.1 LRRK2 and Rab GTPases

Recently, in 2016 several Rab proteins have been shown to be endogenous phosphorylation substrates for the LRRK2 kinase (Steger et al., 2016; reviewed by Kiral, F. R., 2018). A subgroup of up to 14 Rab GTPase proteins has been identified as direct substrates for LRRK2 and these are Rab3A, Rab3B, Rab3C, Rab3D, Rab5A, Rab5B, Rab5C, Rab8A, Rab8B, Rab10, Rab12, Rab29, Rab35 and Rab 43 (Steger et al., 2016; Steger et al., 2017).

The LRRK2-dependent phosphorylation site within the Rab's lies in a conserved switch-II motif. For Rab8A is in Thr72, for Rab10 Thr73 and for Rab12 Ser106. This site could alter the ability of Rabs to interact with cognate effectors (Steger et al., 2016; Steger et al., 2017; Jeong, G. R., et al., 2018). For example the phosphorylation of Rab8 and Rab10 by LRRK2 prevent interactions with Rabin-8 (a GDP/GTP exchange factor), and GDI (Jeong, G. R., et al., 2018).

In the context of Parkinson's disease, all pathogenic LRRK2 mutations significantly stimulate Rab protein phosphorylation (Lis, P., et al., 2018). In a related study, Rab proteins reduce cytotoxicity related to α-synuclein aggregation (reviewed by Kiral, F. R., 2018); possibly providing a mechanism for LRRK2-mediated protection against alpha-synuclein toxicity (Daher et al. 2014).

3.2 Rab10

Generally Rab proteins have restricted function and localization. However, Rab10 appears to be rather anomalous and is ubiquitously expressed. Rab 10 was cloned from Madin-Darby Canine kidney (MDCK) cells (Chavrier et al., 1990) and its function was similar to Rab8 in terms of polarized exocytosis (Peränen et al., 1996). This Rab GTPase is implicated in exocytic mechanisms in adipocytes (Sano et al., 2008), neurons (Wang et al., 2011) and endophagocytosis in other cell types (Cardoso et al., 2010) and many others such as regulating actin binding and endosomal regulation, affecting axonal growth, pathfinding, and regeneration, membrane protein recycling etc depending on the cell type concerned and the interacting proteins involved (reviewed by C. Lin, B. Tang, 2017). The sub-cellular localization of Rab10 is varied, and has been found in the endoplasmic reticulum (ER) (English,Voeltz, 2013), in the Golgi/TGN (Chen et al., 1993; Leaf, Blum, 1998), in the endosomes and/or phagosomes (Cardoso et al., 2010) as well as in primary cilia (Babbey et al., 2010).

In 2010 Lu's laboratory, published a paper showing that Rab10 facilitates Toll-like receptor-4 (TLR4) signaling by controlling its trafficking onto the plasma membrane (Wang et al., 2010). Initially, they observed that Rab10 expression is up-regulated upon TLR4 activation by LPS. Using Immunofluorescence in Raw 264.7 macrophage cells, they found that Rab10 and TLR4 co-localized in endomembrane compartments. In addition, silencing of Rab10 expression reduced the production of LPS-induced pro-inflammatory mediators in macrophages, when Rab10 overexpression enhanced TLR4 signaling and functions. According to this paper, the TLR4 cell surface expression was modulated via Rab10 silencing by promoting its translocation. In the same paper, using an in vivo model of human acute respiratory distress syndrome (ARDS), they demonstrated that after LPS stimulation, altered Rab10 expression in macrophages influences disease severity. These findings suggest that continuous replenishment of TLR4 receptor from the ER and Golgi is a crucial step for TLR4 signaling and this process is mediated by a Golgi-associated Rab10 in a GTPase-dependent manner (Wang et al., 2010).

4. TLRs

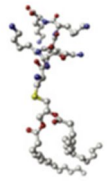
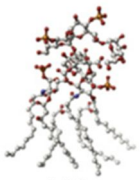
Toll-like receptors consist a family of single-pass type I transmembrane protein receptors (reviewed in Moresco et al., 2011). They took their name (Toll) after the identification of the original gene in *Drosophila melanogaster* by Gay, N. J. and Keith, F. J, in 1991. These receptors are prototype pattern-recognition receptors (PRRs), that sense pathogen-associated molecular patterns (PAMPs) from microorganisms such as lipopolysaccharide (LPS), peptidoglycan, flagellin, and microbial nucleic acids, or damage-associated molecular patterns (DAMPs) such as released molecules upon cellular stress or tissue damage, oxidative stress and heat shock proteins, unmethylated double-stranded DNA (CpG) and single-stranded RNA (ssRNA) (O'Neill LA, 2008; Yu Liu et al., 2014). In figure 8, the ligands of TLR2 and TLR4 can be shown. Activated TLRs could regulate inflammatory cytokines and chemokines enrolling intracellular signaling pathways to regulate the inflammatory response (Brown J, et al., 2011; Yu Liu et al., 2014).

Toll-like receptors are integral membrane glycoprotein receptors with a molecular weight from 89 to 150 kDa. They are characterized by three distinct components, an N-terminal domain, which functions as a ligand recognition domain, a single transmembrane helix and a C-terminal cytoplasmic signaling domain (Matsushima N., et. al. 2009). The ligand recognition domain for PAMPs and DAMPs is an extracellular leucine-rich repeats domain (16-28 LRRs), almost 20 uncharged, hydrophobic residues constitute the transmembrane domain and the intracellular domain is also known as TIR or Toll/Interleukin-1 (IL-1) receptor (Wang Y., et. al. 2013; Botos I., et. al. 2011; Matsushima N., et. al. 2007).

In 1996, Lemaitre et al. discovered that Drosophila mutants in the Toll gene are more sensitive to fungal infections, and the role of TLRs in innate immunity became evident (Lemaitre et. al. 1996). The first ever TLR identified in human, was TLR4 (Medzhitov et al. 1997). Since then, 10 TLR family members have been discovered in humans, and at least 13 in mice. TLR3, TLR7, TLR8, and TLR9 are nucleic acid-sensing TLRs and are localized within endosomal compartments, while the other TLRs are localized at the plasma membrane (Blasius and Beutler 2010; McGettrick and O'Neill 2010; reviewed in Kian-Huat Lim and Louis M. Staudt 2013).

The trafficking of TLRs is orchestrated in part by ER resident proteins, such as UNC93B and PRAT4A; whereas for some TLRs, co-receptors including CD14 and MD2, facilitate the ligand binding (Blasius and Beutler 2010). Moreover, TLR signaling is turned off by various negative regulators such as IPAK-M, MyD88 short, FADD, SHP1 and SHP2 (Flannery and Bowie 2010; Kawai and Akira 2010). Deregulation of this signaling pathway cascade can lead to several human diseases, like auto-immune diseases and lymphoma malignancies (Ngo et al. 2011).

Receptor	PAMPs	DAMPs
TLR2	Lipoproteins	Snapin
	Peptidoglycan	Hyaluronic acid
	Lipoteichoic acid	Hsp 70
	Lipoarabinomannan	HMGB1
	Glycosylphosphatidylinositol	Gp96
	Phenol-soluble modulin	
	Zymosan	
	Glycolipids	
TLR4	Lipopolysaccharide	Hsp 22, 70, 72
	Taxol	HIF-1α
	Viral glycoproteins	HMGB1
	rSV fusion protein	Fibronectin
	MMTV envelope protein	ECM components
		Fatty acid
		mmLDL
		Fibrinogen

Receptor	TLR2/1 or 2/6	TLR4
Ligands	Lipopeptides	LPS
Source	Gram-positive bacteria, fungi	Gram-negative bacteria
Examples	 Pam_2CSK_4	 LPS

Liu Y., et. al., Clinical reviews in allergy and immunology, 2014

Moresco E., et. al. Current Biology, 2011

Figure 8. Ligands of TLR2 and TLR4.

4.1.1 TLR2

As it has been described above, TLR2 and TLR4 are cell-surface TLRs. TLR2 is expressed by a variety of cell types, including immune cells such as monocytes, myeloid DCs, mast cells, T lymphocytes, B lymphocytes, synovial fibroblast like cells and epithelial cells (Huang QQ and Pope RM, 2009). TLR2 is a member of the MyD88-dependent receptor family that can bind a wide range of ligands, both exogenous and endogenous DAMPs that are depicted in figure 8 above (reviewed in Kian-Huat Lim and Louis M. Staudt, 2013).

Following the simultaneous binding of ligands in the LRRs of receptor chains domain, TLR2 form heterodimers, either with TLR1 or TLR6 (Lorne E., et. al, 2010). Upon activation, heterodimers interact with lipopeptides through a hydrophobic pocket. This pocket is formed near the center of the dimer (reviewed in Moresco E., et. al. 2011).

TLR2 is implicated in a variety of auto-immune diseases, as well as in other diseases, including synucleionopathies. TLR2 has increased levels of expression in synovial fibroblasts and macrophages in Rheumatoid arthritis (RA) patients (Radstake TR, et. al. 2004). In addition, TLR2 promotes angiogenesis, cell adhesion, and invasion through a key pathway in the pathogenesis of RA (Saber T., et. al. 2011). Human synovial fibroblasts and peripheral blood mononuclear cells (PBMCs) from RA patients revealed increased levels of IL-6, IL-17, as well as Heat shock glycoprotein 96 (Gp96) expression in the synovial fluid of the joints in RA (Chovanova L., et. al. 2013; Tang CH, et. al. 2010; Huang QQ, et. al. 2012).

Recently, some studies changes in expression of TLR2 in Systemic lupus erythematosus (SLE). According to Komatsuda A. et. al. the mRNA levels of TLR2 are significantly increased in PBMCs derived from SLE patients, while in experimental animal models, C57BL TLR2 deficient mice developed a less severe disease and fewer immunological alterations (Komatsuda A., et. al. 2008; Loser K., et. al. 2010). In addition, in systemic sclerosis (SSc), a connective tissue disease of presumed auto-immune origin, stimulation of DC subsets by ligands for TLR2 results in increased secretion levels of IL-6, IL-10 and TNFa, but decrease of IL-12 (Van Bon L., et. al. 2010). Furthermore, in salivary gland epithelial cells, acinar cells and salivary-infiltrating mononuclear cells of Sjogren's syndrome patients, TLR2 has higher expression levels and increased production of IL-23/IL-17 from PBMCs of patients (Kawakami A., et. al. 2008; Kwok SK, et. al. 2012; Spachidou MP, et. al. 2007). Elevated expression of TLR2 is also observed in PBMCs and keratinocytes from psoriasis patients (Carrasco S., et. al. 2011).

In multiple sclerosis (MS), which is a chronic inflammatory disease of the central nervous system, elevated TLR2 expression in patients (PBMC and CSF-mononuclear cells) and rodent experimental auto-immune encephalomyelitis models has been observed, while in TLR2-null mice remyelination is increased (Sloane JA, et. al. 2011; Andersson A., et. al. 2008). In the above models, a TLR2 ligand (HMGB-1) is evident in active lesions, suggesting TLR2 signaling may play a role in MS pathogenesis. In addition, TLR2 has been shown to play a significant, central role in the pathogenesis of auto-immune diabetes (Filippi CM, et. al. 2011; Devaraj S., et. al. 2011; Ururahy MA, et. al. 2012).

4.1.2 TLR2 in synucleionopathies and PD.

This implication between immune response and CNS is both beneficial and detrimental to health. As it has already been mentioned, microglial cells, as well as macrophages, play an important role in this interaction. Microglial cells, express TLRS that can detect changes in the environment of neurons, and thus their activation and inflammatory response are related to synucleionopathies and PD. In 2010, Lin H, et. al. PGN-induced iNOS, COX-2 and proinflammatory cytokine expression is mediated through the TLR2/MyD88/PI3-kinase/AKT pathway, This pathway initiates IKKα/β and NF-κB activation in BV-2 microglia (Lin H., et. al. 2010).

TLR2 is increasingly implicated in Parkinson's disease pathogenesis, recognizing PAMPs or DAMPs, leading to the release of inflammatory and toxic molecules, and neuroinflammation (Dzamko, et. al. 2017; Kim, et. al. 2013; Panaro, et. al. 2008). This receptor is expressed in neurons and migroglial cells associated with a-synuclein accumulation (Trudler D., et. al. 2010; Dzamko, et. al. 2017). a-synuclein oligomers are considered as an endogenous ligand for TLR2 (Kim, et. al. 2013). Recently, studies revealed that a-synuclein triggers TLR2 in human monocytes inducing interleukin-1β (IL-1β) production (Codolo G., et. al. 2013; Drouin-Ouellet et. al. 2015). In addition, a few reports have revealed an upregulation in several TLRs in models of PD and other synucleinopathies, as the (thy1)-[A30P]-αSYN, the (PLP)-αSYN, and Thy1.2-αSyn mice (Stefanova, et. al. 2007; Letiembre, et. al. 2009; Watson, et. al. 2012).

It is known that TLR2 activation in neurons and microglia leads to the production of TNFa and IL-10. TNFa presents deleterious effects on dopaminergic neurons, while IL-10 seems to be protective in PD (Borrajo, et. al. 2014; Dzamko, et. al. 2017; Fellner, et. al. 2013; Neves, et. al. 2015;

Johnston, et. al. 2008; Yang, et. al. 2015; Zhu, et. al. 2017). In 2016, Silva et. al. demonstrated the production of less cytokines like TNFa, IL-1β, IL-6, IL-10, after stimulation with TLR2 agonist in whole blood cultures from PD patients. However, the percentages of monocytes ($CD14^+/CD16^+$ or $CD14^+/CD16^-$) had similar expression between patients and controls (Silva, et. al. 2016).

As it has already been mentioned, PD is an age-related disease. However, Rocha S., et. al., suggest that TLR2 deficiency in periphery is independent of age of the patients, as well as the age at PD onset, or duration (Rocha S. et. al. 2018). In addition, in 2017, Xiaoyuan Li, et. al. revealed the association between two genetic variants (rs3804099 and rs3804100) of TLR2 as risk factors of sporadic PD in Han Chinese population (Xiaoyuan Li, et. al. 2017).

4.1.1 TLR4 in neurodegeneration and PD.

TLR4 was the first characterized TLR by Medzhitov in 1997 (Medzhitov R, et. al. 1997). As for TLR2, TLR4 is a cell surface receptor expressed by a wide range of cells like monocytes, myeloid DCs, mast cells, T and B lymphocytes, synovial fibroblast-like cells and epithelial cells as well as by microglia, enabling them to sense pathogens (Huang QQ and Pope RM, 2009; Hutchinson M. R., et. al 2008). TLR4 initiates both myeloid differentiation factor 88 (MyD88) (dependent and Toll/interleukin (IL)-1R domain containing adapter), inducing interferon signaling, leading to pathogens' reduction (Wang Y., et. al 2018).

The ligands of Toll-like receptors such as LPS, taxol and fibronectin are depicted in figure 8. Exogenous ligands like LPS, enhance the production of pro-inflammatory cytokines and chemokines in PBMCs of patients in several immunological and neurodegenerative diseases. After the binding of ligands in the LRRs of receptor chains domain, TLR4 forms homodimers. Firstly, a required co-receptor, MD-2 has to be formed. After that, LPS induces the formation of two TLR4-MD-2 dimers. Thus, TLR4 dimerization is actually formed by two dimers, as follows: The first dimer (TLR4-MD-2) after LPS interaction with a hydrophobic pocket within MD-2 is linked with the other dimer (TLR4-MD-2) via the surface of this TLR4 (Nagai, Y., et. al. 2002; Park, B.S., et. al. 2009; reviewed by Moresco, et. al. 2011). TLR4 is transported from the plasma membrane to the endosome for ubiqutination and to the lysosome for degradation, so important ways for negative regulation of TLR4 signaling are the downregulation of its expression or the degradation of TLR4. This receptor was shown to have a complex signaling arrangement, as it activates two pathways: either the MAL-MYD88 pathway inducing NF-κB signaling or the TRAM-TRIF pathway inducing IRF3 signaling (Wang Y., et. al 2007).

TLR4 is implicated in a variety of auto-immune diseases, as well as in other diseases, including neurodegeneration. In autoimmune diseases, TLR4, as well as TLR2, are increased in synovial fibroblasts and macrophages in Rheumatoid arthritis (RA) patients (Radstake TR, et. al. 2004). In addition, TLR4 response to LPS triggers appeared enhanced in PBMCs of early onset RA patients, which leads to increase of IL-6 and TNFa production (Kowalski ML, et. al 2008). Double knock-out mice ($IL1rn^{-/-}Tlr4^{-/-}$) protected against severe arthritis, with lower numbers of Th17 cells and less IL-17 cells. In macrophages and fibroblasts, TLR4 activation by LPS led to increased

collagen-induced arthritis in mice (Abdollahi-Roodsaz S., et. al 2008; Hou Y, et. al 2013).

Recently, some studies revealed the expressional change of TLR4, as well as for TLR2, in Systemic lupus erythematosus (SLE). Martina Kirchner et al. showed that TLR4 cell surface expression was decreased dramatically on $CD14^+$ monocytes in SLE patients (Kirchner M, et al 2013). In those patients IL-10 production is down-regulated upon TLR4 activation, while TNFa is decreased by TLR2 activation rather than TLR4 (Tsao JT., et al 2012). TLR-4 deficient mice develop fewer alterations in cytokine and chemokine production, and a less severe disease (Loser K, et al. 2010). In transgenic mice with monoclonal anti-dsDNA, severe SLE syndromes through the high production of IL-10 and IFN-gamma, both in vivo and in vitro after TLR4 activation by LPS (Lee TP, et al. 2010). As for TLR2, stimulation of patients' DC subsets by ligands for TLR4 results in increased secretion levels of IL-6, IL-10 and TNFa, but decrease of IL-12 (Van Bon L., et al. 2010). Besides DCs, TLR4 is also expressed on surface of fibroblasts and is considered as a potential therapeutic target for SSc (Fineschi S., et al. 2008).

For Sjogren's syndrome patients, psoriasis patients, the pathogenesis of auto-immune diabetes and multiple sclerosis, TLR4 presents the same pattern of immunological alteration and pathology with TLR2. In addition, TLR4 signaling pathway also plays a role in the response to interferon-beta (IFNβ) treatment in MS patients (Bustamante MF, et al. 2011; Kawakami A., et al. 2008; Kwok SK, et al. 2012; Spachidou MP, et al. 2007; Carrasco S., et al. 2011; Sloane JA, et al. 2011; (Filippi CM, et al. 2011; Devaraj S., et al. 2011; Ururahy MA, et al. 2012).

4.2.1 TLR4 in neurodegeneration and PD.

As it has already been mentioned, microglial cells, as well as macrophages, express TLRS that can detect changes and project this through signaling molecules to neurons, and thus their activation and inflammatory response are related to neuronal pathology and PD. The expression of TLR4 in the PD brain is associated with neuroinflammation and neurodegeneration (Dzamko et al., 2017; Shin et al., 2015). According to Fellner et al. TLR4 seems to be activated by α-synuclein, while TLR4 activation and TNFa expression presents deleterious effects in neurons and microglial cells (Fellner et al., 2013; Borrajo et al., 2014; Dzamko et al., 2017; Neves et al., 2015; Johnston et al., 2008; Yang et al., 2015; Zhu et al., 2017).

In 2011, Stefanova et al. suggested that TLR4 activation promotes the clearance of microglial a-synuclein in a-synucleinopathies, is a mediator of the microglial phagocytosis of a-synuclein in a model of multiple system atrophy and is implicated in a-synuclein-induced inflammatory responses (Stefanova et al., 2011). In another multiple system atrophy study, the hypothesis is also supported by Brudek et al., who measured increased levels of TLR4 mRNA in the SN, striatum, cortex, and nucleus dentatus (Brudek et al., 2013). Four years later, Drouin-Ouellet et al. using flow cytometry and western blots to detect TLR4 expression in blood and brain samples of Parkinson's disease patients and mice overexpressing human a-synuclein, they demonstrated that TLR4 expression is increased and overexpression of a-synuclein leads to a progressive microglial response (Drouin-Ouellet et. al. 2015).

In the aspect of cell lines, TLR4 signaling proved to have a critical role in the activation of BV-2 cells and the induction of inflammation in this cell model, after stimulation with MPP^+. MPP+ activate BV-2 cells for mimicking PD inflammation by the inflammatory response (and NF-κB activation and pro-inflammatory secretion) of TLRs like TLR2 and TLR4 to MPP^+ (Zhou P et al 2016). These findings are also supported by Lawrimore and Crews, who treated microglia-like BV2 and retinoic acid-differentiated neuron-like SH-SY5Y cells with a TLR4 agonist (LPS) to examine pro-inflammatory immune signaling, kinase, and transcription factor activation. Their results support that TLR4 signaling in those cell lines, is crucial for the complex brain neuroimmune signaling (Lawrimore CJ and Crews FT 2017).

In macrophages, An et al. observed decreased TLR4 expression following LPS and CpG DNA stimulation (An et al., 2002). Using flow cytometry, different groups reported downregulation of TLR4 expression after LPS treatment on RAW264.7 cells and mouse peritoneal macrophages (Akashi et al., 2000; Rhule, et al., 2006). In addition, exposure to the polysaccharide from the roots of Actinidia eriantha (AEPS) contributes in to down-regulation of TLR4 gene, in RAW264.7 cells and evokes response through TLRs/NF-κB signaling pathway (Sun H., et al. 2015). Similarly, Wang et al who have previously identified a lysosome-associated GTPase Rab7b, demonstrated that this Rab negatively regulates TNFa, IL-6 and IFNβ after LPS stimulation and colocalizes in LAMP-1– positive subcellular compartments with TLR4 decreasing TLR4 expression in RAW264.7 cells (Wang et al. 2007).

5. PFFs

The question of the propagation of a-synuclein pathology is a large field of investigation, and is thoroughly reviewed in this recent report (Peelaerts et al., 2018). The first reported efficient seeding of aggregation and fibrilization of endogenous a-synuclein in primary neuronal cultures generated from wt mice, by recombinant a-synuclein pre formed fibrils or PFFs, was in 2012 (Luk KC. et al. 2012). The same group demonstrated this in wt animals in vivo, by stereotaxic injections targeting the dorsal striatum, a region interconnected with multiple CNS nuclei, including midbrain DA neurons. With immunohistochemistry they show deposits of phosphorylated asyn (pSyn129), a pathological marker, in the injection sites, and abundant asyn pathology in TH^+ (tyrosine hydroxylase) SNpc DA neurons. Analyses revealed that the pathological transfer of endogenous mouse αsyn in the brain (i.e. cell-to-cell transmission) followed intraneuronal connectivity. In addition through behavioral tests they proposed that α-Syn pathology is sufficient to generate the principal behavioral and pathological characteristics of sporadic PD (Volpicelli-Daley LA et al. 2011; Luk KC. et al. 2012). Moreover, synthetic αsyn pre formed fibrils, can act as ligands for TLRs in cell culture and change their signaling and membrane trafficking (Watson MB et al 2012). While there is still some discussion in the literature about the specific forms and structures of a-synuclein fibrillar preparations (Melki, 2018), we used these in our experiments as a model for extracellular pathological forms of a-synuclein.

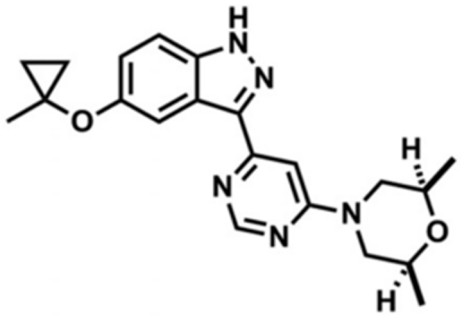
6. LRRK2 kinase inhibitors

As it has already been mentioned, certain mutations in LRRK2 are causative for PD. At least seven LRRK2 point mutations clearly segregate with familial disease (Paisan-Ruiz, et al., 2004; Zimprich, et al., 2004; Kachergus, et al., 2005; Bonifati, 2007). The most common one, G2019S, occurs within the LRRK2 kinase activation loop and leads to increased LRRK2 kinase activity (West, et al., 2005; Greggio, et al., 2006). More recent evidence points to a role for LRRK2 even in idiopathic forms of PD (di Maioi et al., 2016) Thus, the development of potent and selective inhibitors of LRRK2 is crucial as a potential mechanism leading to disease modification.

Except of PD pathogenesis, the development of such inhibitors could reveal clues for the evaluation of LRRK2 pleiotropy beyond neurodegenerative diseases (Fell, et al. 2015). Although a few potent inhibitors of LRRK2 kinase activity with improved selectivity, have been identified, like LRRK2-IN-1, GSK2578215A and HG-10-102-01, their utility is restricted to in vitro assays (Deng, et al., 2011; Reith, et al., 2012; Choi, et al., 2012). In 2012 and 2015, two LRRK2 kinase inhibitors, GNE-7915 and GNE-0877 were tested in rodents and nonhuman primates. While these inhibitors offered greatly improved selectivity as well as brain penetrance, some potential off-target effects remained (Estrada, et al., 2012; Fuji, et al., 2015).

6.1 MLi-2

In 2015, Fell et. al., discovered of cis-2,6-dimethyl-4-(6-(5-(1-methylcyclo-propoxy)-1H-indazol-3-yl)pyrimidin-4-yl)morpholine or MLi-2, a highly potent, and selective LRRK2 kinase inhibitor with activity in the central nervous system, meaning it crosses the blood brain barrier. Testing its pharmacological properties they found that MLi-2 has greater than 295-fold selectivity for almost three hundred kinases. Measuring the dephosphorylation of pS935 LRRK2, in MLi-2 treated mice revealed a dose-dependent inhibition, central and peripheral over a 24-hour period. In addition, MLi-2 administration has shown some potential side effects, including morphologic changes in the lung, consistent with enlarged type II pneumocytes, similar to the lung phenotype observed in LRRK2 KO animals (Matthew J. Fell, et. al. 2015; Ekstrand, et. al., 2007).

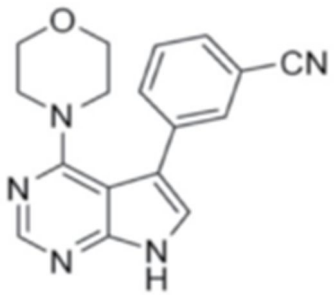


Matthew J. Fell, et. al., J Pharmacol Exp, 2015

Figure 9. MLi-2 structure.

6.2 PF-475

In 2014, Henderson et al. identified PF-06447475 (PF-475), a highly potent, brain penetrant and selective LRRK2 inhibitor (Henderson et al. 2014). While there was some evidence that LRRK2 signaling played a role in a-synuclein neurodegeneration, LRRK2 kinase inhibitors had not been tested previously for efficacy in models of a-synuclein-induced neuronal loss. Thus, Daher et al., injected human a-synuclein with adeno-viral vectors in the substantia nigra of wt and G2019S-LRRK2 rats, for 4 weeks. Administration of PF-475 in rats produced a reduction of neurodegeneration and neuroinflammation that had been increased in response to a-synuclein overexpression (Daher, et al. 2015). Similarly, in differentiated human neuronal-like cells, the inhibitor blocked the pS935 LRRK2 kinase phosphorylation, reduced ROS generation, and reversed apoptosis signaling induced by rotenone treatment (Mendivil-Perez, et al. 2016).



Daher, et. al., Journal of Biological Chemistry, 2015

Figure 10. PF-475 structure.

6.3 BX795+amlexanox

BX795 is a potent and relatively specific inhibitor of two kinases: TANK-binding kinase 1 (TBK1) and IκB kinase ε (IKKε). These kinases regulate the production of Type I interferon. BX795 blocks the production of interferon β in macrophages under the stimulation of LPS (Clark K., et al. 2009). A subset of Toll-like receptors such as TLR4, activate a distinct signaling pathway, in which IKKε and TBK1 are required (Kawai T., and Akira S., 2006; Takeuchi O., and Akira S., 2008). The amlexanox inhibitor is an inhibitor of TBK1 showing greater selectivity in comparison to BX795 (Minegishi, Y., et al. 2016). In addition, Dzamko et al. showed in 2012, that stimulation of the Toll-Like Receptor (TLR) pathway in bone marrow-derived macrophages (BMDMs) or RAW264.7 macrophages induces phosphorylation of LRRK2 at Ser910 and Ser935 (sites that regulate the binding of 14-3-3 to LRRK2) and this phosphorylation is induced by IKK-related (IKKε and TBK1) kinases both in vivo and in vitro (Dzamko N., et al. 2012).

b. Materials and Methods

1. *Cell lines*

The RAW264.7 macrophage like cell line (a kind gift from Dr. Matt LaVoie; Harvard University) were cultured in DMEM (Dulbecoo's modified Eagle medium, Sigma; MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (10% FBS) and penicillin/streptomycin.

BV2 microglia like cell line (a kind gift from Dr. Matt LaVoie; Harvard University) were cultured in RPMI media supplemented with 10% FBS and penicillin.

All cells are maintained at 37 ºC in 5% CO2.

2. *Stably silenced for LRRK2 RAW264.7 and BV2 cells.*

LRRK2 stably silenced (KD) and control (scr) RAW264.7 and BV2 cell lines were a kind gift from Dr. Matthew J. LaVoie and were made as described in Schapansky, et al. (2014). The selection antibiotic was added to the culture medium, 2 µg/µl puromycin dihydrocloride from Streptomyces alboniger (Sigma P8833).

3. *Antibodies and reagents*

i. *Western blot*

The antibodies we used for total LRRK2 were purified rabbit monoclonal antibody to LRRK2 MJFF2 C41-2 from abcam (ab133474) and rabbit monoclonal antibody to LRRK2 UDD3 30(12) from Abcam (ab133518).

For phosphorylated LRRK2 we used rabbit monoclonal antibody to LRRK2 UDD2 10(12) phosphoS935 from Abcam (ab172382) and for autophosphorylated LRRK2 we used purified rabbit monoclonal antibody to LRRK2 MJFR-19-7-8 phosphoS1292 from Abcam (206035).

The antibodies we used for Rab10 were for total Rab10 rabbit monoclonal antibody to Rab10 clone EPR13242 from Abcam (ab181367) and for phosphorylated Rab10 purified rabbit monoclonal antibody to Rab10 clone MJF-R211 phosphoT73, where threonine 73 is the site phosphorylated by LRRK2.

For TLRs total protein detection, we used purified rat monoclonal antibody IgG2B for TLR2 from R&D systems (MAB1530) and purified rabbit monoclonal antibody IgG for TLR4 from R&D systems (MAB27591).

To evaluate the equal loading in western immunoblotting analysis, we used mouse monoclonal antibody to β-actin from Origene (TA811000) and mouse monoclonal antibody to GAPDH from EMD Millipore (MAB374).

ii. FACS

For the assessment of plasma membrane levels of TLR2 and TLR4 by flow cytometry, we used FACS buffer (1% FBS, 0.2% FBS in PBS 1x) and the antibodies were purified rat monoclonal antibody IgG2B for TLR2 from R&D systems (MAB1530) and purified rabbit monoclonal antibody IgG for TLR4 from R&D systems (MAB27591).

The secondary antibodies we used for detection were CF568 goat anti-rat IgG (H+L) from BIOTIUM (20096) and CF555 goat anti-rabbit IgG (H+L) antibody from BIOTIUM (20033).

4. Pharmcological inhibitors

The pharmacological kinase inhibitors we used for LRRK2, were MLi-2 at a concentration of 100nM, which was kindly supplied by Dr. Dario Alessi; University of Dundee, PF-06447475 used at a concentration of 100nM, supplied from Sigma; BX-795 and amlexanox, inhibitors of TBK1, 1 μM, kindly supplied by Dr. Rebecca Matsas; Helenic Pasteur Institute.

5. Western analysis

Cells were collected in DMEM, washed 2 times with cold 1x PBS and centrifuged at 4°C for 5min at 5000 rpm each time. Then resuspended in lysis buffer [30mM Tris/HCl pH=8, 150mM NaCl, 5mM MgCl2, glycerol 5% protease and phosphatase inhibitors (Roche)]. After 30 min incubation on ice, the cell lysates were collected and centrifuged at 4°C for 10min at 13000 rpm. The protein concentration was measured, using a Bradford Protein Assay Reagent (Biorad).

Equal amounts of protein (40-50μg) were then boiled in sample buffer for 6 min and separated by SDS-PAGE. After electrophoresis, proteins were transferred on nitrocellulose membranes (Amersham) 400 mA, 1.5-2h, on ice and then the membranes were blocked in 5% milk diluted in 1x PBST or 5% BSA in TBST (for probing with phospho-specific antibodies) for 1 h at room temperature. The membranes were then incubated overnight at 4ºC with the desired primary antibodies diluted in 3% PBST/BSA or 5% milk in TBST for b-actin and GAPDH. After the incubation, membranes were washed five times with PBST 1x or TBST 1x over 30

min, and incubated with HRP-conjugated secondary antibodies (Pierce) for 1h at room temperature. After five washes with PBST or TBST, immunoreactive bands were detected using the DuraWest substrate (Pierce) or the Clarity Western ECL Substrate (Biorad).

In order to probe the same membranes with different antibodies for total and phosphorylated or autophosphorylated protein, we used a stripping buffer containing 2% SDS, 62.5 mM Tris-HCl, pH=6.8 and mM 2-mercaptoethanol. After 25 min incubation at 55 oC under agitation, the membranes were again blocked in 5% milk diluted in 1x PBST or 5% BSA in TBST, for probing with phospo antibodies, for 30 min at room temperature. We incubated the membranes overnight with primary antibodies at 4 oC as above and then we followed the same procedure for detection as described above.

6. *Flow Cytometric Analysis*

Two days prior to treatment, 500.000 cells for the control and LRRK2 silenced RAW cell lines were plated. As ligands for the TLR's, we used, LPS 2μg/ml or 100ng/ml, purchased from Sigma, and 1μg/ml human a-synuclein PFFs kindly provided by Dr. Andy West; University of Alabama. To detect cell surface expression of TLR4 and TLR2, cells were collected in DMEM and PBS, and resuspended in FACS buffer. 0.5 to 2 million cells were used for each condition and were incubated with antibodies against mouse TLR4 and TLR2 for 30 min on ice, washed, incubated with anti-Rb and anti-Rat accordingly. If the cells were not measured immediately they were fixed in 2% PFA, and stored at 4 oC. The FACS acquisition was performed with the Cytomics FC500 (Beckman Coulter) cytometer. The data were analyzed using the FlowJo software 8.7 (Tree Star, Inc., Ashland, OR).

7. *Microscopy*

To assess morphological changes in LRRK2-KD and scr RAW264.7 macrophage cells following TLR activation with exogenous a-synuclein PFF's, we acquired bright-field images of living cultures at different time points following treatment. The conditions were as follows: un-treated cells, cells treated with PFF's for 6h, cells treated with PFF's overnight, and treated cells plus the LRRK2 inhibitor MLi-2 or the TBK1 inhibitor amlexanox, at the indicated concentrations. Images were acquired using a 10X objective, and 6-10 representative fields were obtained. Cells were scored qualitatively relative to the morphology of un-treated cells.

c. Results

1) *Western Immunoblotting*

A residual LRRK2 expression is observed in cells stably silenced for LRRK2.

Raw264.7 cells, both control and stably silenced for LRRK2, were assessed for LRRK2 expression. LRRK2 expression was also examined after exposure in LPS or LPS and PF-475 kinase inhibitor. The time points of the exposures were 1.5h and 3h. The results are shown in figure 11.

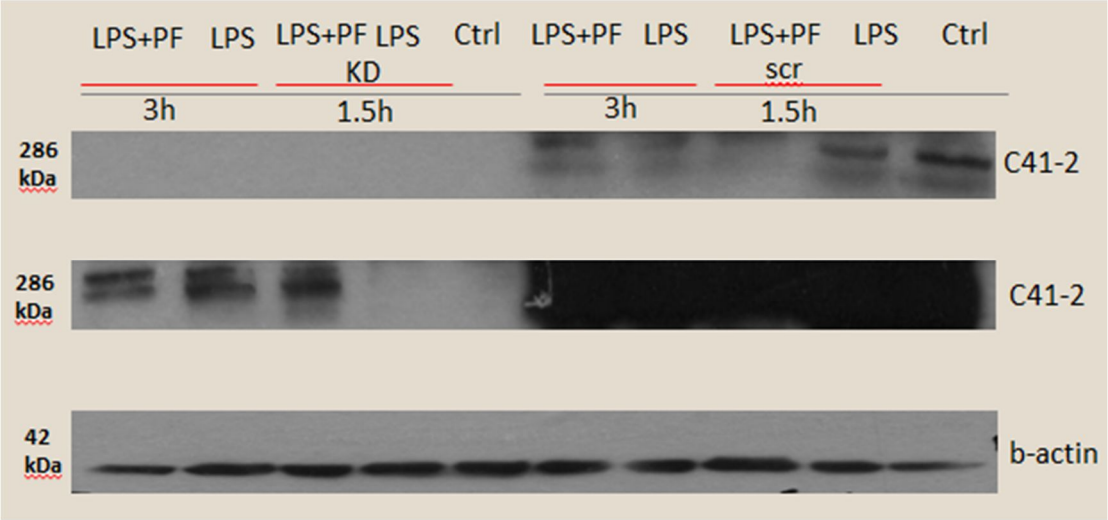


Figure 11. LRRK2 expression in control and stably silenced cells upon different treatments and time points. On the second line overexposure is shown, indicating low levels of LRRK2 expression.

As it becomes evident, high levels of LRRK2 are not observed in stably silenced cells but only in control cells. However, overexposure of the membrane reveals a residual expression of LRRK2 in KD for LRRK2 cells.

LPS +/- PF-475 effects in total LRRK2, LRRK2 phosphorylation and auto-phosphorylation, and total Rab10 in control cells.

Control cells were assessed for differences in phosphorylation and auto-phoshporylation levels of LRRK2 after exposure to LPS with or without the PF-475 LRRK2 inhibitor. The effects were studied after 2h and 4h exposure. The results are shown below:

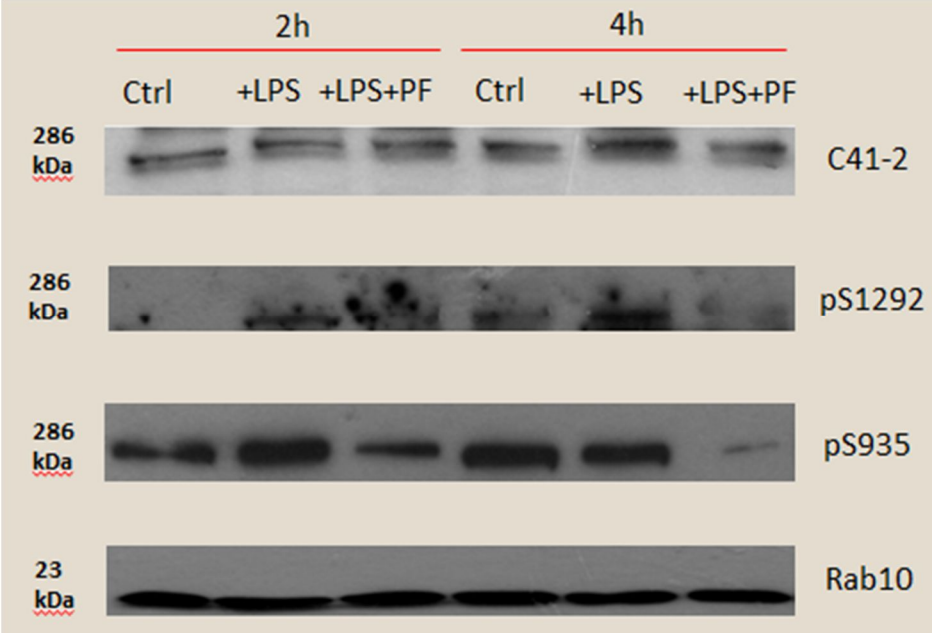


Figure 12. Total LRRK2, phosphorylated LRRK2, and total Rab10 expression upon different exposures and time points in control cells.

For LRRK2 auto-phosphorylation, detected with pS1292 antibody, LPS exposure leads to an increase in auto-phosphorylated LRRK2, while addition of the kinase inhibitor prevents this increase. In terms of phosphorylated LRRK2 (pSer935) levels, an increase is observed at 2 hours exposure to LPS, which is blocked by treatment with PF-475 at 2 and 4 hours. Total Rab10 levels, aren't changed by the different exposures.

2) *Flow cytometrical analysis*

LPS alters the trafficking of TLR4 in RAW264.7 cells.

We examined the regulation of TLR4 trafficking in control cells, which normally express LRRK2, by Flow cytometry, measuring the cell surface expression (we will refer to this as cse) of TLR4. Comparing untreated cells and cells treated with LPS, a significant reduction in TLR4 cse upon LPS treatment was observed in one hour following treatment.

We tested different time points of LPS exposure, in a time course of one hour, one hour and a half and three hours. LPS induces a time-dependent but transient loss of TLR expression on the plasma membrane, with the expression returning to control levels by 3h after treatment. These results are depicted in figures 13, 14.

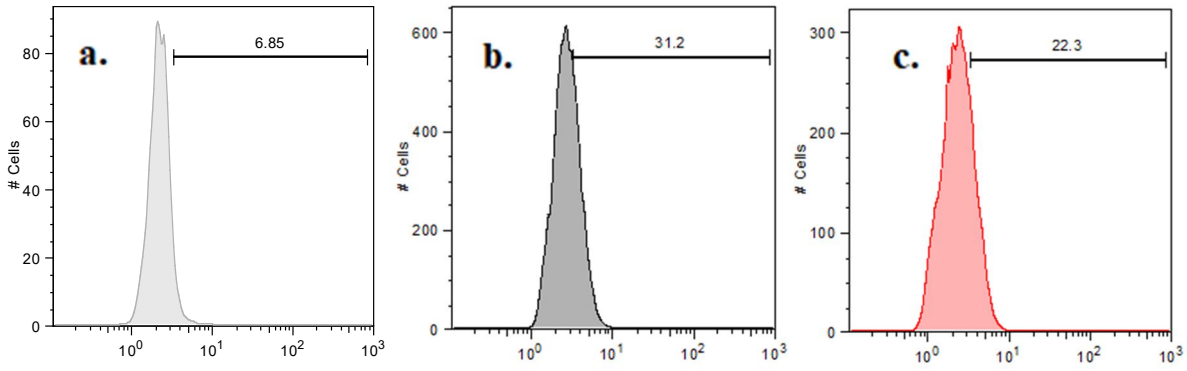


Figure 13. Treatment with LPS reduces the levels of TLR4 on the cell surface after 1 hour. a. unstained scr cells in order to test the specificity and the intensity of the signal, b. non-treated cells, c. LPS-treated cells for 1 hour.

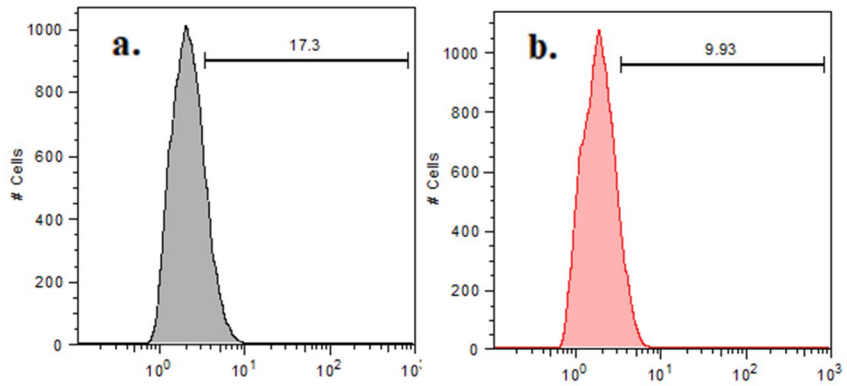


Figure 14. Treatment with LPS reduces the expression levels of TLR4 on the cell surface after 1.5 hour. a. untreated cells and b. treated cells with LPS for 1.5 hour. c. untreated and d. treated with LPS cells for 3 hours.

The decrease of TLR4 at the plasma membrane is dependent on LRRK2 kinase activity.

Next, we determine if the alteration in TLR4 trafficking by LPS could be altered using LRRK2 kinase inhibitors. For this purpose, we treated the control cells with the kinase inhibitor MLi-2 together with LPS. Following the same conditions as above, for one hour, one hour and a half and three hours, we measured the expression of TLR4 in the cell surface. Our results revealed that, MLi-2 rescues the reduction of TLR4 cse by LPS treatment. The same pattern was observed in an incubation time of one hour and a half, when LPS treatment elicited a greater reduction in cse. In contrast, in three hours LPS didn't affect TLR4 cse and the inhibitor was close to the baseline and even lower. This indicates that LRRK2 kinase activity participates in the regulation of TLR4 plasma membrane trafficking.

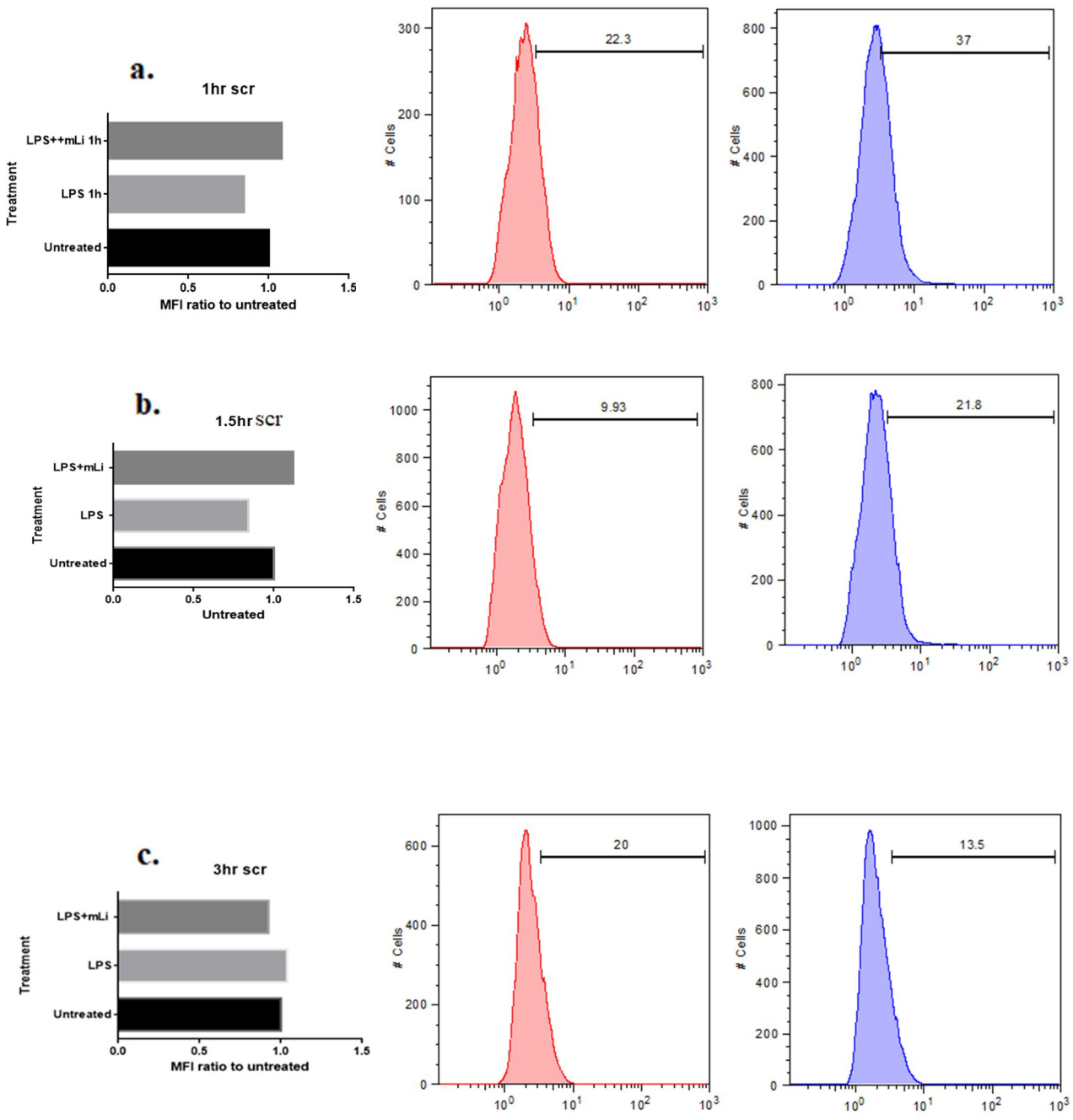


Figure 15. Inhibition of LRRK2 kinase, rescues the reduction of TLR4 cse by LPS in control RAW264.7 cells. a. MFI ratios to untreated at 1 hour, b. MFI ratios to untreated at 1.5 hour, c. MFI ratios to untreated at 3 hours. (red=LPS-treated cells, blue=LPS+MLi-2-treated cells).

MLi-2 alone doesn't affect TLR4 trafficking in control cells.

A critical control is to determine if treatment with the kinase inhibitor alone could have an effect on TLR4 membrane trafficking and especially in replenishment of the receptor onto the plasma membrane. In a time course of 1 hour, we measured by flow cytometry the percentage of TLR4 in the cell surface. We found that MLi-2 alone did not alter TLR4 cell surface expression, significantly.

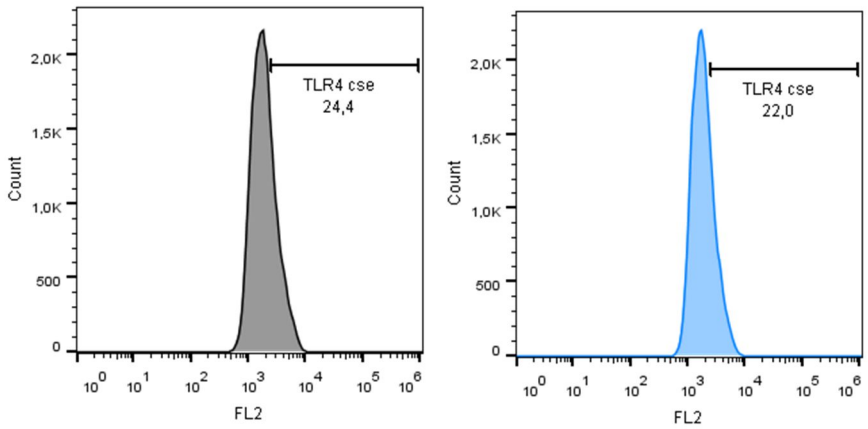


Figure 16. TLR4 cse isn't affected by MLi-2 alone treatment in scr cells. (black=non-treated cells, blue=MLi-2-treated cells).

LPS increases TLR4 in the plasma membrane in stably silenced for LRRK2 cells.

We next performed the same experiments in cells stably silenced for LRRK2, to provide further evidence about the interaction between LRRK2 and TLRs membrane trafficking. In order to compare control and knock down cells, we measured TLR4 cse through flow cytometry under the same conditions. As it is depicted in figure 17, LPS increases the TLR cse of LRRK2-KD cells in every time point (1h, 1.5h, 3h) we treated the cells, compared to untreated cells.

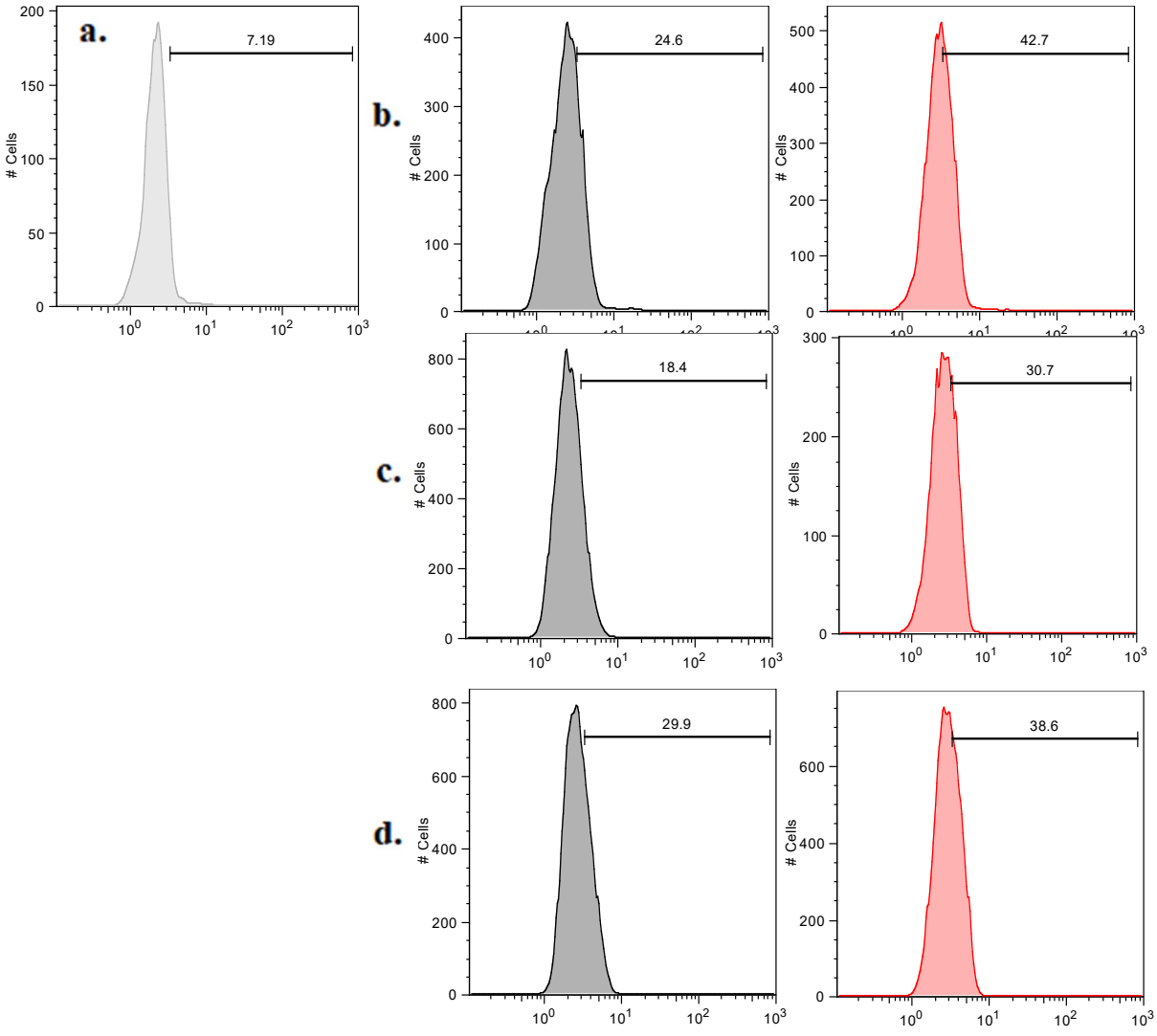


Figure 17. Percentages of TLR4 expression in the plasma membrane of LRRK2 stably silenced cells a. unstained cells as internal control of the staining, b. 1 hour LPS-treated cells, c. 1.5 hour LPS-treated cells and d. 3h LPS-treated cells (grey=unstained cells, black=non-treated cells and red=LPS-treated cells).

MLi-2 treatment affects the increase of TLR4 at the plasma membrane, which can be explained by residual LRRK2 expression.

In order to check if the alterations induced by LPS are blocked in LRRK2 deficient cells, we add the MLi-2 kinase inhibitor for LRRK2 in LPS-treated cells. Again the incubation time was the same as for the control cells. The results revealed that MLi-2 changes the alterations induced by LPS in a smaller scale than in controls, with no difference between the time points. These results could be explained by the fact that, even the stably silenced for LRRK2 cells show a low basal level of LRRK2 expression, as shown by Western immunoblots using antibodies for LRRK2 (figure 11). These data indicates that deficient for LRRK2 cells are characterized by increase of TLR4 at the plasma membrane following LPS treatment, and the pattern seems to be the opposite compared to control cells.

In these figures, we present the percentages of LPS and, LPS and MLi-treated cells together with the MFI ratios to untreated cells, for all time points in cells deficient for LRRK2.

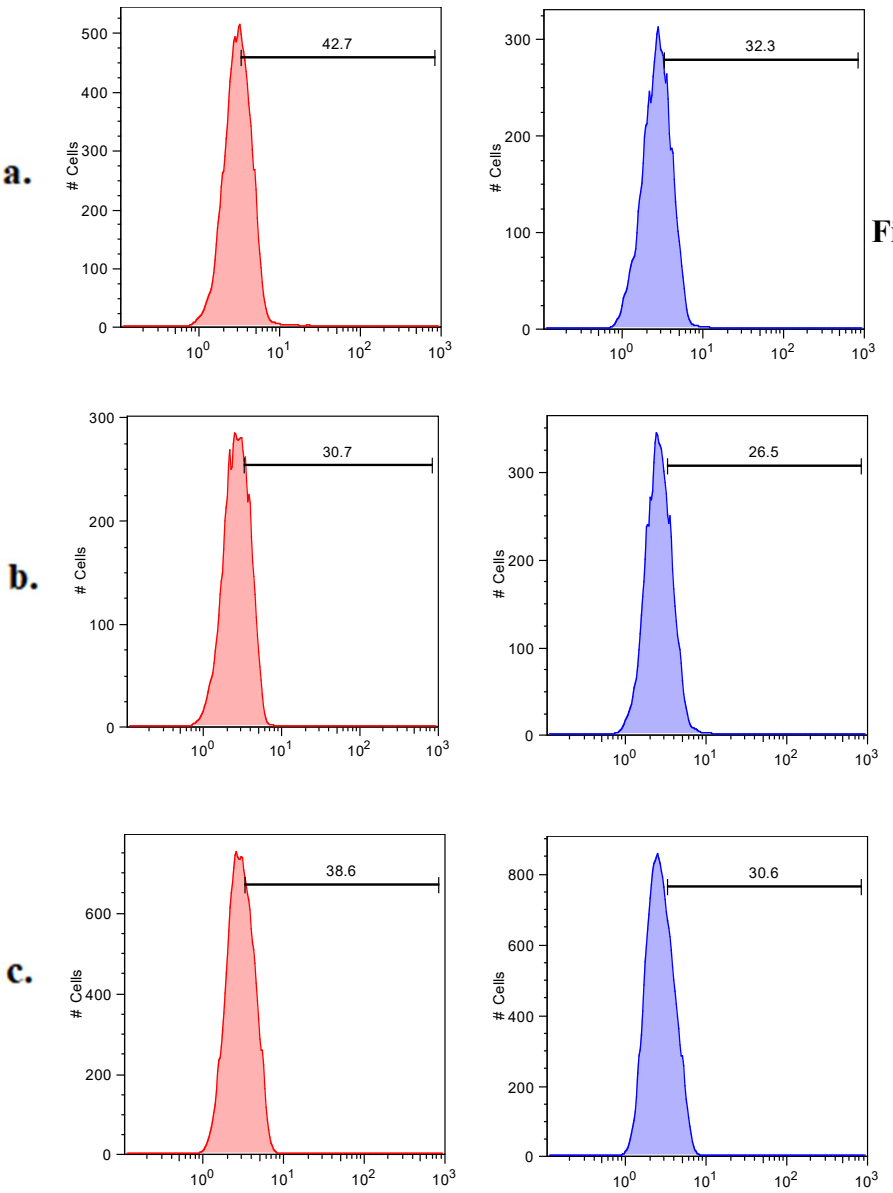


Figure 18 . Lps- induced increase in TLR4 cse is restricted by MLi-2 of the treatment. a.treatment with LPS+MLi for 1 h, b. treatment for 1.5 h,. c. treatment for 3h (red=LPS treated, blue=LPS+MLi treated cells).

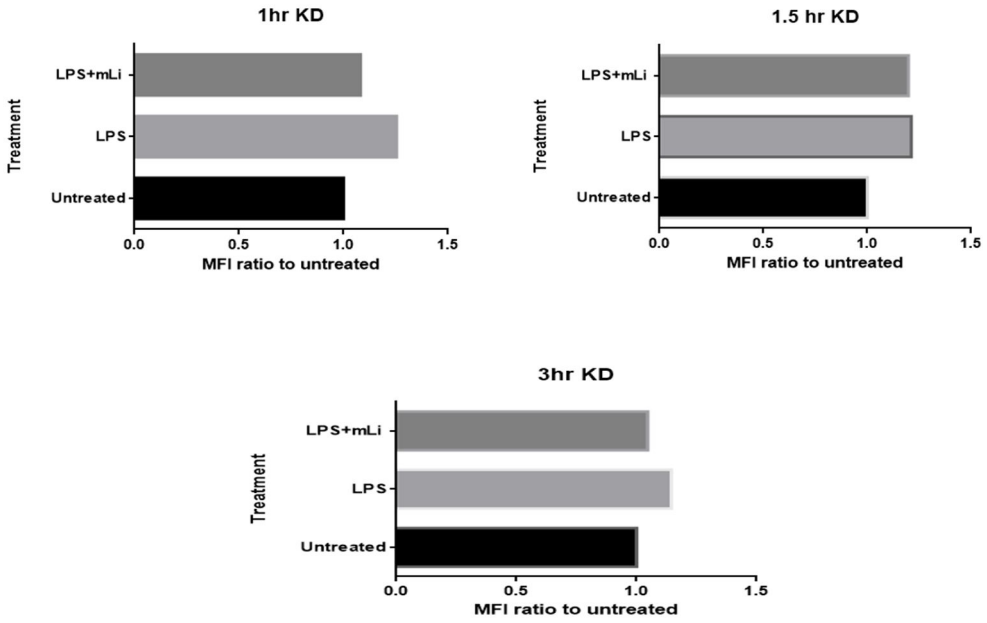


Figure 19. MFI ratios to untreated for the three time points treatment in LRRK2 deficient cells.

Human a-synuclein PFFs alter TLR4 cell surface expression, while MLi-2 rescues this effect.

It is known that pre-formed fibrils of a-synuclein are ligands for Toll like receptors such as TLR4 and TLR2, as well (Béraud D., et al. 2011). Based on that, we examined plasma membrane expression of TLR4 and TLR2 following treatment with a-synuclein PFFs with and without LRRK2 kinase inhibitors. Thus, following treatment with 1μg/ml PFFs and 100nM MLi-2, we measured TLR4 cse at different time points. We incubated the treated cells for 6 hours and 16 hours. At 6 six hours following treatment is in agreement with those we had with LPS in scr cells, that TLR4 plasma membrane expression is reduced. In addition, treatment with MLi-2 reversed the effect of PFFs.

In figure 20 the percentages of TLR4 cse, as well as, MFI ratios to non-treated cells are depicted.

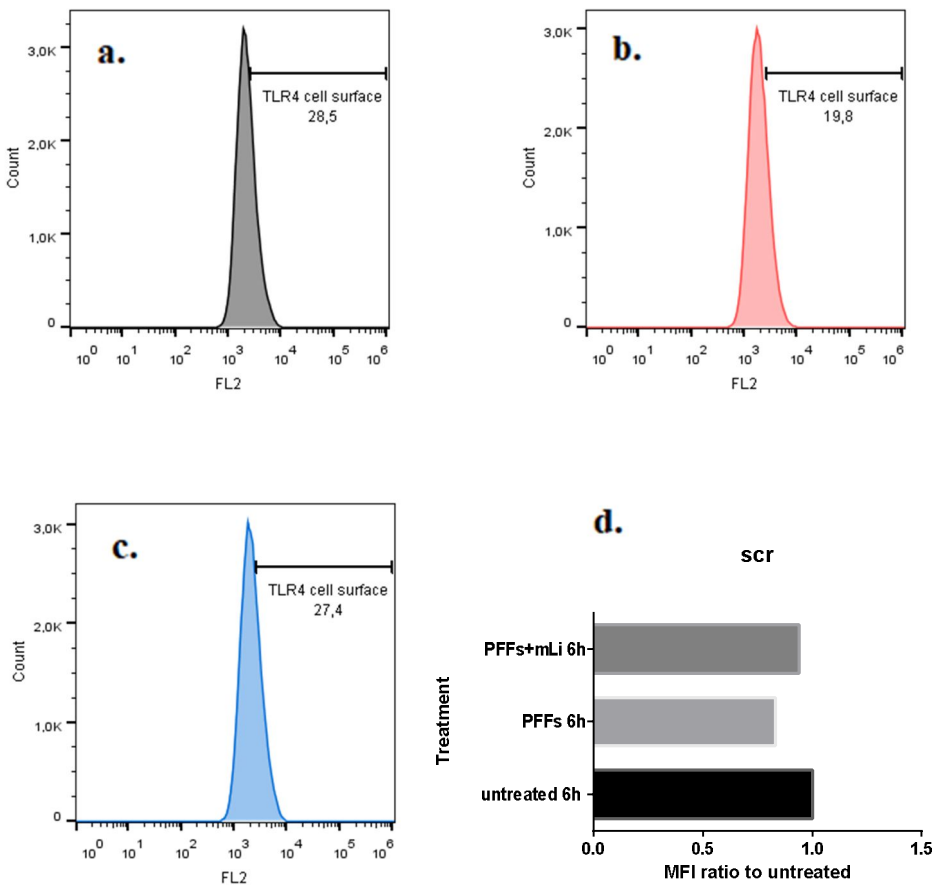


Figure 20. Treatment with PFFs alters TLR4 cse in control cells by decreasing it at 6 h compared to non-treated cells. Addition of MLi-2 reverses the loss of TLR4 cse. a. non-treated cells, b. treatment with PFFs, c. treatment with PFFs+MLi-2, d. MFI ratios to untreated. (black=non-treated cells, red=PFFs-treated cells, blue=PFFs+MLi-2-treated cells).

Human a-synuclein PFFs promote a non-significant increase TLR4 sce in O/N treatment, while MLi decreases TLR4 cse in control cells.

In contrast with 6 hours incubation, treatment with PFFs for 16 hours did not affect the levels of TLR4 expression in the plasma membrane, significantly. On the other hand, addition of MLi-2 together with PFFs alters TLR4 cse by significantly decreasing it. These data together, suggest that TLR4 cse changes not only by addition of different ligands and inhibitors, but is also depending on time. One possibility is that certain cytokines produced by these cells after longer exposure to PFF's may in turn alter the trafficking of TLR4; this is one area that will be followed up in future studies.

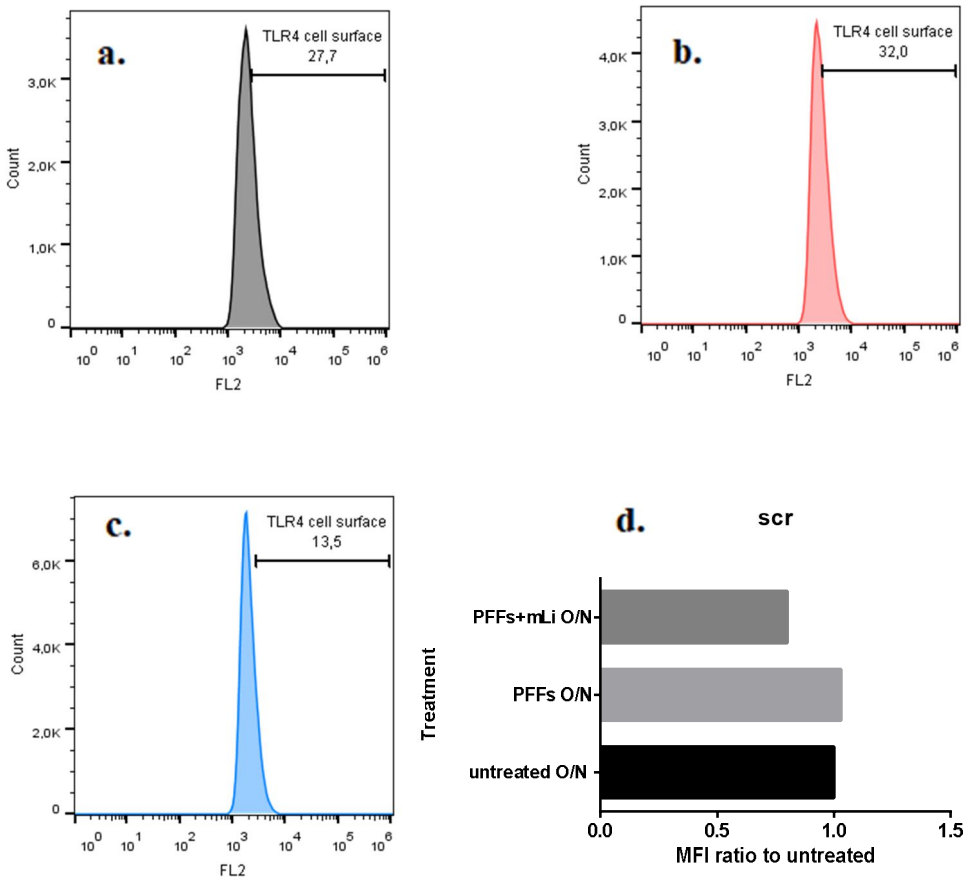


Figure 21. Treatment with PFFs does not alter TLR4 cse in LRRK2 control cells O/N, MLi-2 reduces its expression levels in the membrane. a. non-treated cells, b. treatment with PFFs, c. treatment with PFFs+MLi-2, d. MFI ratios to untreated. (black=non-treated cells, red=PFFs-treated cells, blue=PFFs+MLi-2-treated cells).

Human a-synuclein PFFs don't alter TLR4 cse after 6 hours in LRRK2-KD cells.

To further examine the role of LRRK2 in TLR trafficking, we performed similar experiments in cells silenced for LRRK2. Addition of a-synuclein PFFs in stably silenced cells for LRRK2 had no significant effect in the expression of TLR4 in the plasma membrane after 6 hours. The same pattern is observed after treatment with PFFs and MLi-2.

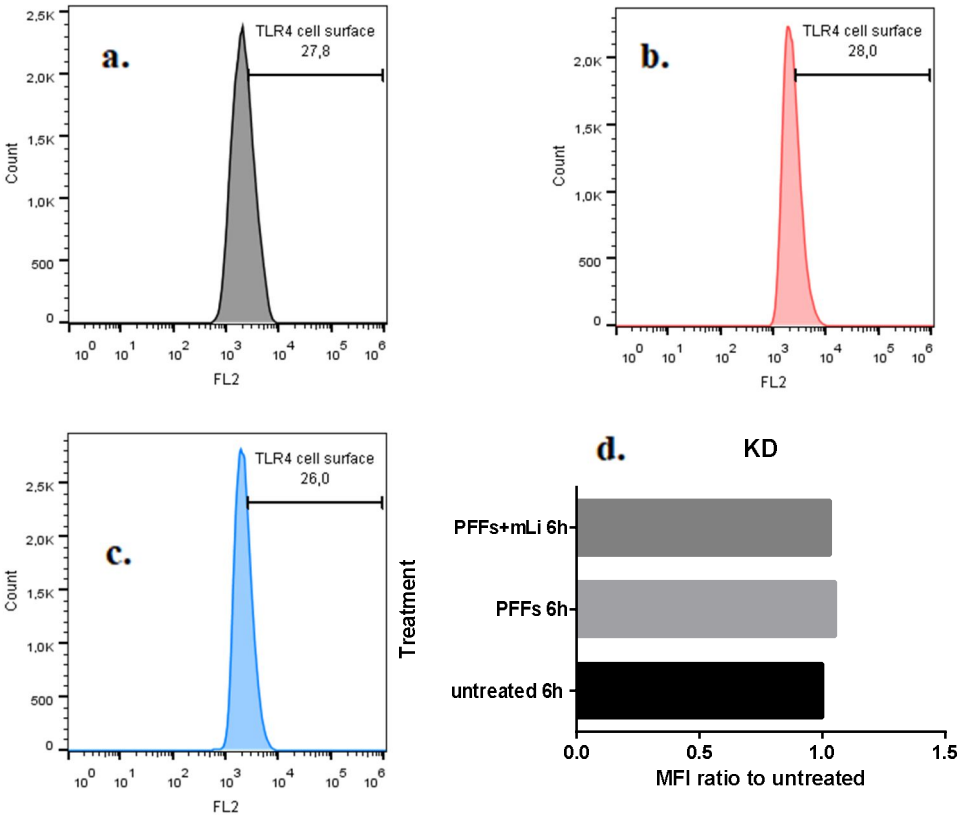


Figure 22. Treatment with PFFs does not alter TLR4 cse in LRRK2 deficient cells at 6 h, as well as MLi-2 a. non-treated cells, b. treatment with PFFs, c. treatment with PFFs+MLi-2, d. MFI ratios to untreated. (black=non-treated cells, red=PFFs-treated cells, blue=PFFs+MLi-2-treated cells).

TLR4 cse reduction by human a-synuclein PFFs is altered by MLi-2 after 16 hours.

We next examined TLR4 cse following longer exposure to a-synuclein PFFs. Treating stably silenced for LRRK2 cells with human a-syn fibrils for 16 hours, had the same effect that we had observed in control cells incubated with fibrils for 6 hours, a loss of TLR4 cse. Treatment with PFFs and the MLi inhibitor for 16h partially rescues the loss of TLR4. This suggests that in silenced cells, which we have seen maintain a low level of expression, the LRRK2-dependent regulation of TLR4 expression is delayed compared to scr control cells. This is likely due to the residual LRRK2 expression in these lines, even despite the inclusion of selection antibiotic at all times. These observations are depicted in figure 23.

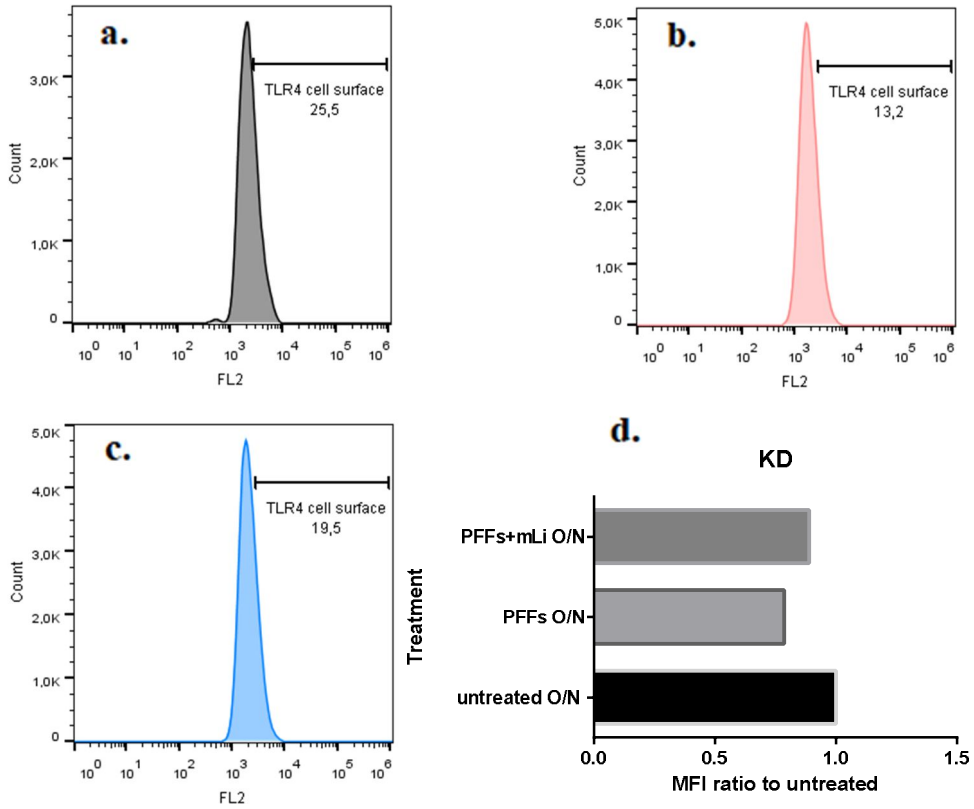


Figure 23. Treatment with PFFs alters TLR4 cse in stably silenced cells by decreasing it at 6 h compared to non-treated cells. Addition of MLi-2 partially increases TLR4 cse compared to PFF-treated cells alone. a. non-treated cells, b. treatment with PFFs, c. treatment with PFFs+MLi-2, d. MFI ratios to untreated. (black=non-treated cells, red=PFFs-treated cells, blue=PFFs+MLi-2-treated cells).

We also checked the expression of TLR2 with flow cytometry. As TLR2 is related to neurodegeneration, as it is described in section 4.1.2, we wanted to check if the activity of LRRK2 can affect potential alterations in trafficking induced by its ligands. A-synuclein pre-formed fibrils are known ligands for TLR2 (Gustot A., et al, 2015). Thus, we used PFFs in the same concentration 1μg/ml, and MLi-2 kinase inhibitor for LRRK2, 100nM.

Human a-synuclein PFFs don't alter TLR2 cse, while addition of MLi-2 causes a reduction in TLR2 cse in control cells after 6h.

Treatment with PFFs, which are ligands for TLR2, for six hours revealed no significant change in TLR2 cell surface expression. This pattern changes after the incubation of control cells with MLi-2 inhibitor, as well.

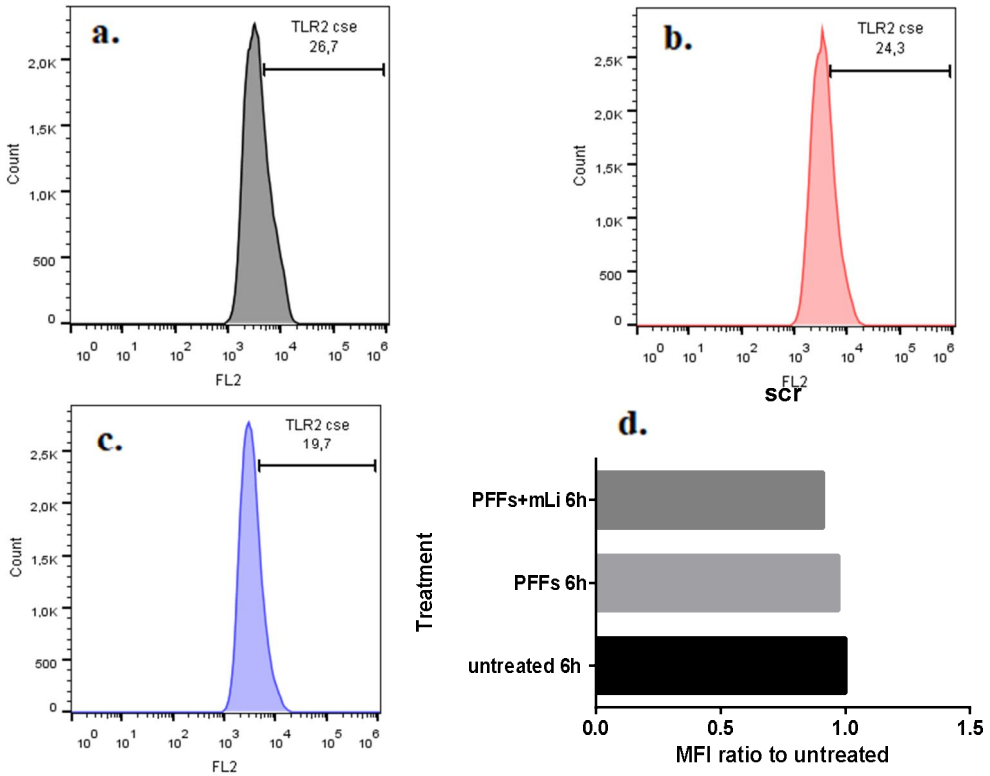


Figure 24. Treatment with PFFs does not alter TLR2 cse in LRRK2 control cells at 6 hours, MLi-2 reduces its expression levels in the membrane. a. non-treated cells, b. treatment with PFFs, c. treatment with PFFs+MLi-2, d. MFI ratios to untreated. (black=non-treated cells, red=PFFs-treated cells, blue=PFFs+MLi-2-treated cells).

Human a-synuclein PFFs don't alter TLR2 cse, while addition of MLi-2 causes a reduction in TLR2 cse in control cells O/N.

Treating the cells with fibrils for 16 hours revealed that we have the same pattern with 6 hours in the aspect of PFFs alone, while MLi-2 inhibitor causes the increase of TLR2 expression in the plasma membrane.

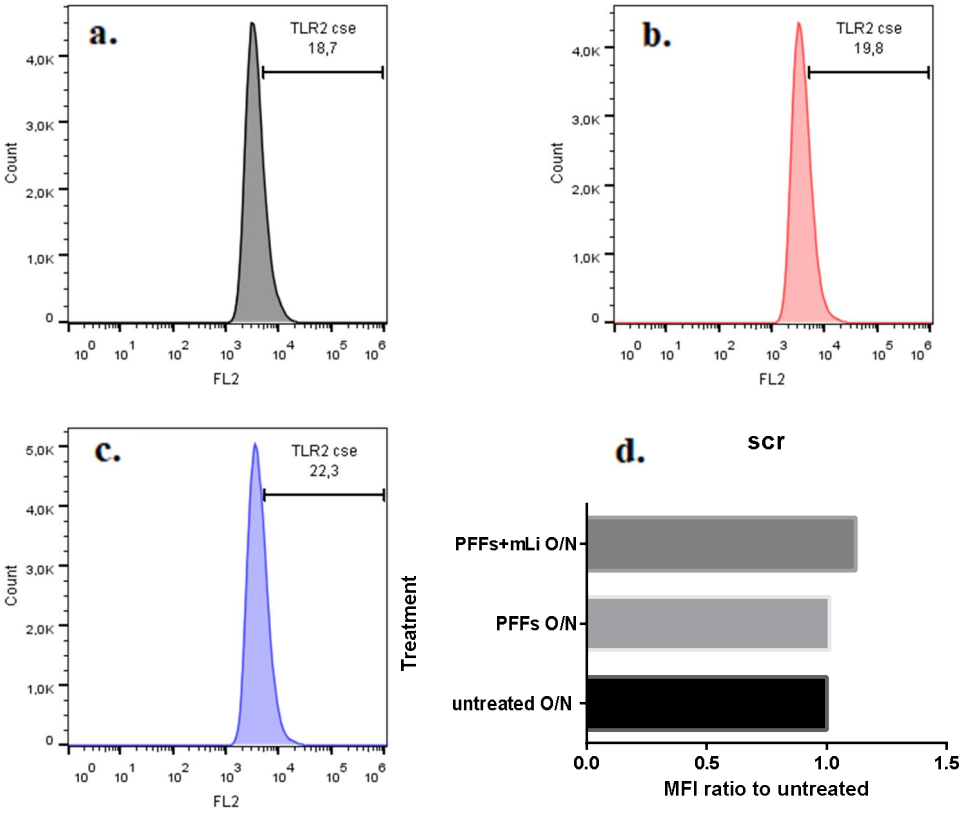


Figure 25. Treatment with PFFs does not alter TLR2 cse in LRRK2 control cells O/N, MLi-2 increases its expression levels in the membrane. a. non-treated cells, b. treatment with PFFs, c. treatment with PFFs+MLi-2, d. MFI ratios to untreated. (black=non-treated cells, red=PFFs-treated cells, blue=PFFs+MLi-2-treated cells).

Human a-synuclein PFFs alter TLR2 cell surface expression, while MLi-2 doesn't rescue this effect in KD cells after 6h.

In contrast with LRRK2 control cells, in stably silenced for LRRK2 cells the addition of PFFs alters the expression of TLR2 in the cell membrane. Moreover, treatment with MLi-2 not only doesn't rescue this effect but cause a higher decrease after 6 hours incubation.

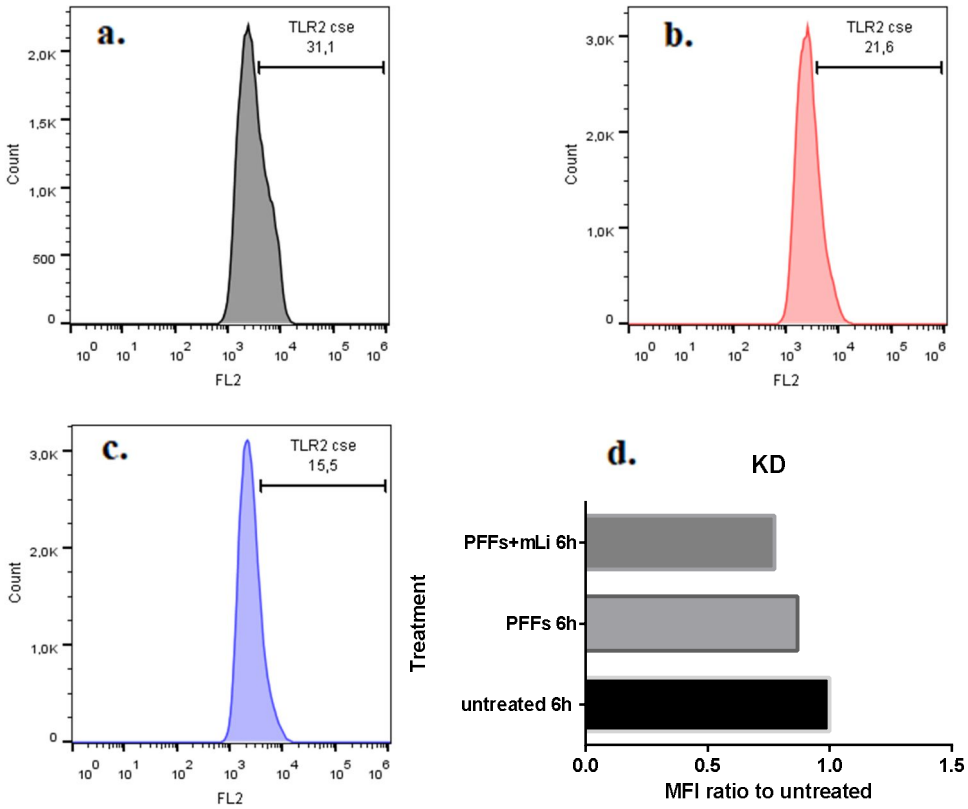


Figure 26. Treatment with PFFs alters TLR2 cse in KD cells by decreasing it at 6 h compared to non-treated cells. Addition of MLi-2 militates thereduction of TLR2 cse again. a. non-treated cells, b. treatment with PFFs, c. treatment with PFFs+MLi-2, d. MFI ratios to untreated. (black=non-treated cells, red=PFFs-treated cells, blue=PFFs+MLi-2-treated cells).

Human a-synuclein PFFs treatment in KD cells, has the same results in TLR2 cell surface expression, at 6 and 16 hours.

Both treatments with human a-synuclein PFFs and human a-synuclein PFFs+MLi-2 have the same effects on TLR2 cse at 16 hours, as they have at 6 hours.

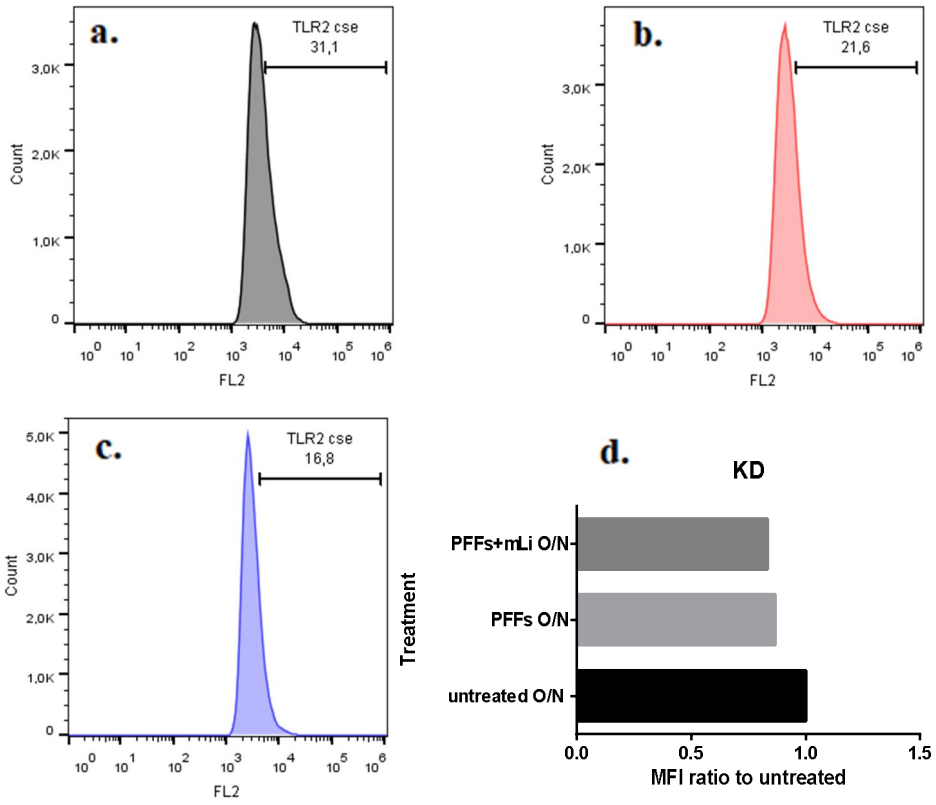


Figure 27. Treatment with PFFs alters TLR2 cse in KD cells by decreasing it at 16 h compared to non-treated cells. Addition of MLi-2 militates the reduction of TLR2 cse again. a. non-treated cells, b. treatment with PFFs, c. treatment with PFFs+MLi-2, d. MFI ratios to untreated. (black=non-treated cells, red=PFFs-treated cells, blue=PFFs+MLi-2-treated cells).

3) Morphology of activated microphages-microscopy

Immune cell like macrophages and microglia undergo different morphological changes when activated. As an initial investigation of this phenotype in the RAW macrophage cell line, we qualitatively assessed cells following treatment with a-synuclein PFFs. For this purpose, using cells in the same density, we treat them with a-synuclein PFFs (1μg/ml), MLi-2 (100nM) kinase inhibitor for LRRK2 and the TBK1 inhibitor amlexanox (100nM). In living cells, we took pictures after 6 hours and 16 hours treatment. Activated macrophages are derived from circulating monocytes or resident tissue macrophages and migrate through the extracellular space in response to chemotactic agents, cytokines and bacterial endotoxins. Activated macrophages appear larger, and more elongated compared to the round inactivated cells. In the aspect of their chemical characteristics, activated macrophages have increased metabolism, increased levels of lysosomal proteins, greater proteases, cytokine, growth factors, chemotactic factors, ROS and NOS release (McWhorter F. Y., et al. 2013). In figure 28 control untreated cells are depicted.

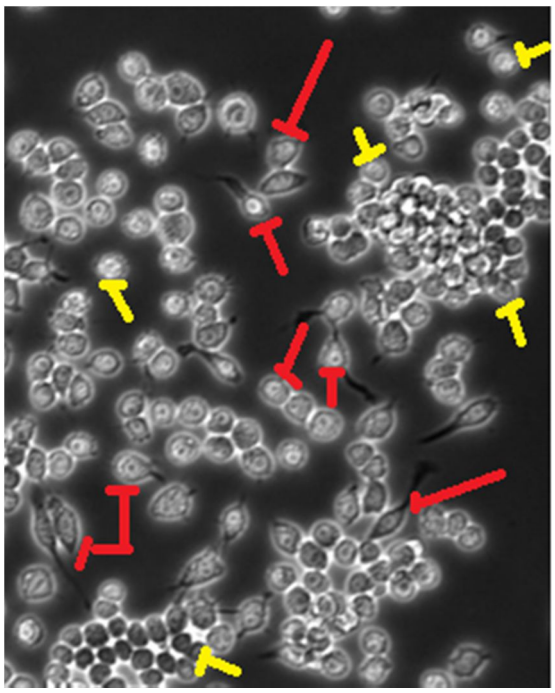


Figure 28. Control non-treated RAW264.7 cells. With red some activated macrophage cells are indicated, while in yellow some of the inactivated cells are indicated.

In figure 29 there are four representative pictures of RAW264.7 scr untreated cells with activated versus inactivated macrophages being in different ratios in each picture. We will compare the following figures with different treatments with this figure. Here, the majority of cells assume a small, round shape, in clusters of cells.

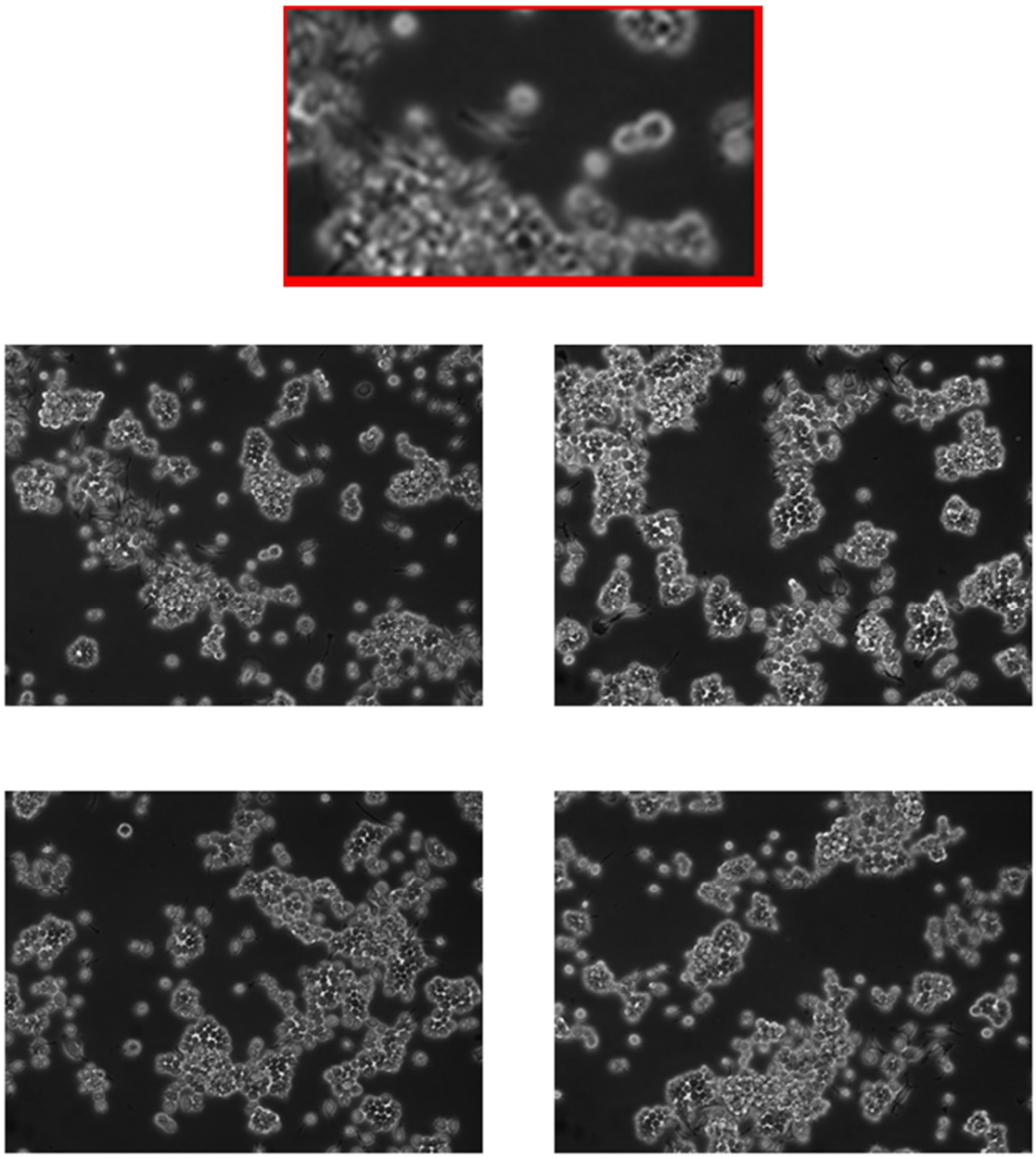


Figure 29. Different pictures of the same well of non-treated control cells, 6 hours (Brightfield 20x).

Treating the cells with 1µg/ml PFFs for 6 hours we took pictures again. What we observed was that more macrophages assumed an activated shape (elongated, flat, and larger).

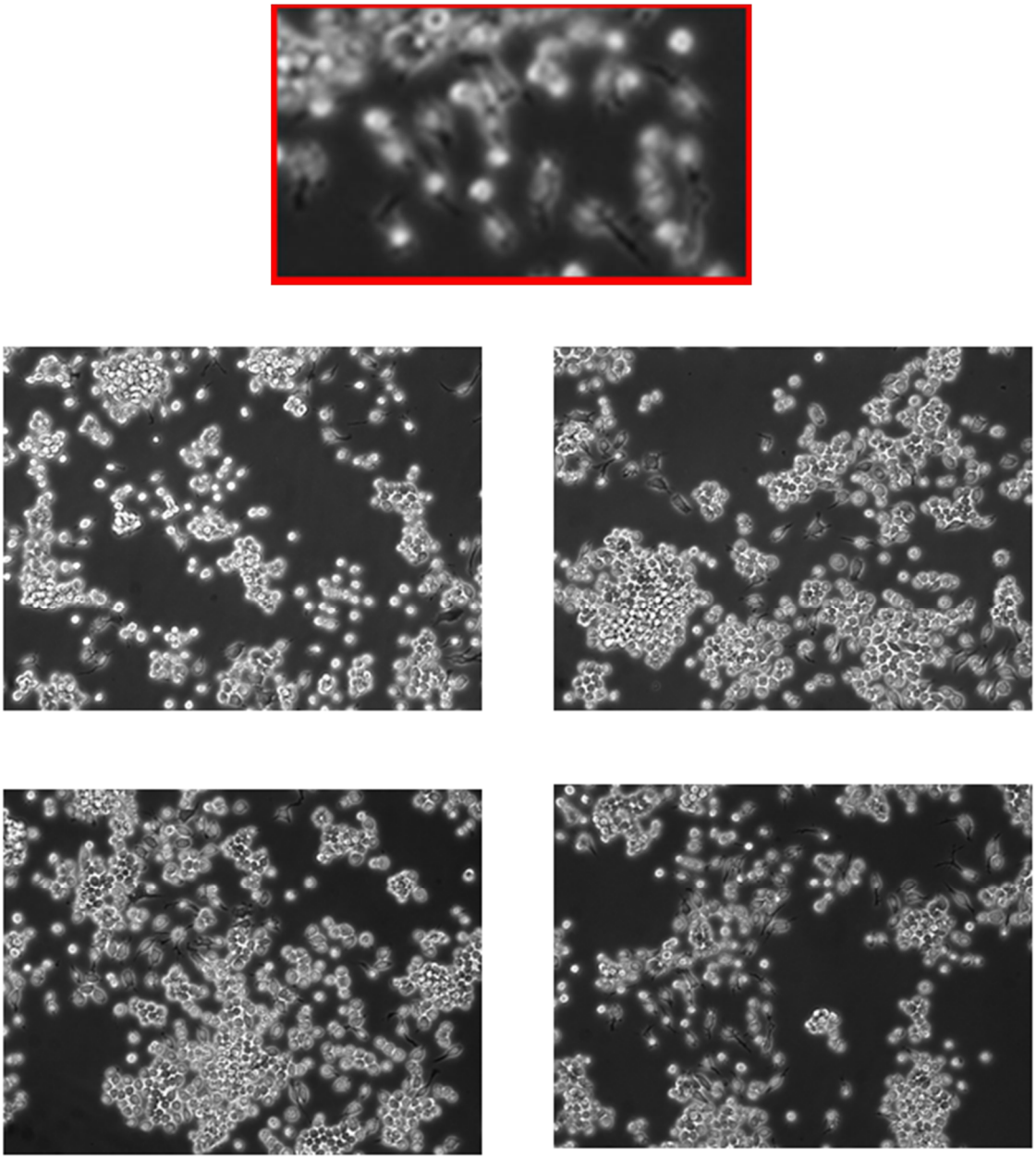


Figure 30. Control cells after treatment with PFFS (1µg/ml) for 6 hours (Brightfield 20x).

In parallel cultures, together with the addition of PFFs we treated the cells with MLi-2 kinase inhibitor for 6 hours. Comparing with the previous figure with PFFs treatment, fewer cells showing an activated morphology are observed in total. A representative image appears in Figure 31. This is consistent with previous findings in cultured microglial cells exposed to LPS and treated with a kinase inhibitor; in these cells the activation was suppressed by kinase inhibition (Moehle et al., 2012).

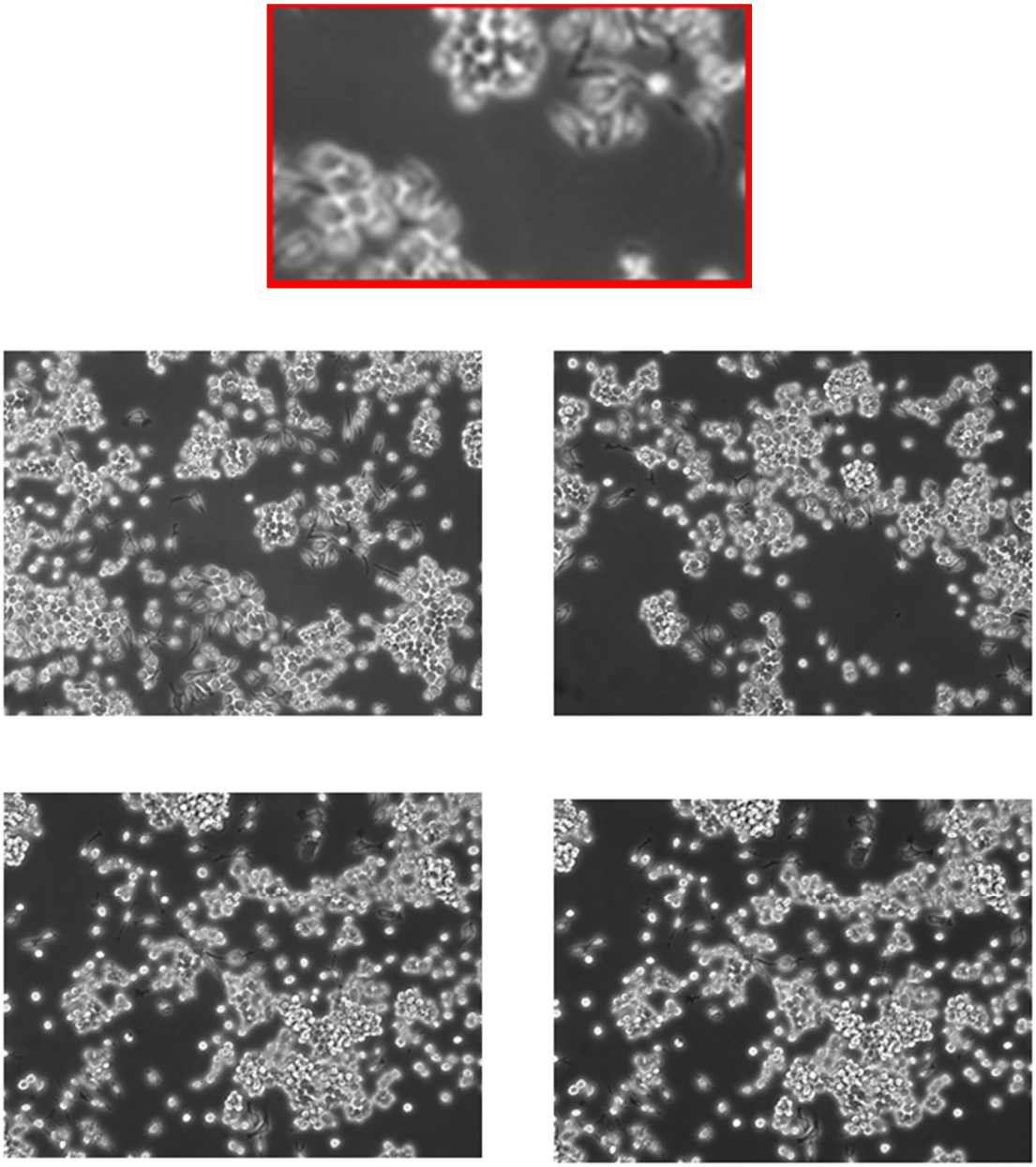


Figure 31. Control cells after treatment with PFFS (1µg/ml) and MLi-2 (100nM) for 6 hours (Brightfield 20x).

Addition of the TBK inhibitor amlexanox (100nM), together with the PFFs, provokes no qualitative difference in the number of cells compared to the PFFs alone, but the activated macrophages seem larger and elongated that any other condition at 6 hours. This suggests that they are in a different functional stage.

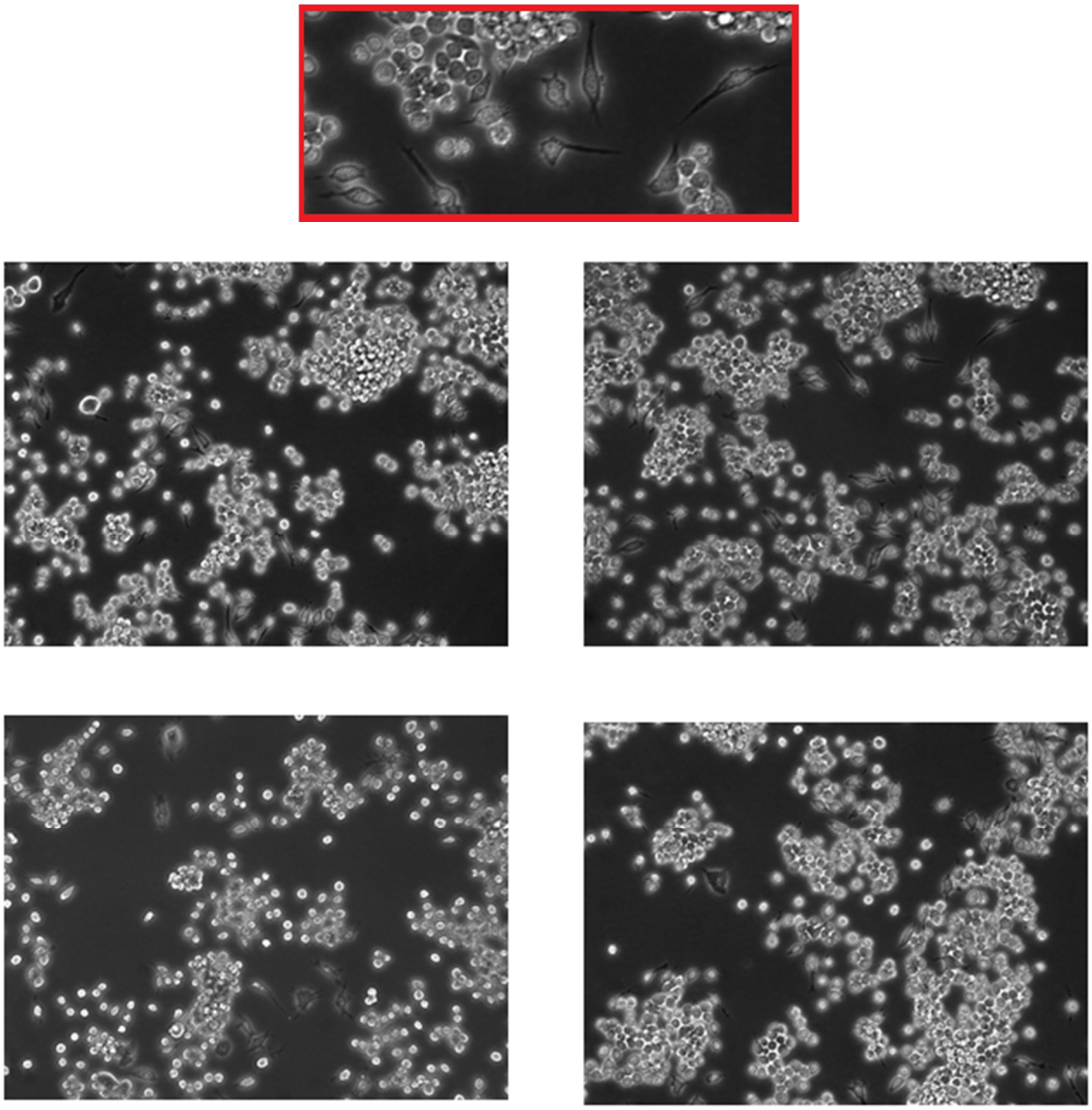


Figure 32. Control cells after treatment with PFFS (1µg/ml) and amlexanox (100nM) for 6 hours (Brightfield 20x).

Morphology of control cells and observation of activated macrophages at 16 hours upon different treatment.

Even without any treatment, differences in shape and activation state of macrophage cells were observed between 6 hours and 16 hours. After 16 hours, macrophages appeared more activated in comparison with control cells at six hours.

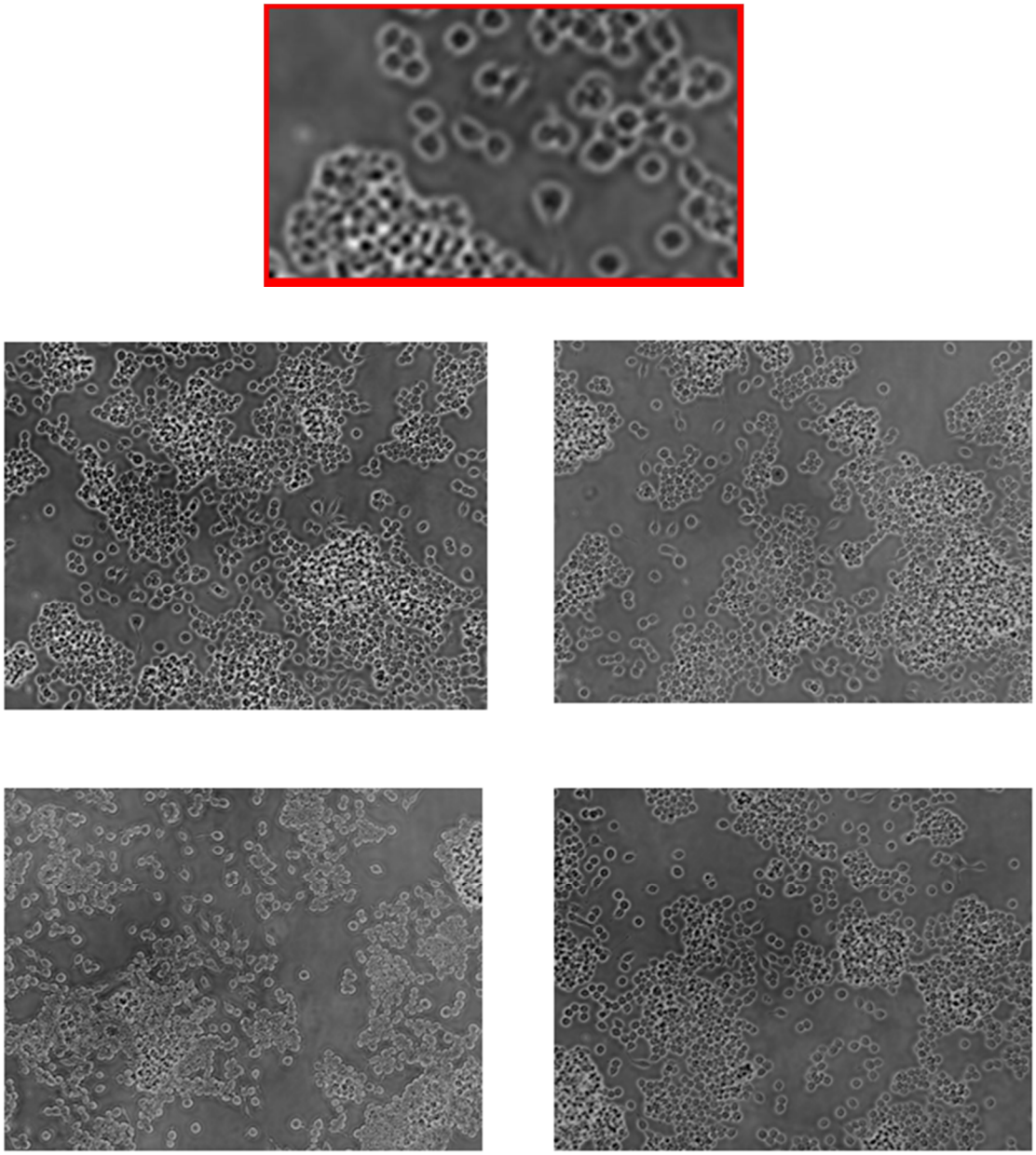


Figure 33. Different pictures of the same well (6-well plate) of non-treated control cells, 16 hours (Brightfield 20x).

In control cells at 16 hours treated with PFFs, and treated with PFFs and MLi-2, more activated macrophages are observed compared with control non-treated cells at 16 hours; it's also evident that they are more compared to the same condition at 6 hours, however, at 16 hours treatment with MLi-2 inhibitor doesn't appear to alter the extent of macrophage activation in comparison to PFF treatment alone.

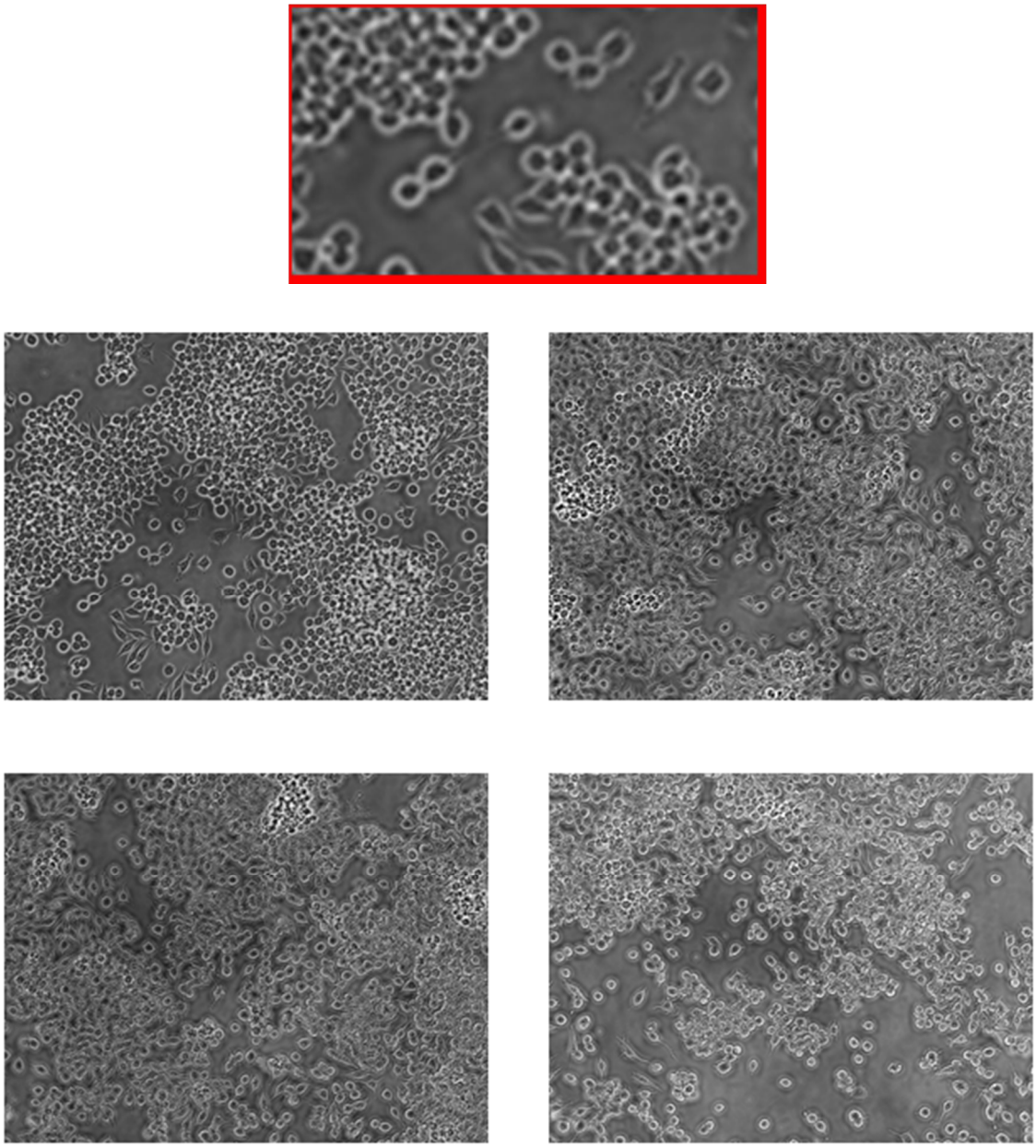


Figure 34. Control cells after treatment with PFFS (1µg/ml) for 16 hours (Brightfield 20x).

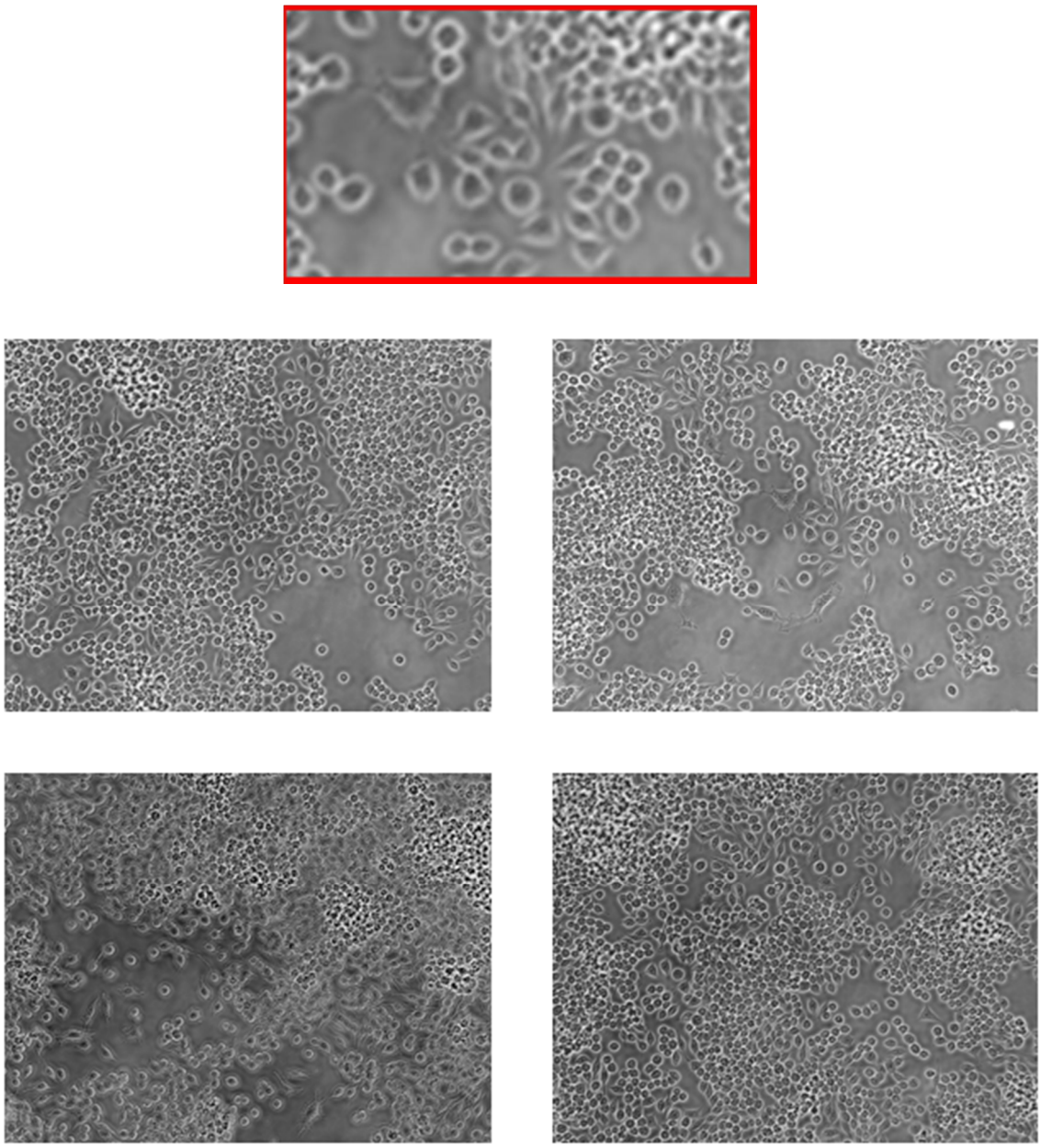


Figure 35. Control cells after treatment with PFFS (1µg/ml) and MLi-2 (100nM) for 16 hours (Brightfield 20x).

As with the 6 hours treatment, treatment with PFFs and amlexanox for 16 hours macrophages appeared more elongated compared with the other conditions for the same time course, suggesting again that their activation state may be greater.

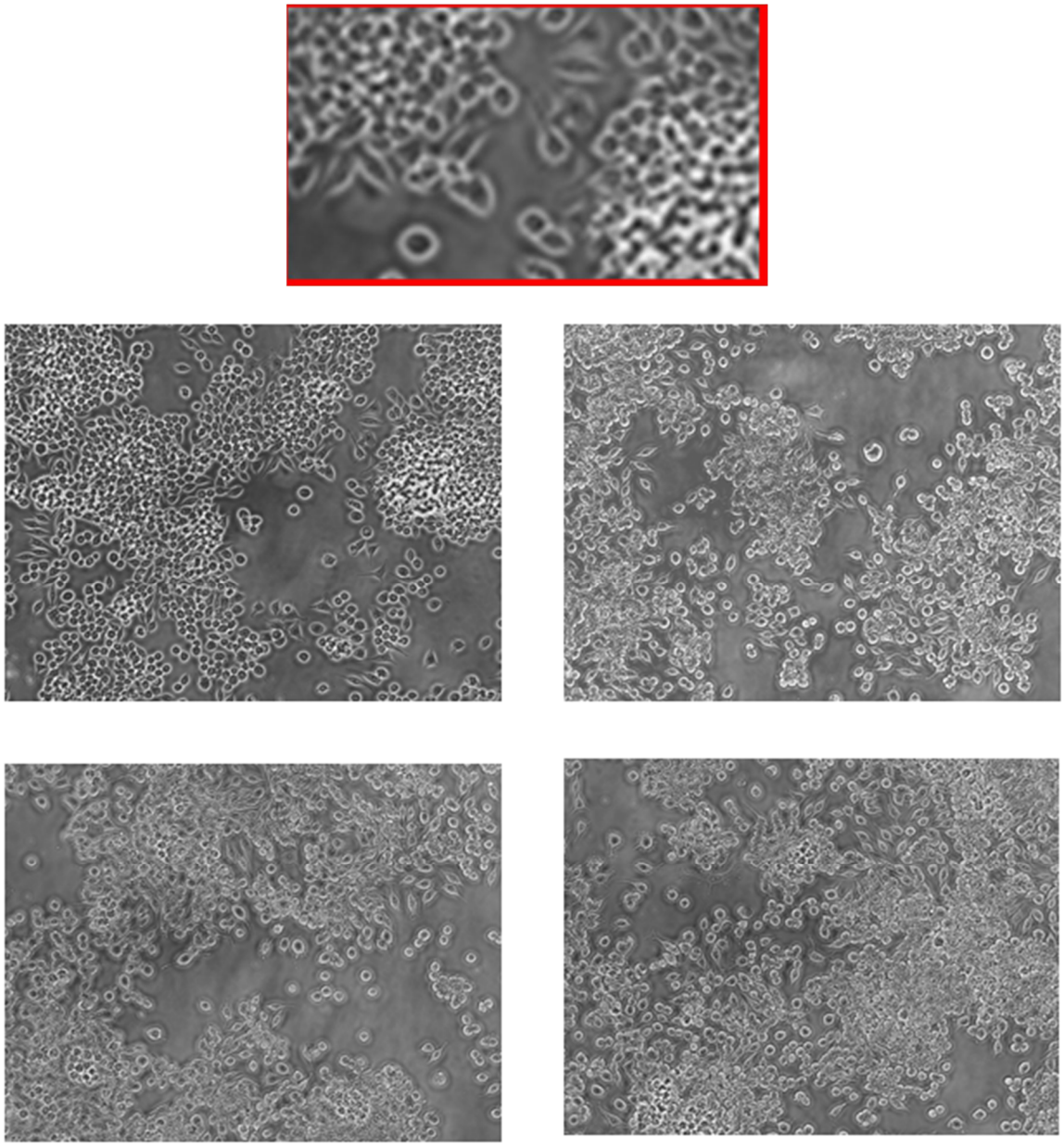


Figure 36. Control cells after treatment with PFFS (1µg/ml) and amlexanox (100nM) for 6 hours (Brightfield 20x).

Morphology of stably silenced for LRRK2 cells and observation of activated macrophages at 6 hours upon different treatment.

In untreated cells stably silenced for LRRK2 more macrophages having an activated appearance were observed than in untreated in control cells, as it is shown in figure 37.

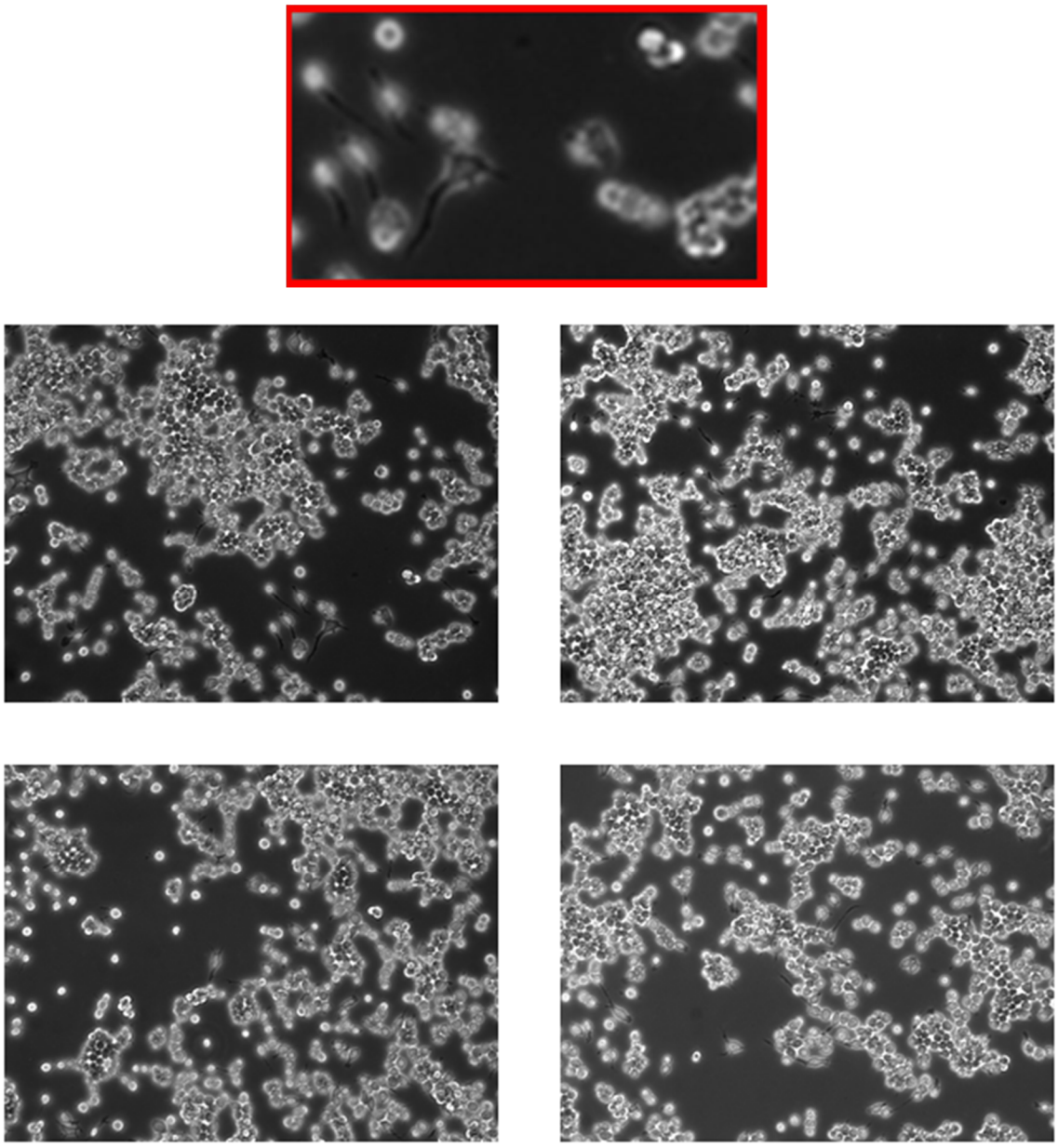


Figure 37. Different pictures of the same well of non-treated KD cells, 6 hours (Brightfield 20x).

Silenced cells treated with PFFs, PFFs and MLi-2 inhibitor, and PFFs and amlexanox presented the same phenotype, which is characterized by more activated macrophages than in non-treated cells. We observed larger numbers of macrophages with an activated, morphology, with elongated features, similar to scr cells treated with amlexanox in both 6 and 16 hour-treatment.

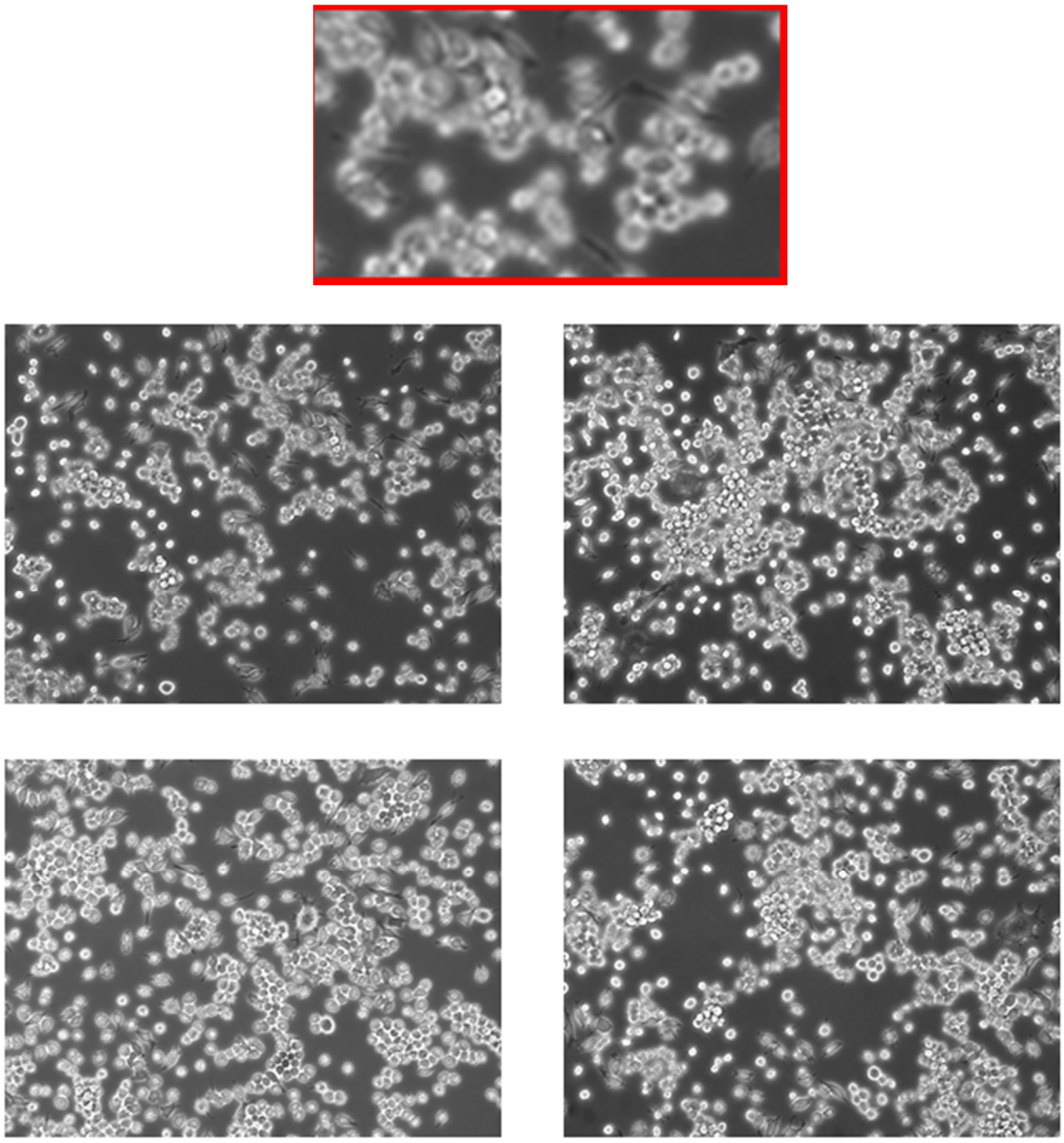


Figure 38. Control cells after treatment with PFFS (1µg/ml) for 6 hours (Brightfield 20x).

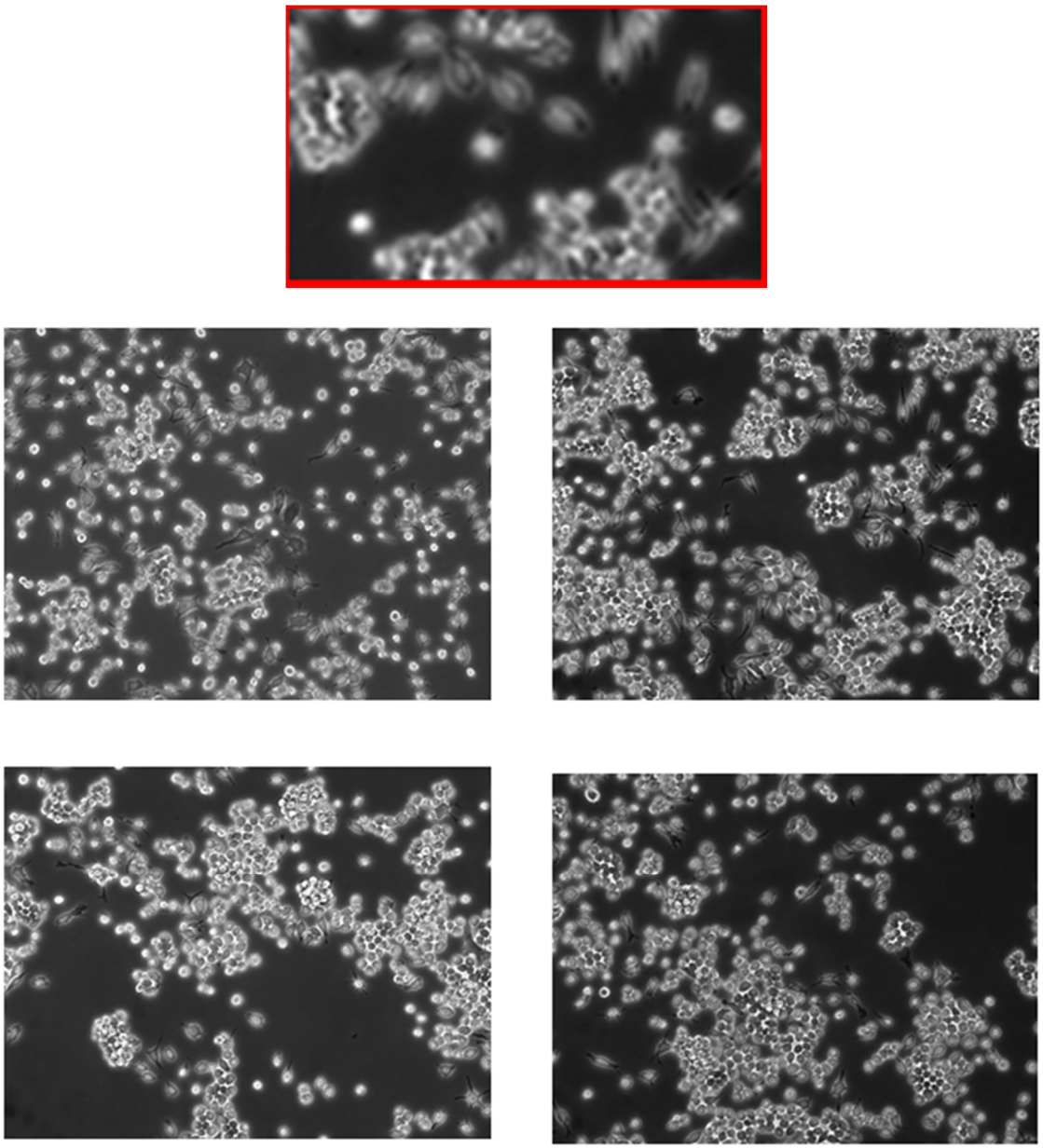


Figure 39. KD cells after treatment with PFFS (1µg/ml) and MLi-2 (100nM) for 6 hours (Brightfield 20x).

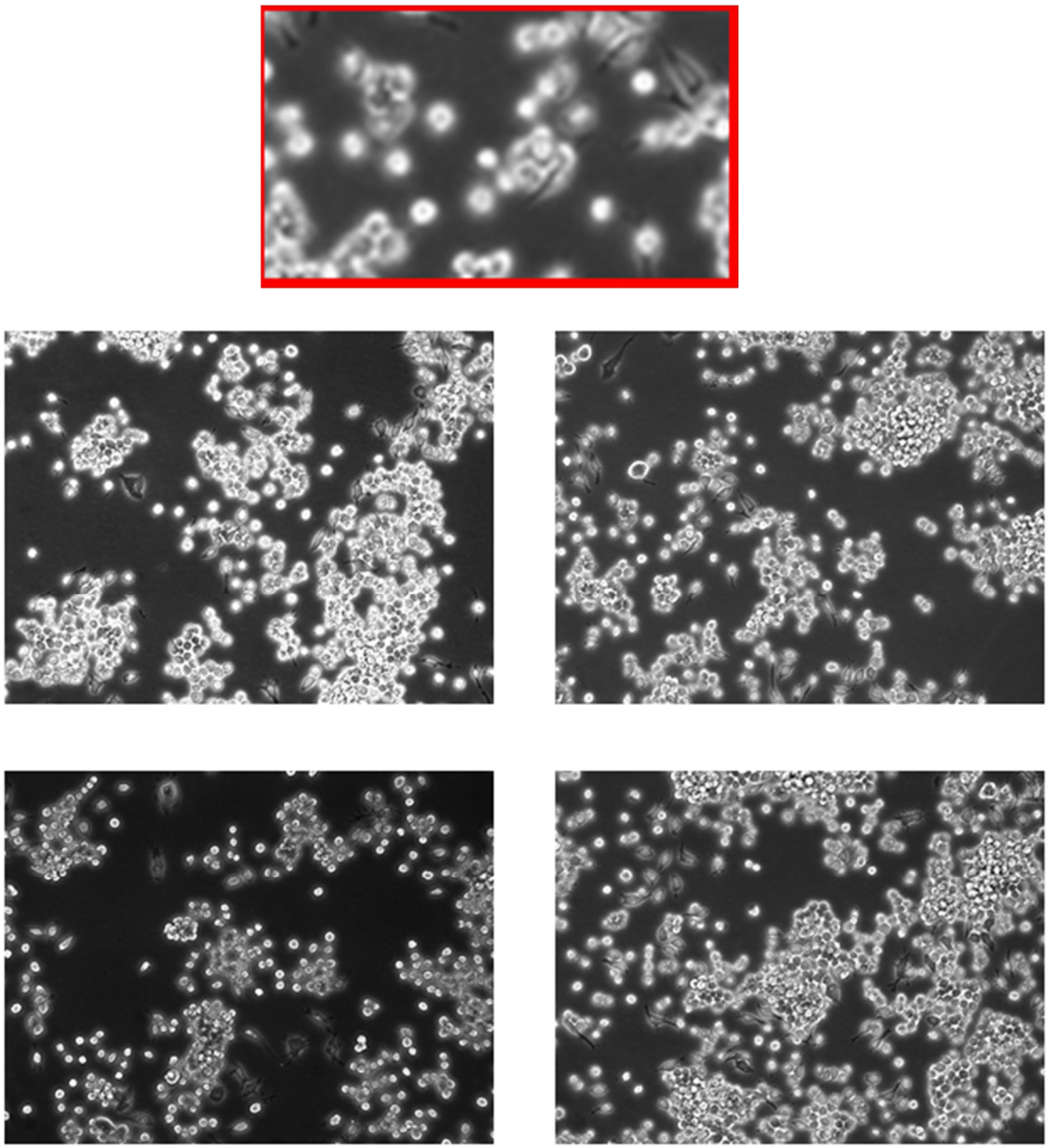


Figure 40. KD cells after treatment with PFFS (1µg/ml) and amlexanox (100nM) for 6 hours (Brightfield 20x).

Morphology of stably silenced for LRRK2 cells and observation of activated macrophages at 16 hours upon different treatment.

Compared to untreated KD cells at 6 hours, we observed large numbers of activated macrophages, however fewer cells with an enlarged appearance at 16 hours compared to 6 hours.

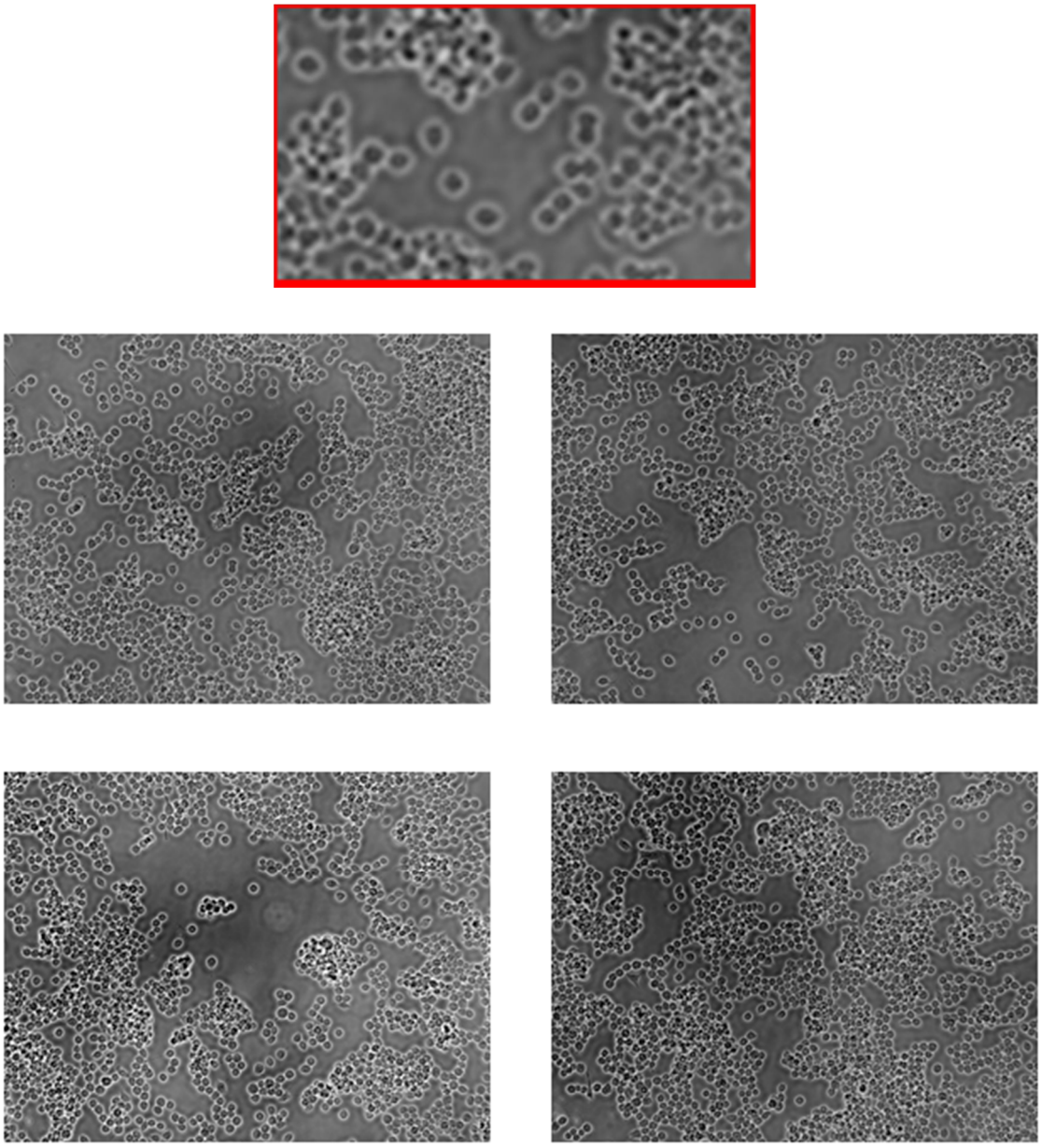


Figure 41. Different pictures of the same well of non-treated KD cells, 16 hours (Brightfield 20x).

Addition of PFFs in KD cells, led to a phenotype of larger activated macrophages, which was not altered by MLi-2 and amlexanox.

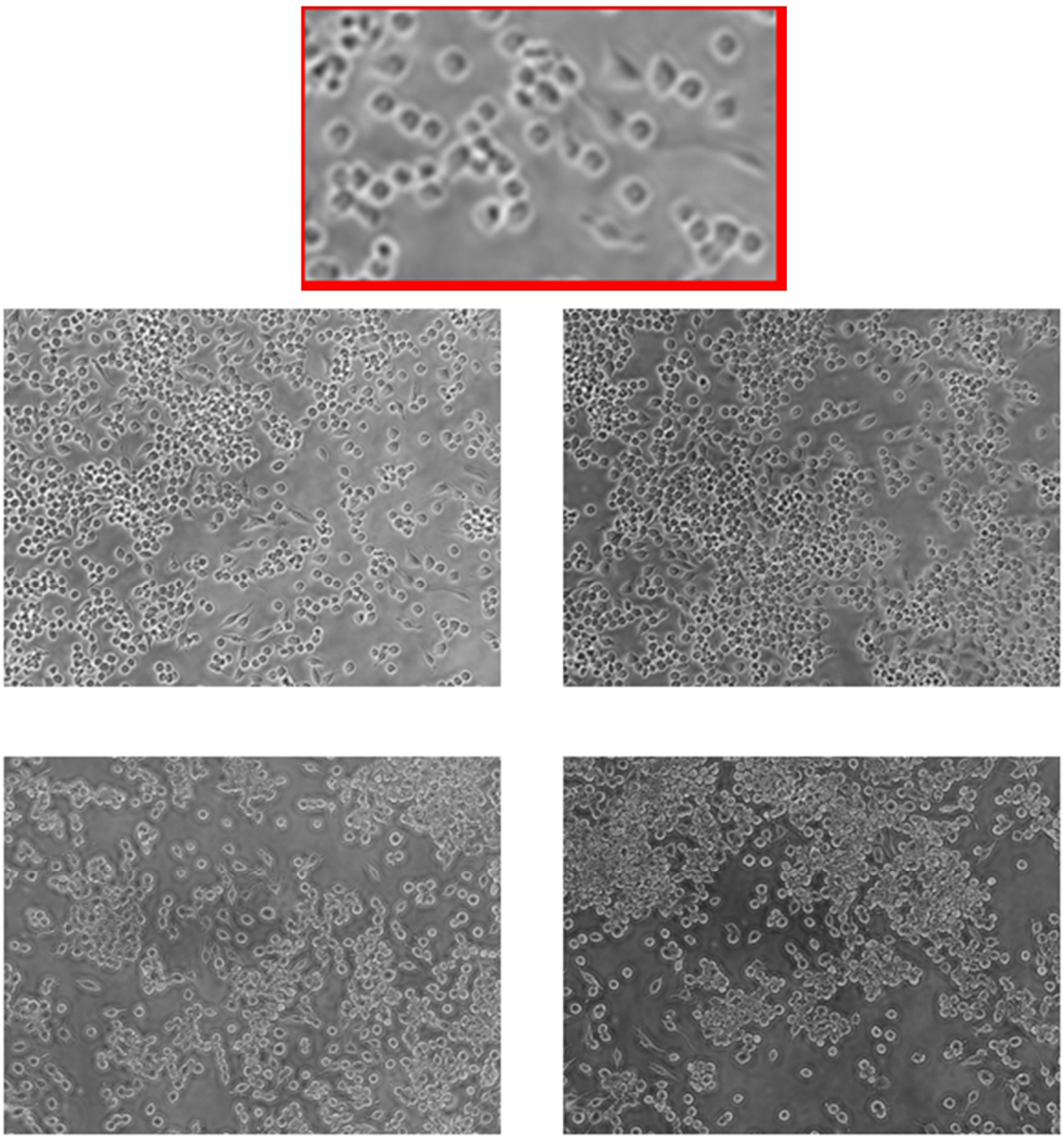


Figure 42. KD cells after treatment with PFFS (1μg/ml) for 16 hours (Brightfield 20x).

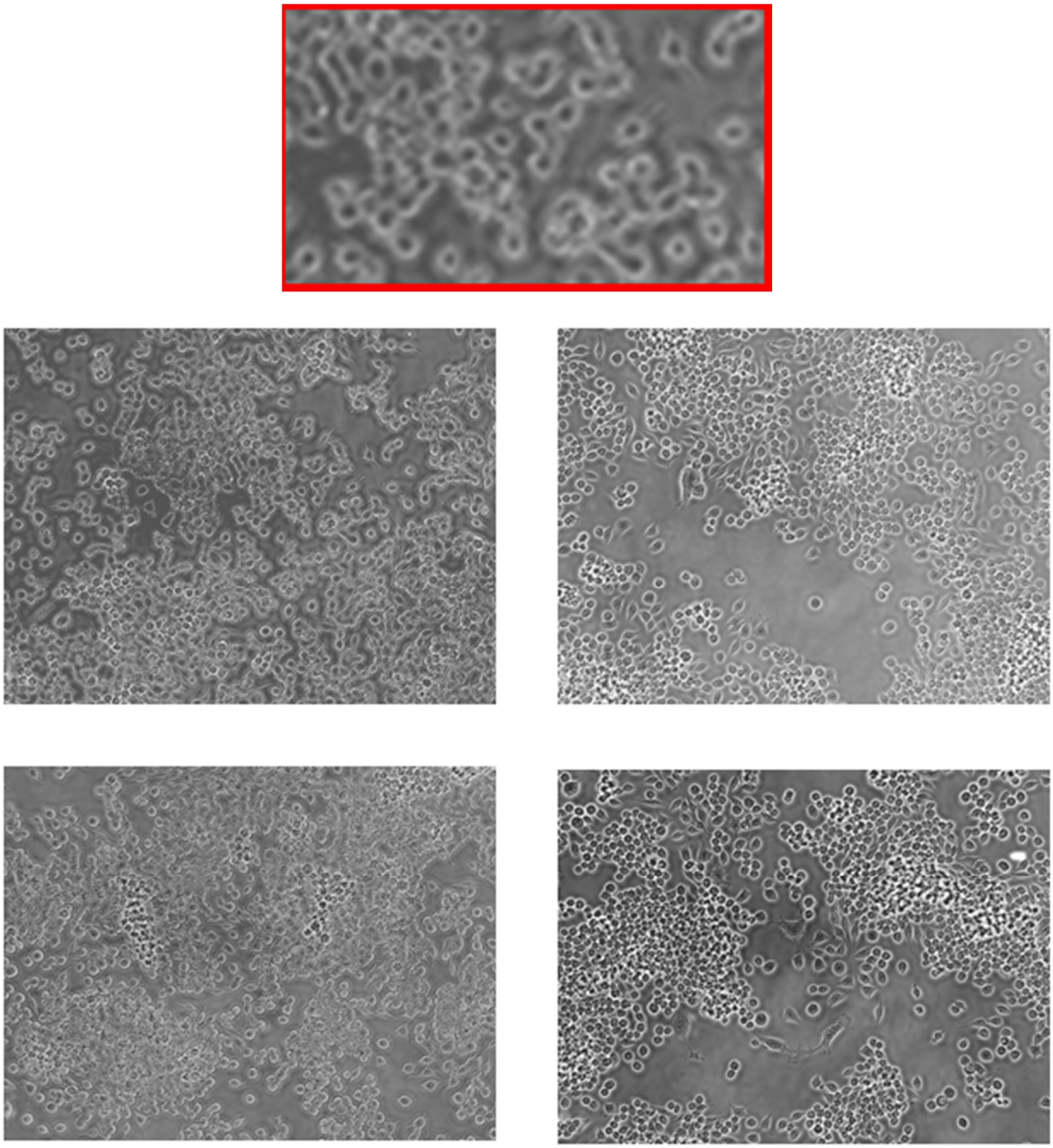


Figure 43. KD cells after treatment with PFFS (1µg/ml) and MLi-2 (100nM) for 16 hours (Brightfield 20x).

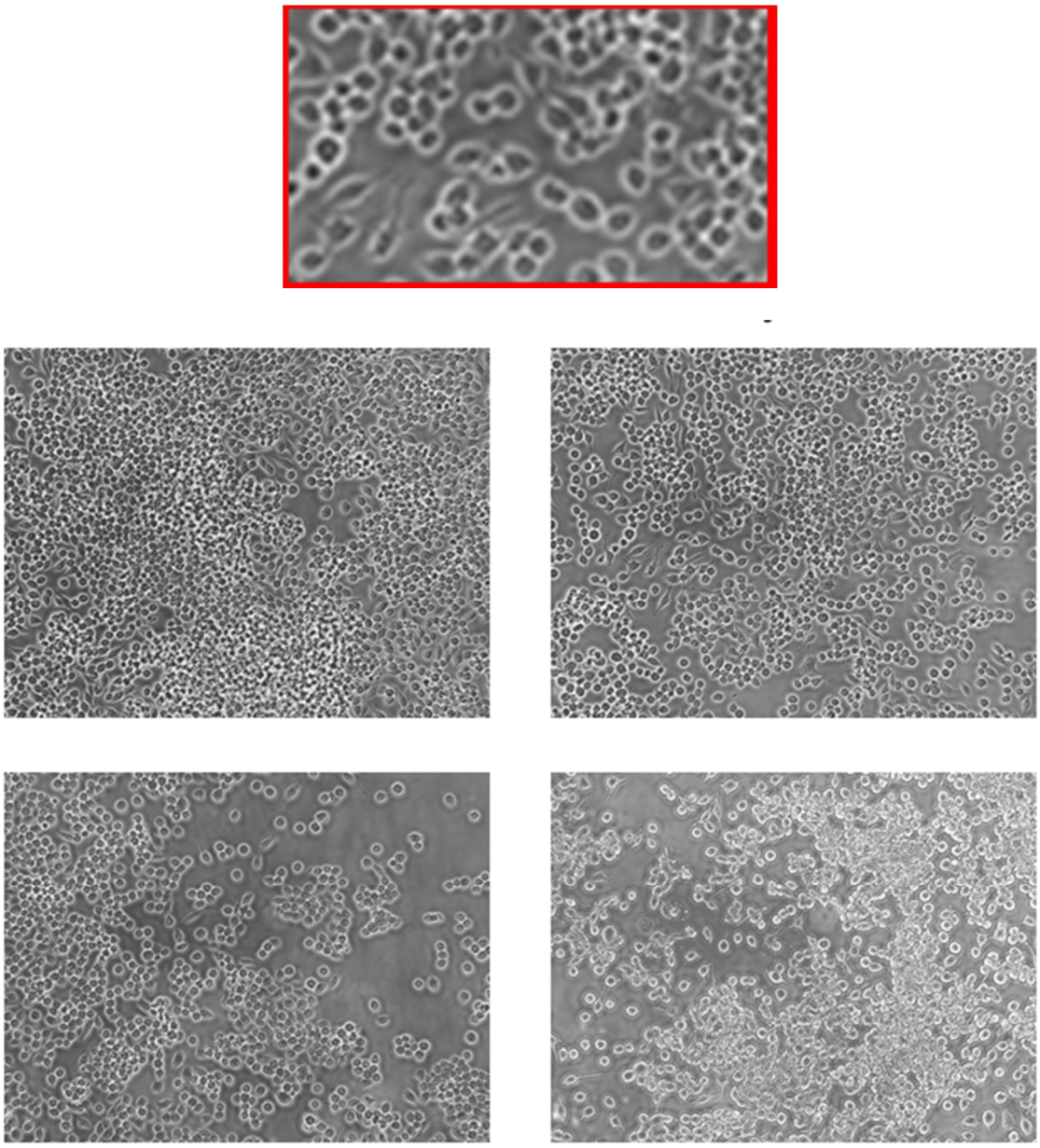


Figure 44. KD cells after treatment with PFFS (1µg/ml) and amlexanox (100nM) for 16 hours (Brightfield 20x).

d. Discussion

LRRK2 missense mutations are the most common known genetic cause of PD and variants in LRRK2 are considered generally as pathogenic or risk factors. In addition, as the expression of LRRK2 is robust within the immune system and the Parkinson's disease pathogenesis has been connected to immunologic factors, the study of this interaction is of vital importance. In this context, TLRs, which regulate inflammatory cytokines and chemokines enrolling intracellular signaling pathways to regulate the inflammatory response, are considered of high interest in PD. As a result, it is crucial to understand their regulation including plasma membrane trafficking. Studies of LRRK2 roles in membrane trafficking have revealed that although LRRK2 interacts with proteins involved in endocytosis and exocytosis of synaptic vesicles, by the WD40 domain (Picoli et. al. 2011). Steger et al. found that Rab10 is a direct phosphorylation substrate of LRRK2, and the phosphorylation site is within its conserved switch-II motif. Related to this, in 2010 a study showed that Rab10 facilitates Toll-like receptor-4 (TLR4) signaling by controlling its trafficking onto the plasma membrane after activation with LPS (Wang et al., 2010). In this project, our goal was to determine if LRRK2 phosphorylation of Rab10 was involved in the regulation of TLR trafficking. While we found that inhibition of LRRK2 activity did alter the trafficking of TLRs, we were not able to detect any changes at the LRRK2-dependent phospho-site in Rab10 (T73). This suggests that perhaps a novel site is involved, or that LRRK2 phosphorylation of a different Rab also participates in TLR trafficking. Follow up studies addressing these questions are ongoing.

In this study, we used flow cytometric analysis in order to assess if LPS or a-syn fibrils (PFFs) alter the trafficking of TLR4 and TLR2. LPS exposure in RAW264.7 cells, revealed a reduction in TLR4 cell surface expression, with a pattern dependent on time. The same effect was observed following PFFs exposure. The above results for LPS exposure are consistent with those of Wang et al. 2010. As phosphorylation of Rab10 by LRRK2 inhibits its function we predicted that blocking this phosphorylation in our model would prevent the regulation of TLR trafficking by Rab10. Several inhibitors have been used in order to block the kinase activity of wt or mutant LRRK2 in order to determine the role of LRRK2 enzymatic activity in physiologic as well as in pathogenic conditions. Many different classes of inhibitors are being developed and tested in models of LRRK2-PD in order to determine their potential for use as therapeutic agents. Although, is important to highlight that broad kinase inhibition may be harmful, as it has already been mentioned in the introduction LRRK2 knock-out mice or mice with a knock-in of a kinase inactive form of LRRK2, display progressive kidney abnormalities (Hinkle, K. M. et al. 2012). However, LRRK2 kinase activity and its potential toxicity need further investigation.

In order to investigate if LRRK2 kinase inhibitors block the alteration mediated by Rab10 in TLR trafficking, we used MLi-2 kinase inhibitor. Indeed, addition of MLi-2 together with LPS exposure, leads to a progressive but transient increase of TLR4 at the plasma membrane in RAW264.7, in a time dependent manner. The same effect is observed after PFFs exposure at six hours.

Wang an colleagues found that overexpression of Rab10 reduced the extent of LPS-induced transient down-regulation of surface TLR4 expression on bone marrow-derived macrophages in a GTPase dependent way (Wang et. al. 2010). If we are able to detect phosphorylation of Rab10, but at a different site than T73, its role in TLR trafficking will be confirmed. Future studies in our system will assess the GTPase dependency as well by expressing mutant forms of Rab10, in cells exposed to LPS or PFF's.

e. References

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