CONTROL OF THE CA^{2+}-NFAT-IL-2 PATHWAY BY LRRK2 IN ASPERGILLUS-STIMULATED DENDRITIC CELLS

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A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

NUS GRADUATE SCHOOL FOR INTEGRATIVE SCIENCES AND ENGINEERING

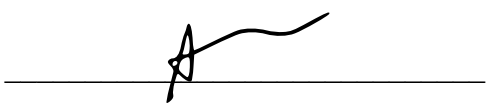
NATIONAL UNIVERSITY OF SINGAPORE

2016

Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



Wong Yoke Wei, Alicia

3 February 2016

Acknowledgements

I would like to start off my acknowledging the one person that I am most indebted to, Dr Teresa Zelante, who has been mentoring, inspiring and encouraging me throughout this project. She is someone that I really look up to not only as a scientist, but also as a friend, and I am truly blessed that our lives crossed paths four years ago. Grazie assai di tutto.

I would also like to thank my supervisors, Dr Florent Ginhoux and Dr Paola Castagnoli, and TAC chairperson, Prof Lu Jinhua, for their valuable time, input and support. My deepest gratitude also goes to Prof Luigina Romani for the precious opportunity to spend a wonderful year in her laboratory and the beautiful city of Perugia, Italy.

In addition, I am grateful to the members from the lab of Dr Paola Castagnoli (SIgN) for their generous help, expertise and friendship, as well as members of the lab of Prof Luigina Romani (University of Perugia) for being so welcoming and accommodating during my time in Italy. With all of them I have shared much laughter and memories. I regret that they are too numerous to be named individually.

I would also like to thank A*STAR for awarding the A*STAR Graduate Scholarship, without which this research project and overseas attachment would not have been possible.

Lastly, I owe who I am today to my parents, my family and my friends (be it from Singapore or abroad). Thank you for encouraging me, for enriching my life and for believing in me all this time.

Siderate la vostra semenza:	*Consider the seed from which you sprang;*
fatti non foste a viver come bruti,	*you were not made to live like unto brutes,*
ma per seguir virtute e canoscenza.	*but for the pursuit of virtue and of knowledge.*

- Dante Alighieri, Inferno, Canto XXVI, lines 118-120

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Summary

The Parkinson's Disease-associated protein, Leucine-rich Repeat Kinase 2 (LRRK2), is expressed in myeloid cells such as macrophages and dendritic cells (DCs) and is possibly involved in the host immune response against pathogens. In the context of inflammatory bowel disease, LRRK2, together with the ncRNA repressor of the nuclear factor of activated T cells (NFAT) (NRON) complex has been reported to be a negative regulator of NFAT. However, as of now the role of LRRK2 and the NRON complex has not been looked at in the context of fungal infection.

This project investigates LRRK2 and the calcineurin/NFAT pathway in DCs. DCs were found to express LRRK2 and that LRRK2 indeed negatively regulate the NFAT pathway in response to *Aspergillus*. In steady state DCs, LRRK2 and NFAT were found to be localized on endosomes and lysosomes. When DCs were stimulated with *Aspergillus*, LRRK2 expression was found to be decreased on both the gene and protein level, and this is possibly achieved by the degradation of LRRK2 by the non-canonical autophagy induced by fungi. In addition, the NFAT pathway itself was found to be regulated by phagocytosis and early stages of autophagy, but not by late stages of autophagy. The degradation of LRRK2 by autophagy was further investigated by looking at LRRK2 expression in lysosomes of DCs and by electron microscopy. *Aspergillus*-stimulated DCs were found to have an increase presence of multilamellar bodies, and that these multilamellar bodies expressed LRRK2. Also, components of the NRON complex were knocked down in DCs to see how this affects DC IL-2 production in response to *Aspergillus*, and it was observed, contrary to expectations, that the NRON complex components were not regulating NFAT pathway negatively as LRRK2. When other cytokines produced by *Aspergillus*-stimulated DCs were looked at, it was found that the different NRON complex

components were also influencing the cytokine response is that downstream of other signaling pathways other than the Ca^{2+}-NFAT-IL-2 axis.

The results shed new light on the complexity of control of the NFAT pathway by LRRK2 and the endosome-lysosome network, and hint at a more complicated role for the NRON complex protein members in the immune response of DCs to *Aspergillus*.

(358 words)

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List of Abbreviations

3-MA	3-methyladenine
ACK	Ammonium-Chloride-Pottasium
ANK	N-terminal ankyrin repeat
ATG	Autophagy-related gene
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
CARD9	Caspase recruitment domain-containing protein 9
CD	Cluster of differentiation
cDC	Classical DC
CLR	C-type lectin receptor
CMA	Chaperone mediated autophagy
COR	C-terminal of ROC
CSE1L	Chromosome segregation 1-like
CSF	Colony stimulating factor
DAPI	4',6-diamidino-2-phenylindole
Dyrk1a	Dual-specificity tyrosine kinase 1a
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Flt3L	Fms-like tyrosine kinase 3 ligand
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSK-3	Glycogen synthase kinase 3
HANDs	Human Immunodeficiency Virus-1 associated neurocognitive disorders
HBSS	Hanks' Balanced Salt Solution
HEK293T	Human Embryonic Kidney cells
HIV-1	Human Immunodeficiency Virus-1
HMW	High molecular weight
HSC70	Heat shock cognate protein 70
IBD	Inflammatory bowel disease
IFNγ	Interferon gamma
IL	Interleukin
IMDM	Iscove's Modified Dulbeccos Medium
IQGAP	IQ motif-containing GTPase activating protein 1
ITAM	Immunoreceptor tyrosine-based activation motif
KPNB1	karyopherin (importin) β 1
LAMP-1	Lysosomal-associated membrane protein 1
LC3	Microtubule-associated 1A/1B-light chain 3
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LRRK2	Leucine-rich repeat kinase 2
MAPK	Mitogen-activated protein kinase
MHCII	Major histocompatibility complex class II
MIP-2	Macrophage inflammatory protein type 2
MKKK	MAP kinase kinase kinases
MVB	Multivesicular body

MWCO	Molecular weight cut off
NAADP	Nicotinic acid adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
NFAT	Nuclear factor of activated T cells
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
NKT cells	Natural killer T cells
NLR	NOD-like receptor
NRON	Noncoding repressor of NFAT
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
pDC	Plasmacytoid DC
PHA	Phytohemagglutinin
PKA	Protein kinase A
PKB	Protein kinase B/Akt
PLCγ2	Phospholipase Cγ2
PMSF	Phenylmethanesulfonylfluoride
PP2A	Protein phosphatase 2A
PPP2R1A	Protein phosphatase 2 regulatory subunit A
PRR	Pathogen recognition receptor
PSMD11	26S proteasome non-ATPase regulatory subunit 11
PVDF	Polyvinylidene fluoride
RFP	Red fluorescent protein
RIPA buffer	Radioimmunoprecipitation assay buffer
ROC	Ras of complex proteins
SPAG9	Sperm-associated antigen 9
SYK	Spleen tyrosine kinase
TBP	TATA binding protein
Th cell	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNPO1	Transportin-1
ULK1	Uncoordinated-51 (UNC-51)-like kinase
WGP	Whole glucan particle

Chapter 1. Literature Review

1.1. The Ca^{2+}/calcineurin/NFAT pathway

The nuclear factor of activated T cell (NFAT) family proteins were first discovered as transcription factors that bind to the human IL-2 promoter in T cells (Shaw et al., 1988). The NFAT family comprises five members, namely NFAT1 (also known as NFATc2 or NFATp), NFAT2 (also known as NFATc1 or NFATc), NFAT3 (also known as NFATc4), NFAT4 (also known as NFATc3 or NFATx), and NFAT5 (also known as TonE-BP or NFATL1) (Crabtree & Olson, 2002). Of the five members, the first 4 are regulated by Ca^{2+} signaling. Normally, NFAT resides in the cytoplasm in its phosphorylated, inactive state. In addition, cytoplasmic NFAT is bound by calcineurin, a phosphatase complex composed of three subunits: a catalytic A subunit, a regulatory B subunit and calmodulin. In innate immune cells, response to lipopolysaccharide (LPS) binding to toll-like receptor 4 (TLR4) or particulate β-glucan binding to dectin-1, SYK activates phospholipase Cγ2 (PLCγ2), inositol triphosphate (IP_3) and subsequent Ca^{2+} flux into the cell (Fric et al., 2012). NFAT signaling is initiated when the free concentration of intracellular Ca^{2+} rises above the threshold of 400nM. This concentration of intracellular calcium is considered to be sufficient to activate calcineurin by withdrawal of an inhibitory peptide from this complex. Activated calcineurin catalyzes the dephosphorylation of NFAT, allowing NFAT to translocate to the nucleus and induce transcription of target genes (Wu et al., 2007) (Figure 1). NFAT has been found to mediate diverse functions in numerous other immune and non-immune cell types as reviewed in Fric et al. (2012) (Table 1). NFAT phosphorylation for export back to the cytoplasm is rapidly accomplished by first nuclear priming kinase, dual-specificity tyrosine kinase 1a (Dyrk1a) or protein

kinase A (PKA), and then glycogen synthase kinase 3 (GSK-3). It is this efficient mechanism of NFAT nuclear export that enables discrimination of stimuli that lead to brief *versus* prolonged Ca^{2+} signals (Wu et al., 2007).

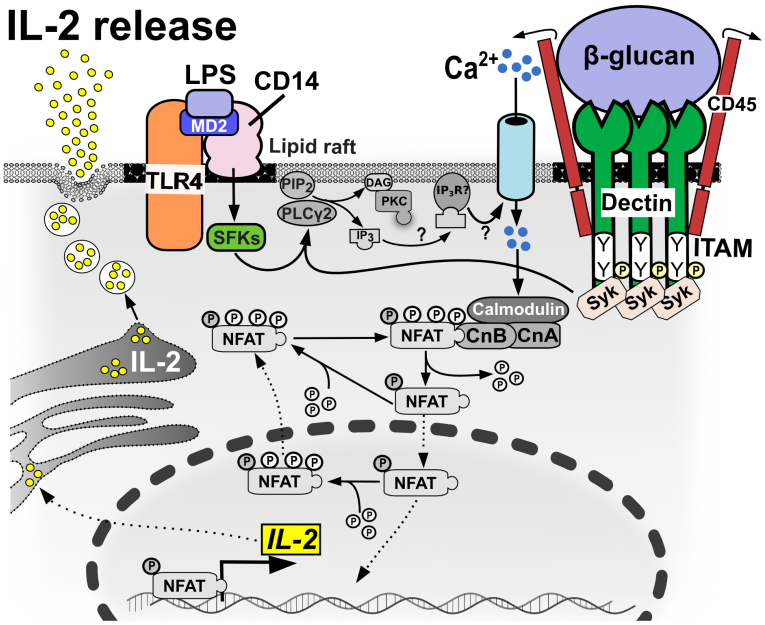


Figure 1: The NFAT pathway in DCs (Fric et al., 2012). NFAT is a transcription factor. At resting state, NFAT is phosphorylated and is located in the cytoplasm of the cell. Ligand binding to PRRs, TLR4 or dectin, initiates downstream signaling which leads to calcium flux. The increase of calcium concentration in the cell activates calmodulin and the calcineurin complex, leading to the activation of NFAT by dephosphrylation. NFAT translocation to the nucleus and gene transcription. One of the cytokines produced by DCs downstream of NFAT signaling is IL-2.

One of the cytokines expressed downstream of the NFAT pathway is interleukin-2 (IL-2).

1.2. IL-2

IL-2 is a cytokine first identified in the 1970s as the factor in conditioned medium derived from phytohemagglutinin (PHA)-stimulated human blood lymphocytes that could generate T cells from bone marrow cells suspensions (Morgan et al., 1976). IL-2 is produced mainly by T lymphocytes, but also to some extent by

natural killer (NK) cells and natural killer T (NKT) cells. This cytokine is now known to be important not just for T cell survival, but all facets of lymphocyte function. For example, IL-2 is important in influencing $CD8^+$ T cells in all aspects of an immune response, such as their initial expansion, contraction, formation of memory, and also their secondary expansion (Boyman & Sprent, 2012).

Table 1: Different functions of NFAT transcription factors in myeloid cells (Fric et al., 2012)

Cell Type	NFAT	Function	Ref.
Dendritic Cells	NFAT1 NFAT2	Regulation of DC life cycle/apoptosis of terminally differentiated DCs in response to LPS	(Zanoni et al., 2009)
		Regulation of IL-2, IL-10 and IL-12 expression	(H. S. Goodridge et al., 2007; Granucci et al., 2003; Granucci et al., 2004; Zanoni et al., 2005)
		Activation of NK Cells	(Granucci et al., 2004; Granucci et al., 2006)
Macrophages	NFAT1 NFAT2 NFAT4 NFAT5	Regulation of IL-6, IL-10, and IL-12 and TNFα	(Elloumi et al., 2012; H. S. Goodridge et al., 2007; Granucci et al., 2006; Liu et al., 2011)
		Regulation of multiple TLR-induced genes, eg. *Nos2* (inducible nitric oxide synthase (iNOS))	(Buxade et al., 2012)
Mast Cells	NFAT1 NFAT2	Regulation of mast cell activation during hypoxia	(Walczak-Drzewiecka et al., 2008)
		Enhanced mast cell survival after FcεRI activation due to regulation of A1 expression	(Kitaura et al., 2004; Xiang et al., 2001)
		Regulation of IL-13 and TNF expression	(Klein et al., 2006)
Neutrophils	NFAT2 NFAT4	Regulation of IgE-mediated expression of cyclooxygenase and release of prostaglandin	(Vega et al., 2007)
		Resistance to *C. albicans* infection – genes involved include IL-10, Cox2, Egr1, and Egr2	(Greenblatt et al., 2010)
Eosinophils	NFAT2 NFAT4	Degranulation, cytokine release, apoptosis	(Jinquan et al., 1999; Meng et al., 1997)
Basophils	NFAT2	Regulation of IL-4 production	(Qi et al., 2011; Schroeder et al., 2002)
Megakaryocytes	NFAT2 NFAT3	Role in megakaryocyte differentiation	(Kiani et al., 2007)
		Mediates CD154 expression	(Crist et al., 2008)
Osteoclasts	NFAT2	Master regulator of osteoclastogenesis	(Negishi-Koga & Takayanagi, 2009; Takayanagi, 2007)
NK cells	NFAT1 NFAT2	Inducible NFAT expression	(Aramburu et al., 1999)
		Mediates CD16-induced activation of cytokine genes	(Dybkaer et al., 2007; Wang et al., 2007)
		Role in cytotoxicity and cell proliferation	(Dybkaer et al., 2007; Kim et al., 2010)

1.2.1. Dendritic cells and IL-2

It has been shown that dendritic cells (DCs) also have the ability to produce IL-2 (Granucci et al., 2001; Wuest et al., 2011; Zelante et al., 2012). DCs are instrumental in the immune system for inducing pathogen-specific responses. Two types of DCs are reported in literature: Plasmacytoid DCs (pDCs), and classical DCs (cDCs) as reviewed by Merad et al. (2013). In particular, pDCs are part of a small DC subset distinct from the cDCs in their morphology and resembling plasma cells. cDCs, on the other hand, encompass all DCs that are not pDCs, such as the cluster of differentiation (CD)8^+ and $CD11b^+$ resident lymphoid DCs, tissue-migratory DCs, and Langerhans cells. cDCs function as sentinels, hence they are sensitive to environmental stimuli.

In the laboratory, murine DCs are often cultured *in vitro* from bone marrow-derived progenitors using cytokines such as colony stimulating factor (CSF)-2 (also known as granulocyte-macrophage colony-stimulating factor (GM-CSF), or fms-like tyrosine kinase 3 ligand (Flt3L)). DCs generated by culturing in GM-CSF was thought to be the semi- and non-adherent fraction of the culture that is enriched in $CD11c^+$ Major histocompatibility complex class II^+ ($MHCII^+$) cells resembling tissue DCs (Inaba et al., 1992). However, it was recently reported that this $CD11c^+MHCII^+$ semi- to non-adherent fraction actually composed of two cell populations – DCs and macrophages – that differ from each other in terms of functionality and gene expression (Helft et al., 2015). Culturing bone marrow progenitors in Flt3L, on the other hand, it is possible to generate three types of cDC subsets: $B220^-$ $CD11b^{high}CD172a^{high}CD24^{low}Clec9A^-$ cDCs,

$CD11b^{low}CD172a^{low}CD24^{high}Clec9A^{+}$ cDCs, and $B220^{+}$ pDCs (Merad et al., 2013).

In line with their role as sentinels of the body, DCs express several pathogen recognition receptors (PRRs). Dectin-1 is one such PRR that is classified as a C-type lectin receptor (CLR) and is found on numerous immune cells of both human and mice. β-glucans (either β(1→3)- or β(1→6)-linked glucans) are the only ligand of dectin-1 (Brown, 2006). Upon binding to β-glucans, dectin-1 clusters at the β-glucan particle and induces the formation of a "phagocytic cup". Signaling is then initiated by SRC kinase-mediated phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM)-like motif located on the cytoplasmic tail of dectin-1. This recruits SH2-domain-containing protein spleen tyrosine kinase (SYK). These events occur within 1 minute of binding of β-glucan to dectin-1, and are dependent on that the β-glucan is particulate or immobilized on a surface. Soluble β-glucans do not induce these events (Goodridge et al., 2011). SYK then activates the caspase recruitment domain-containing protein 9 (CARD9) pathway. Dectin-1 also activates the RAF pathway. Both these pathways activate canonical and non-canonical nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and downstream gene expression (Geijtenbeek & Gringhuis, 2009). Another pathway that is activated by dectin-1 in DCs is the Ca^{2+}/calcineurin/nuclear factor of activated T cells (NFAT) pathway (Goodridge et al., 2007).

NFAT signaling has been shown to regulate cytokine IL-2 expression in DCs stimulated *in vitro* with whole glucan particles (WGP) (Fric et al., 2014), which are a particulate form of β-glucan derived from the yeast

Saccharomyces cereviseae. Also, it has been reported that the NFAT pathway is activated in DCs in response to live *Candida albicans* and zymosan, another type of particulate β-glucan, binding to dectin-1 and this leads to the production of cytokines including IL-2 (Goodridge et al., 2007). Given that IL-2 is important for the survival and activation of T cells (Boyman & Sprent, 2012), and that major T helper (Th) cell populations that arise as a response to fungal infection include inflammatory Th1 and Th17 cell types as well as the immunosuppressive T regulatory (Treg) cell type (Romani, 2011), these point to a possible role of DC-derived IL-2 in fungal immunity.

In contribution to understanding how DC-derived IL-2 is involved in the emergence of the Th response in fungal infections *in vivo*, the laboratory recently discovered that the environmental fungus, *Aspergillus fumigatus*, induces IL-2 expression production in lung $CD103^+$ DCs through the Ca^{2+}-Calcineurin-NFAT signaling pathway. This expression of IL-2 in DCs was found to be important for T cell proliferation and modulation of Th17 polarization, as well as for controlling lung inflammation and susceptibility to invasive pulmonary Aspergillosis. Single-cell mass cytometry by time-of-flight (CyTOF^{TM} mass cytometer) analysis of expanded pulmonary T cells revealed that the conditional lack of IL-2 expression in DCs leads to the expansion of a subset of T cells with a phenotype similar to that of IL-17^+ T memory stem cells were increased, and that express a higher amount of β-catenin (Figure 2). These results are the first to identify a biologically relevant *in vivo* function for IL-2 production by DCs as well as a new function for the Ca^{2+}-Calcineurin-NFAT–IL-2 signaling pathway as a regulator of T_H17 cell plasticity and functionality. β-catenin stabilization is known to exert a powerful effect on

the prevention of inflammatory disease, yet we demonstrated that not only does it act to enhance survival of existing Treg cells and promote unresponsiveness in precursors of T effector cells, but it also associates with IL-23s ability to differentiate and stabilize a pool of memory stem cells that represent a durable source of mostly pathogenic T_H17 cells. Thus, although IL-23 has been already described as being pathogenic in T_H17-dependent innate immunity (Wu et al., 2013), our data provide the first evidence that, in mature T_H17 cells, a balance between IL-2 and IL-23 productions by $CD103^+$ DCs regulates T_H17-cell longevity and function in the lung. Targeting the IL-23–IL- 17 immune axis might represent therapeutically useful means of controlling immunopathology in infection (Zelante et al., 2015).

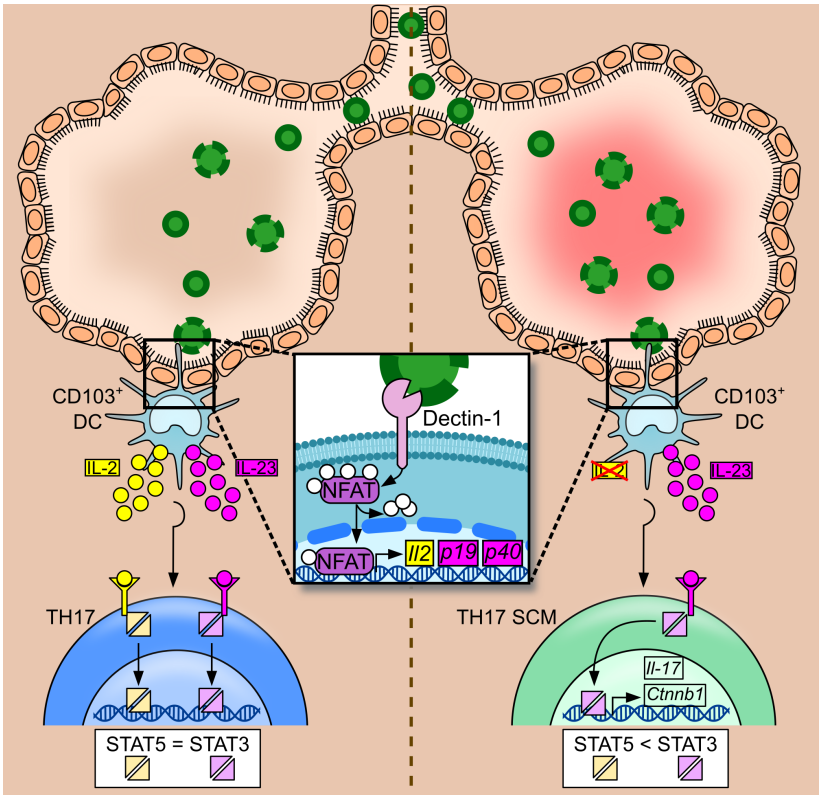


Figure 2: The influence of cytokines IL-2 and IL-23 on Th17 polarization by $CD103^+$ DCs. IL-2 and IL-23 from $CD103^+$ DCs are important for the induction of Th17 cell responses in fungal infection (left). This IL-2 production from $CD103^+$ DCs is mediated by the NFAT pathway (inset). In the absence of IL-2, Th17 cells had a phenotype more similar to $IL\text{-}17^+$ T memory stem cells (right). It was found that IL-2 affects the polarization of Th17 cells through the modulation of STAT phosphorylation (Zelante et al., 2015).

1.2.1.1. D1 cells

In 1997, an *in vitro* cell model of DCs, called D1 cells, was described. These cells were generated by culturing single cell suspensions of spleens obtained from C57BL/6 mice in conditioned medium produced by NIH-3T3 cells overexpressing rGM-CSF. The survival of D1 cells is dependent on the cytokine, GM-CSF. D1 cells, like bone-marrow derived DCs, have the phenotype of immature DCs. They express CD11c, FcγII/III receptor and F4/80. D1 cells also express major histocompatibility complex class I (MHC class I) and MHC class II, as well as co-stimulatory molecules CD40 and B7.1 (Winzler et al., 1997). While their PRR and cytokine receptor expression has not been reported in literature, D1 cells have been successfully induced to undergo maturation by microbial stimuli such as LPS (Schuurhuis et al., 2000; Winzler et al., 1997), β-glucan (Fric et al., 2014; Yu et al., 2015), bacteria (Granucci et al., 2001) and fungi (Zelante et al., 2015), as well as inflammatory cytokines such as tumor necrosis factor-α (TNFα) or IL-1β (Winzler et al., 1997). Maturation leads to D1 cells upregulating their MHC class II, B7.2 and CD40 surface expression (Winzler et al., 1997).

1.3. LRRK2

1.3.1. LRRK2 and its expression in the immune system

First discovered in genome-wide linkage studies of patients of Parkinson's Disease (Funayama et al., 2002; Paisán-Ruíz et al., 2004), the leucine-rich repeat kinase 2 (LRRK2) protein is part of the ROCO protein family (Lewis, 2009). It is 2527-amino acids long and consists of six defined domains – the N-terminal ankyrin repeats domain (ANK), leucine-rich repeats domain (LRR), Ras of complex proteins domain (ROC), C-terminal of ROC domain (COR), a serine/threonine kinase domain, and lastly a C-terminal WD40 domain (Figure 2). Currently, little is known about the function of these domains. The function of the ANK and LRR domains in the LRRK2 protein is unclear, but it has been speculated that the ANK domain is involved in protein polymerization (Lu et al., 2010). The ROC domain, and the COR domain have GTPase activity (Deng et al., 2011; Smith et al., 2005; West et al., 2007). The WD40 domain is a conserved protein interaction domain, shown to be needed for the autophosphorylation of the LRRK2 protein, and could be important in the dimerization and function of LRRK2 (Greggio et al., 2008).

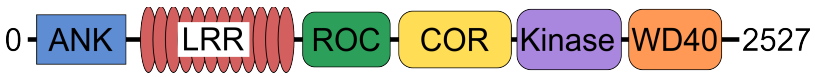


Figure 3: Schematic diagram of the LRRK2 protein domains. The LRRK2 protein consists of 6 domains – the N-terminal ankyrin repeats domain (ANK), leucin-rich repeats domain (LRR), Ras of complex proteins domain (ROC), C-terminal of ROC domain (ROC), a kinase domain, and lastly a WD40 domain (adapted from Lewis (2009)).

The majority of studies on LRRK2 have been focused on linking various point mutations in the various domains of LRRK2 with Parkinson's Disease (as reviewed by Mata et al. (2006)), and the contribution of mutated LRRK2 protein to neuronal toxicity (Jorgensen et al., 2009; Smith et al., 2005; West et al., 2007). Studies have also shown a possible involvement of the immune system in Parkinson's Disease pathogenesis. Inflammation is thought to lead to the neurodegeneration and neurotoxicity seen in Parkinson's Disease (Liu, 2006; Whitton, 2007). Also, it is known that patients of Parkinson's Disease are often hospitalized for various types of infections such as urinary tract infection and pneumonia (Gerlach et al., 2011). In addition, LRRK2 has now been genetically linked to two other chronic inflammatory diseases, Crohn's Disease (Barrett et al., 2008) and leprosy (Sun et al., 2011; Zhang et al., 2009), and is being studied as a potential therapeutic option for Human Immunodeficiency Virus-1 (HIV-1) associated neurocognitive disorders (HANDS) (Marker et al., 2012). These studies give initial clues to the involvement of LRRK2 in the immune system.

Studies have shown that in addition to being expressed in many body tissues including regions of the brain (Paisán-Ruíz et al., 2004), LRRK2 is also expressed in cell populations of the human immune system. LRRK2 is highly expressed in human peripheral blood mononuclear cell (PBMC) fractions, $CD14^+$ monocytes and $CD19^+$ B cells (Gardet et al., 2010; Hakimi et al., 2011; Thévenet et al., 2011). Interestingly, the mRNA expression of LRRK2 may not correlate with protein levels in the cell, as was found in comparison of the $CD14^+16^-$ and $CD14^+CD16^+$ monocyte populations by Thévenet et al. (2011). While the mRNA level expression of LRRK2 was

comparable between these two monocyte populations, LRRK2 protein was expressed 14-fold more in the $CD14^{+}CD16^{+}$ monocytes than the $CD14^{+}16^{-}$ population (Thévenet et al., 2011). LRRK2 is expressed also in human $CD11b^{+}$ DCs and $CD56^{+}$ NK cells (Gardet et al., 2010), but not in red blood cells or granulocytes (Hakimi et al., 2011). In human T cells, information regarding LRRK2 expression is not consistent among studies. While some report that both $CD4^{+}$ and $CD8^{+}$ cells express LRRK2 (Hakimi et al., 2011; Thévenet et al., 2011), the level of LRRK2 expression differed between studies. Another study found that LRRK2 was not expressed in $CD4^{+}$ T cells, but expression in $CD8^{+}$ T cells were not consistent across human tissue datasets and cDNA library (Gardet et al., 2010).

In mice, LRRK2 expression is reported to be present in bone marrow-derived macrophages (BMDMs) and bone marrow-derived dendritic cells (BMDCs), $CD4^{+}$ T cells (Liu et al., 2011), as well as in B cells (Maekawa et al., 2010) (in particular only in B-2 cells but not B-1 cells (Kubo et al., 2010)). In addition, Hakimi et al. (2011) also looked at LRRK2 expression in mouse BMDMs via western blotting, and found that a high molecular weight (HMW) variant (<290kD) of LRRK2 was present in these cells, as well as the lysates of PBMC fractions, but not detected in the lysate of other cell types. The composition and significance of this LRRK2 HMW variant is currently unknown.

1.3.2. LRRK2 and inflammatory diseases

Other studies looking at the involvement of LRRK2 in inflammatory diseases, as well as in the response to microbes and pathogen-associated molecular patterns (PAMPs) have given some clues regarding the role of this protein in these contexts.

Besides Parkinson's Disease, LRRK2 has been associated with inflammatory bowel disease (IBD), specifically with the chronic inflammatory condition of Crohn's disease (Barrett et al., 2008; Törkvist et al., 2010), but not with another IBD called ulcerative colitis (Gardet et al., 2010; Törkvist et al., 2010). Also, macrophage-differentiated THP-1 cells and PBMCs exposed to the inflammatory cytokine interferon gamma (IFNγ) have increased LRRK2 expression on both the gene and protein level (Gardet et al., 2010). In the context of experimental colitis, sublethally irradiated mice exhibit worse disease symptoms when they were reconstituted with bone marrow from LRRK2-deficient mice. This suggested that LRRK2 could be controlling inflammation, rather than promoting it. Also, the role of LRRK2 in inflammation has been studied in the context of exposure to several microbial PAMPs. It has been shown that LRRK2 mRNA levels are significantly upregulated in BMDMs that have been exposed to a variety of TLR ligands (Poly I:C, LPS, R837 and CpG), while stimulation of TLR2 ligand Pam3CSK4 led to a decrease of LRRK2 mRNA compared to the unstimulated control. LRRK2 protein levels were also upregulated in BMDMs following lentiviral transduction. In contrast, when the NOD-like receptor (NLR) ligands L18 and C12 were used to stimulate BMDMs, they did not seem to induce an increase in LRRK2 protein levels (Hakimi et al., 2011). Similarly, in

microglia, LPS stimulation also causes an upregulation of LRRK2 (Gillardon et al., 2012; Moehle et al., 2012) and this was seen in a dose- and time-dependent manner (Moehle et al., 2012). In support of this, knock down of LRRK2 in microglia attenuates the inflammation response to LPS (Kim et al., 2012). However, contradictory to these studies is the study by Liu et al. (2011) that found LPS induced downregulation of protein levels in BMDMs. The discrepancy might be explained by the different durations of stimulation in these studies – Liu et al. (2011) only performed stimulations in time points up to one hour, while Hakimi et al. (2011), Gillardon et al. (2012) and Moehle et al. (2012) stimulated their cells for much longer periods of 5, 18 and 12 hours respectively. Furthermore, LRRK2 may have different functions in different cell types. In addition, LRRK2 phorphorylation at serine residues S910 and S935 post-TLR stimulation in BMDMs (Dzamko et al., 2012) and as well in microglia in response to HIV-1 Tat protein (Marker et al., 2012) has been shown. While the significance of these phosphorylations are not yet known, it could be a way for microbes to influence LRRK2 function, as suggested by Marker et al. (2012).

Further evidence of LRRK2 involvement in the response to extracellular microbial signals is presented by Moehle et al. (2012), who showed that small molecular inhibitors of LRRK2 kinase function led to attenuation of pro-inflammatory signaling in response to LPS-treated microglia. However this is contradicted by the study done by Gillardon et al. (2012) who found that in FVB mice-derived microglial cells that have been transduced with GTPase-mutated LRRK2, where the GTPase activity is decreased, had significantly increased production of TNFα, but significantly

downregulated secretion of the anti-inflammatory cytokine IL-10 in response to LPS. This result was surprising since there is evidence of counter-regulation of the kinase and GTPase function of LRRK2 between each other (Biosa et al., 2013; Taymans et al., 2011; Xiong et al., 2012).

1.3.3. LRRK2 as a signaling molecule

In addition, given that LRRK2 is a large protein with GTPase activity and kinase function, it is not surprising that is involved in various signaling pathways. For example, LRRK2 has been shown to be involved in pathways that are activated downstream of TLR activation, including the NFκB pathway. It has been shown that LRRK2 phosphorylation at Ser910, Ser935 and Ser955 can be mediated by various components of the NFκB pathway following TLR stimulation (Dzamko et al., 2012).

Also, it has been shown that LRRK2 can activate the NFκB pathway independent of its kinase activity (Gardet et al., 2010). The mitogen-activated protein kinase (MAPK) pathway is another pathway that LRRK2 has been found to interact with. Gloeckner et al. (2009) has shown that LRRK2 is able to phosphorylate MAP kinase kinase kinases (MKKK). Also, LRRK2 Parkinson's Disease mutants, such as the mutants G2019S and I2020T which have an increase in kinase activity (West et al., 2005), have been found to increase phosphorylation of MAP kinase kinase 6 (MKK6) (Gloeckner et al., 2009), MKK4 (Chen et al., 2012) and overactivation of the MAPK pathway, leading to increased autophagy levels (Bravo-San Pedro et al., 2013). Other pathway that LRRK2 is involved in include the Wnt pathway in dopaminergic neuron cutures (Berwick & Harvey, 2012; Sancho et al., 2009) and the

nicotinic acid adenine dinucleotide phosphate (NAADP)-sensitive lysosomal Ca^{2+} signaling in the context of autophagy (Gómez-Suaga et al., 2012). The involvement of LRRK2 in the immune context of both the Wnt and NAADP-sensitive lysosomal Ca^{2+} pathway is currently not known.

1.3.4. LRRK2 in autophagy and protein degradation

Autophagy is a type of lysosomal degradation pathway initially found to be used by single-celled eukaryotes to regulate energy and nutrient usage, but now has been found to occur also in cells of higher organisms. There are three main types of autophagy, including macroautophagy, chaperone mediated autophagy (CMA) and microautophagy (Levine et al., 2011) (Figure 4). Macroautophagy, most often referred to in literature as just autophagy, is noted by the formation of the autophagosome. The autophagosome is a double-membraned structure that arises by elongation of a phagophore around a target until the phagophore fuses with itself. The autophagosome is able to fuse with a lysosome to form the autolysosome, allowing its contents to be degraded (Liu et al., 2012). CMA differs from macroautophagy as it involves selective transport of cytosolic soluble proteins across the lysosomal membrane to be degraded (Orenstein et al., 2013). Microautophagy involves the local deformation of the lysosomal membrane that internalizes parts of the cytoplasm in the process of doing so (Li et al., 2012; Mijaljica et al., 2011).

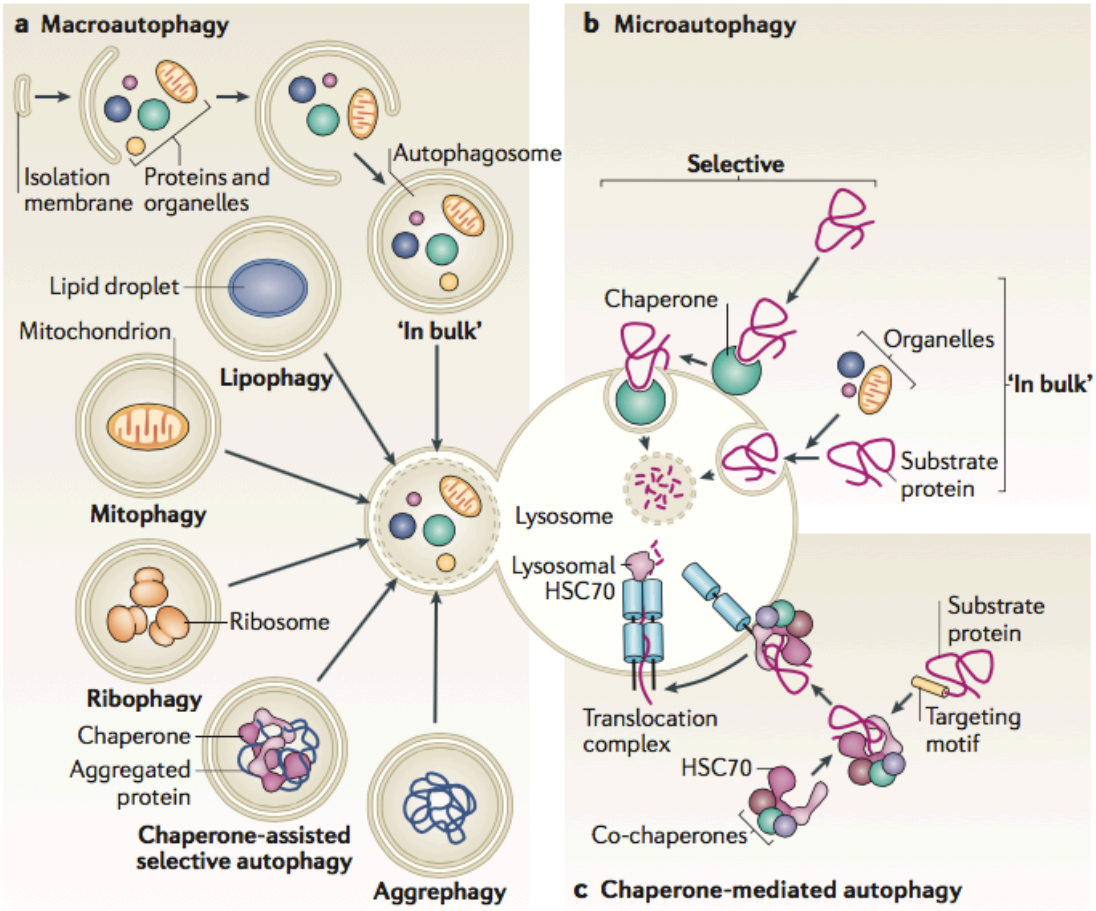


Figure 4: The three different types of autophagy (Cuervo, 2011). Autophagy refers to the cellular process of degradation of intracellular organelles. There are three main types of autophagy – (a) macroautophagy, (b) microautophagy and (c) chaperone-mediated autophagy. (a) Macroautophagy is characterized by the formation of a double membraned autophagophore phagophore that encircles proteins and organelles for degradation. The double membrane autophagophore eventually merges with a lysosome to form a autophagolysosome where the contents will be degraded. (b) Microautophagy is the small invagination of the lysosome membrane to uptake proteins and organelles directly into the lysosome for degradation. (c) Chaperone-mediated autophagy is the uptake of target proteins to the lysosome for degradation in conjunction with a chaperone protein, such as the heat shock cognate protein 70 (HSC70).

In addition, reports of non-canonical autophagy have been emerging in literature. Non-canonical autophagy differs from canonical autophagy in terms of their usage of the autophagy-related gene (ATG) proteins and deviation from the normal formation of the autophagosome. There are a few flavors of non-canonical autophagy that have been reported in literature under specific contexts, such as beclin 1-independent autophagy, autophagy that bypasses the initiation step by the unccordinated-51 (UNC-51)-like kinase-1 (ULK1)

complex, or autophagy that does not require the sequential recruitment of the ATG proteins. Also, the formation of non-canonical autophagy structures, such as the formation of the autophagosome from multiple isolation membranes, or the recruitment of the autophagy-associated protein, microtubule-associated 1A/1B-light chain 3 II (LC3-II), to single-membrane phagosomes during phagocytosis of engaged TLRs, have also been reported (reviewed in Codogno et al. (2012)). It is also reported in literature that there is a non-canonical autophagy in the form of LC3-associated phagosomes that is triggered by Dectin-1 activation and degrades β-glucans in DCs, and that could regulate fungal antigen processing and presentation (Ma et al., 2012).

1.3.4.1. Detection of autophagy

Autophagy can be detected in various ways, and the vast majority of published methods are regarding the detection of macroautophagy.

The most traditional way of detecting autophagy is by looking at subcellular structures via transmission electron microscopy, allowing one to look for characteristic double-membraned autophagosomes. However, the shortcoming of looking at autophagy by transmission electron microscopy is that it is not easy to quantify autophagy by this method in an objective manner (Mizushima et al., 2010; Tanida & Waguri, 2010).

A way to quantify autophagy is to use florescence electron microscopy to detect the formation of cytoplasmic punctate LC3 formations that are associated with autophagosomes, which can then

be counted either manually or with software tools. However, punctate LC3 formations can also occur in a non-autophagy specific manner when LC3 aggregates occur when it is co-expressed with other aggregate-prone proteins (Mizushima et al., 2010; Tanida & Waguri, 2010).

As the use of transmission electron microscopy and fluorescence microscopy for the measurement of autophagy has their specific shortcomings, it is recommended that they be used in conjunction with other methods. Other methods to quantify autophagy involve the use of biochemical assays to detect LC3-I to LC3-II conversion and LC3-II turnover. LC3-I is the cytosolic form of LC3. When autophagy occurs, LC3 localizes to autophagic membranes and is conjugated to phosphatidylethanoamine to become LC3-II. By detecting this LC3-1 to LC3-II conversion by western blot, autophagy can thus be quantified. LC3-II turnover can also be measured by western blot, by measuring the relative density of the LC3-II band in a treated sample when compared to the untreated control (Mizushima et al., 2010; Tanida & Waguri, 2010).

Sequestome 1 (SQSTM1), also known as p62, is an LC3 binding protein that autophagy-dependent for its degradation. p62 protein levels can be easily monitored by western blot. Its degradation can be used as marker for autophagy induction, while its accumulation has been used as indication for inhibition or defective autophagy degradation (Barth et al., 2010; Bjørkøy et al., 2009; Juenemann & Reits, 2012).

1.3.4.2. The link between LRRK2 and autophagy

In Parkinson's disease, the dysregulated clearance of α-synuclein is thought to be one of the mechanisms behind Lewy body pathology. Indeed it has been shown that LRRK2 is important to regulate α-synuclein clearance in neurons (Tong et al., 2010). A possible mechanism for this could be autophagy, as the link between deficient autophagy and the accumulation of LRRK2 and α-synuclein has been shown in neurons (Friedman et al., 2012).

Numerous studies show that LRRK2 and autophagy are linked. Certain mutations of LRRK2 that are associated with Parkinson's disease, such as the G2019S kinase domain mutation (Bravo-San Pedro et al., 2013) and the R1441C GTPase domain mutant (Hakimi et al., 2011), have been shown to result in altered autophagy levels. However it is not clear if LRRK2 is regulating the autophagic process negatively or positively. The knock down or absence of LRRK2 has been shown to increase autophagy in some studies (Alegre-Abarrategui et al., 2009; Tong et al., 2010). However this is contradicted by other studies that show that the knock down or absence of LRRK2 had no effect on autophagic activity (Gardet et al., 2010; Liu et al., 2011), or that autophagy could also be induced by overexpression of LRRK2 (Gómez-Suaga et al., 2012). Furthermore, LRRK2 itself could be degraded by autophagy, specifically by CMA as LRRK2 contains 8 putative motifs specific for CMA-targeted degradation (Orenstein et al., 2013) (Figure 5). In addition, Orenstein et al. (2013) show that LRRK2 could be taken up selectively when incubated with isolated

lysosomes, and that LRRK2 can also degraded in lysosomes. However, while the study showed that the degradation of G2019S mutant LRRK2 is affected compared to the wild-type protein, the study did not define what circumstances promote or inhibit this degradation of LRRK2 by CMA in a physiological setting. Nevertheless, the study by Orenstein et al. (2013) demonstrates that while LRRK2 seems to be involved in autophagy regulation, autophagy itself could also be controlling LRRK2 intracellular protein levels.

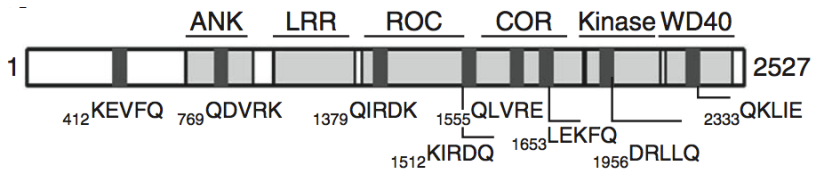


Figure 5: Chaperone mediated autophagy (CMA)-targeting motifs located in the LRRK2 protein (Orenstein et al., 2013). The LRRK2 protein has been reported to contain 8 putative CMA-targeting motifs within its protein sequence, as indicated in the figure.

1.3.5. LRRK2 and endocytosis

Several reports have emerged about LRRK2 involvement in the endocytic-related processes. Firstly, LRRK2 has been shown to localize to endosomic structures such as the endosomes themselves (Schreij et al., 2015), endosomal-autophagic organelles and multivesicular bodies (MVBs) (Alegre-Abarrategui et al., 2009). LRRK2 has also been shown to interact with various proteins that make up the molecular machinery of endosomic processes, such as Rab5b (Yun et al., 2015), Rab7 (Gómez-Suaga et al., 2014), clathrin-light chains and Rac1 (Schreij et al., 2015). In experiments done in neurons, LRRK2 has been shown to be important in synaptic vesicle endocytosis through the regulation of endophilin A in Drosophila (Matta et al., 2012), or

endophilin A1 in mammals (Arranz et al., 2014) by phosphorylation with implications on neurological transmission function.

1.4. NRON complex

As mentioned, LRRK2 is involved in the NFAT pathway, and in this respect Liu et al. (2011) reported that LRRK2 negatively regulates this pathway in collaboration with the Noncoding repressor of NFAT (NRON) complex, which comprises of a Noncoding repressor of NFAT (NRON) and 11 other proteins (CSE1L, KPNB1, TNPO1, EIF3S6, CUL4B, PSMD11, UREB1, DDX3X, IQGAP1, PPP2R1A and SPAG9). Four of these proteins – IQ motif-containing GTPase activating protein 1 (IQGAP1), karyopherin (importin) β 1 (KPNB1), protein phosphatase 2 regulatory subunit A (PPP2R1A) and 26S proteasome non-ATPase regulatory subunit 11 (PSMD11) – when knocked down in cells resulted in NFAT activation, while overexpression led to NFAT suppression instead (Willingham et al., 2005). In T cells, it has been found that NRON and IQGAP are associated with phosphorylated NFAT and calmodulin. NRON is also complexed with inhibitory NFAT kinases casein kinase 1 (CK1), GSK3 and (DYRK). Upon PMA and ionomycin stimulation of T cells, NFAT dissociates from the NRON complex, IQGAP1 and NFAT kinases, and is instead associated with calcineurin and becomes dephosphorylated (Figure 6). It is possible that the NRON complex functions by bringing NFAT close to kinases that maintains it in an inactive state in the cytoplasm, and blocks the interaction between NFAT and calcineurin (Sharma et al., 2011). Given that IQGAP1 is a calmodulin-binding protein, and KPNB1 is a nuclear transport factor (Willingham et al., 2005), the NRON complex could be a ready source of calmodulin and nuclear transport proteins (Sharma et al., 2011). In the study

of Liu et al. (2011), they found that in BMDMs the inhibition of NFAT by LRRK2 is accomplished via the NRON complex. Also that LRRK2 was found to bind 5 of the 11 proteins associated with this complex, and overexpression of LRRK2 enhanced binding of NFAT1 with IQGAP, chromosome segregation 1-like (CSE1L) and transportin-1 (TNPO1). This regulation of NFAT by LRRK2 was found to be independent of its kinase function. Given the importance of the NFAT pathway in the regulation of T cell development and function (as reviewed by Macian (2005)), it was interesting to note that LRRK2 expression is observed to be much lower in T cells than in BMDMs and BMDCs (Liu et al., 2011).

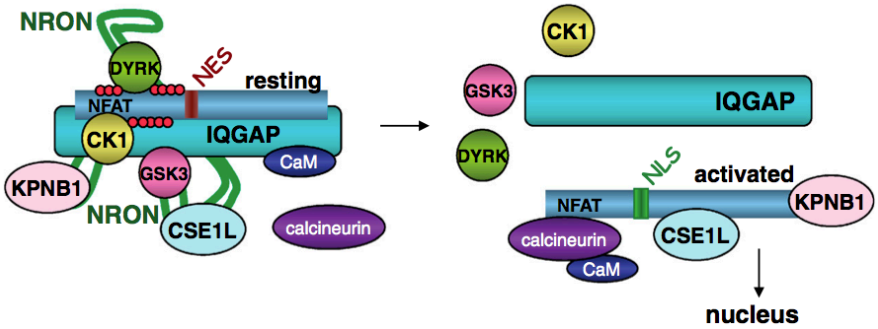


Figure 6: Interaction of the NRON complex with NFAT (Sharma et al., 2011). NRON, IQGAP1, importin and inhibitory kinases associate with NFAT in the cell cytoplasm at resting state (left). When T cells are stimulated, NRON and IQGAP1 dissociate, allowing NFAT kinases to promote NFAT nuclear translocation (right). LRRK2 was reported to interact with components of the NRON complex, including IQGAP, CSE1L and TNPO1. Overexpression of LRRK2 has also been shown to enhance binding of NFAT these proteins (Liu et al., 2011). It is likely that this interaction keeps NFAT nuclear translocation from occurring.

In conclusion, the complexity of the role of LRRK2 in the immune system is only just emerging. While it is starting to be recognized that the dysregulation of the immune system and autophagic processes by LRRK2 mutants could play a role in the pathogenesis of Parkinson's disease, the understanding of the actual physiological role of LRRK2 is poor. Recent studies indicate that LRRK2 is involved in the response to pathogens as LRRK2 expression is altered in response to PAMPs. Also,

given the link between LRRK2, autophagy and endosomes, it could have a role in microbial clearance by autophagy degradation. Furthermore, LRRK2 is implicated very much in the endosomic-autophagic process, and possibly could act as a signaling hub where various pathways converge downstream of PRRs. However how it functions in this context is still not clear. Future studies are needed to dissect the function of LRRK2 in the immune system, which could shed light not only on its role in the defense against pathogens, but also in the general role of LRRK2 in controlling inflammatory responses. These studies could be important to understand if targeting LRRK2 would be a viable therapeutic target for the treatment of both inflammatory, infectious or neurologic diseases.

1.5. Significance of study

A. fumigatus is an ubiquitous environmental fungi that causes life threatening infections in humans of compromised immune status, such as allergic bronchopulmonary aspergillosis and invasive aspergillosis. Invasive aspergillosis in particular causes mortality in 40-90% of cases (Dagenais & Keller, 2009). Given its threat to human health, it is important to understand better the mechanisms behind the immune response to this fungus.

The role of LRRK2 in the immune response to microbes is only just being studied. In light of the recent finding that LRRK2 and the NRON complex are negatively regulating the NFAT transcription factor (Liu et al., 2011), and that LRRK2 levels increase with LPS exposure in microglial cells (Gillardon et al., 2012; Moehle et al., 2012), it will be interesting to see how LRRK2 and NRON complex component levels change in response to fungal stimulation as this is not yet studied in depth. Furthermore, the regulation of the Ca^{2+}-NFAT-IL-2 by LRRK2 and the NRON

complex in DCs and the implications this has on the downstream T cell response in fungal infection has not been explored in detail yet.

Also important to investigate is how LRRK2 levels change with time. As it has been shown that LRRK2 levels increase with time of exposure to LPS, perhaps this increase in LRRK2 levels in longer exposure times could have an effect on autophagy (Moehle et al., 2012) and clearance of pathogens. An increase of LRRK2 levels could also maintain NFAT in its cytoplasmic inactive form, perhaps as a control mechanism to turn down NFAT-cytokine transcription when it is no longer required.

With regards to β-glucan, only Liu et al. (2011) has shown the involvement of LRRK2 in the response to zymosan (a yeast-derived β-glucan) through the NFAT pathway, however their study did not look into autophagy. In addition, while it has been shown that autophagy can be induced by β-glucan (Ma et al., 2012) as well as *Aspergillus fumigatus* (De Luca et al., 2012), these studies do not look at the possible role of LRRK2 in the autophagic process. Hence there remains a gap in knowledge concerning the relationship between β-glucan or fungi, autophagy, and the LRRK2-NFAT pathway. As previous studies suggest that autophagy and LRRK2 could be regulating each other (Alegre-Abarrategui et al., 2009; Gómez-Suaga et al., 2012; Orenstein et al., 2013; Tong et al., 2010), it would be also interesting to see how they interact in the context of fungal infection, and what impact this would have on the Ca^{2+}-NFAT-IL-2 pathway.

Also, it is interesting to note the diverse roles LRRK2 has in signaling, in autophagy and in endosomic processes. A recent report, through *in silico* proteome analysis, has proposed endosomes to serve as a platform where the presence of scaffold proteins mediate the crosstalk of different signaling pathways by recruiting

molecules of these pathways to the endosome. Interestingly, the paper has reported GSK3β, one of the proteins involved in the phosphorylation of NFAT, to be one of these signaling molecules that is able to be recruited to these endosomic scaffolds (Pálfy et al., 2012). This hints at a possible association of the NFAT pathway with endosomes, and it could serve as a way to regulate this pathway.

Chapter 2. Aims

This study explores:

1) The role of LRRK2 and the NRON complex in the NFAT pathway in DCs
2) The possible association of LRRK2 and NFAT with endosomes and lysosomes, and
3) The relationship between the NFAT pathway and autophagy in the immune response to *Aspergillus* in DCs

Chapter 3. Materials and Methods

3.1. Mice

8-week old wild type C57BL/6 mice used for experiments were bred and kept under specific pathogen-free conditions in the Biomedical Resource Centre, Singapore. All experiments and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of A*STAR (Biopolis, Singapore) (Authorization No.: IACUC 110626) in accordance with the guidelines of the Agri-Food and Veterinary Authority (AVA) and the National Advisory Committee for Laboratory Animal Research (NACLAR) of Singapore.

3.2 Expansion of LRRK2$^{-/-}$ bone marrow in mice

Bone marrow harvested from the femurs and tibias of one LRRK2$^{-/-}$ mouse of C57BL/6 background was kindly provided by Prof Zhihua Liu. In order to have enough cells for experiments, the LRRK2$^{-/-}$ bone marrow obtained was expanded by transplantation in wild type C57BL/6 mice for experiments. Prior to bone marrow transplant, recipient mice were lethally radiated with two doses of 6 Gy that were separated by 4 hours before being injected intravenously with 2 to 5×10^6 bone marrow cells in phosphate buffered saline (PBS). Reconstituted mice survived the procedure, and were allowed at least 6 weeks post-transplant for reconstitution to occur before the bone marrow was harvested from their femurs and tibias for DC culture.

3.3. Fungal cells

Aspergillus fumigatus Fresenius, anamorph strain AF293 (MYA-4609, ATCC, Manassas, Virginia, USA) was used for experiments. Conidia were obtained by culturing the fungi in Sabouraud dextrose agar plates. Swollen conidia and hyphae were obtained by allowing conidia to germinate in liquid yeast extract peptone dextrose (YPD) 37°C. In addition, a red fluorescent protein (RFP)-expressing *A. fumigatus* AF293 strain used in this study was obtained from Prof. Eric Pearlman (Case Western Reserve University, Cleaveland, OH, USA).

3.4. Cell culture and stimulation

BMDCs were generated by culturing bone marrow of mice in culture medium containing the cytokine GM-CSF. Bone marrow was extracted by flushing the femurs and tibias of mice with PBS. The collected bone marrow was treated with Ammonium-Chloride-Potassium (ACK) lysing buffer to lyse erythrocytes. The remaining cells post-lysis were cultured in suspension plates with Iscove's Modified Dulbeccos Medium (IMDM) (HyClone, Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing 10% fetal bovine serum (FBS) (Euroclone, Milan, Italy) 2mM L-glutamine (Gibco, Life Technologies, Carlsbad, Carlifornia, USA), 100U/mL Penicillin/Streptomycin (Gibco, Life Technologies, Carlesbad, California, USA), supplemented with 10% supernatant from a GM-CSF-producing B16 melanoma cell line to a final concentration of 20ng/mL GM-CSF to generate BMDCs. Mature BMDCs were harvested after 7 days of culture. Maturation of the DCs were monitored by flow cytometry analysis by staining with fluorochrome-conjugated monoclonal antibodies that are specific for CD11c and MHCII that were purchased from BD Pharmingen (San Jose, CA, USA), eBioscience (San Diego, CA, USA) or

Biolegend (San Diego, CA, USA), examined using the LSR-II (Becton Dickinson, San Jose, CA, USA) and subsequently analyzed using the FlowJo software (Tree Star, Ashland, OR, USA). Cells were considered mature when there was more than 70% $CD11c^{+}MHCII^{+}$ present in culture.

The long term GM-CSF cytokine-dependent DC cell line, D1 (Winzler et al., 1997), was grown in suspension plates with IMDM containing 10% FBS (Australian origin, Gibco, Life Technologies, Carlsbad, California, USA) 2mM L-glutamine, 100U/mL Penicillin/Streptomycin and 55μM β-mercaptoethanol (Gibco, Life Technologies, Carlsbad, CA, USA), supplemented with 30% supernatant from NIH/3T3 cells transfected to produce GM-CSF to a final concentration of 10ng/mL.

In all stimulation experiments, cells were stimulated with *A. fumigatus* in a 1: 10 (fungi:cell) ratio as it was previously determined that the fungal stimulation does not cause significant cytoxicity at this concentration. Prior to stimulation with *A. fumigatus* swollen conidia, cells were harvested and seeded on suspension plate to rest overnight. Pharmaceuticals used in experiments include Cyclosporin A (Cell Signaling Technology, Danvers, MA, USA), FK506 (Cell Signaling Technology, Danvers, MA, USA), 3-methyladenine (Sigma Aldrich, St Louis, MO, USA), Rapamycin (Sigma Aldrich, St Louis, MO, USA), Bafilomycin A1 from *Streptomyces griseus* (Calbiochem, Merck Millipore, Billerica, MA, USA), Cytochalasin D (Sigma Aldrich, St Louis, MO, USA), Leupeptin hemisulfate (Sigma Aldrich, St Louis, MO, USA), and Ammonium chloride (Sigma Aldrich, St Louis, MO, USA). Drugs used at their indicated concentrations have been reported before in literature, and in the laboratory have been shown not to cause cytotoxicity to the cells.

3.5. Intracellular Ca^{2+} mobilization assay

Intracellular Ca^{2+} concentration over time post-stimulation was measured by a Fluo4-NW assay kit. Briefly, D1 (1×10^5 cells/well) were seeded in black 384-well plates from Perkin Elmer and placed in culture at 37 ºC with 5% CO_2 to rest overnight. After resting, cells were incubated for 45 min in darkness with 100 µL Hanks' balanced salt solution (HBSS) containing 20 mM HEPES, 2.5 mM probenecid and Fluo4-NW (Invitrogen, Life Technologies, Carlsbad, CA, USA). Fluorescence was measured with a Victor^4 spectrophotometer (PerkinElmer, Waltham, MA, USA; excitation, 485nm; emission, 535 nm) every 0.5 sec for 80 sec after injection of the stimuli. All experiments were performed at 37 ºC. *F* values were normalized by the first point (*F*0) after the injection of the stimuli, and the percentage (*F*/*F*0*100) is shown.

3.6. NFAT nuclear translocation

D1 NFAT translocation-firefly luciferase reporter cells that were generated in the laboratory were used to assess NFAT nuclear translocation in response to stimulation. These cells were generated by transducing the D1 cells with Cignal Lenti NFAT Reporter with firefly luciferase (SABiosciences, Qiagen, Venlo, Limburg, Netherlands). NFAT nuclear translocation was detected by ONE-Glo™ Luciferase Assay System (Promega, Madison, WI, USA) and the luminescence signal quantified with the GloMax®-Multi Detection System Luminometer module (Promega, Madison, WI, USA).

3.7. Gene knock down by shRNA lentiviral particles

In order to knockdown genes of components of the NRON complex, MISSION® Lentiviral Particles (Sigma Aldrich, St Louis, MO, USA) with the pLKO.1-puro vector containing shRNA sequences targeting NRON, CSE1L, sperm-associated antigen 9 (SPAG9) and PPP2R1A were used. Those targeting CSE1L, SPAG9 and PPP2R1A were commercially available, and two different sequences were purchased for each gene target. The table below details the gene reference identification number (NM ID) and The RNAi Consortium (TRC) clone identification number (clone ID) for the shRNA lentiviral particles targeting these genes (Table 2).

Table 2: Details of commercially purchased MISSION® Lentiviral Particles

Gene target	NM ID	TRC Clone ID
CSE1L	NM_023565	TRCN0000174506 TRCN0000174691
SPAG9	NM_027569	TRCN0000176696 TRCN0000177089
PPP2R1A	NM_016891	TRCN0000012624 TRCN0000012626

shRNA lentiviral particles for targeting NRON were not commercially available, hence two sequences (ACGGTGGGTTTATGACAAATT and ACGGGTGCTGGATGACATATT) were custom designed and packaged into lentiviral particles by Sigma Aldrich (St Louis, MO, USA).

MISSION® pLKO.1-puro Non-Target shRNA Control Transduction Particles (Sigma Aldrich, St Louis, MO, USA) were used as a transduction control.

For the transduction, $1.6x10^4$ D1 cells were seeded and rested overnight in antibiotic-free D1 medium. The next day, the medium was replaced with antibiotic-free D1 medium containing 2μg/mL SureEntry™ transduction reagent (SABiosciences, Qiagen, Venlo, Limburg, Netherlands). The D1 cells were then transduced with MISSION® Lentiviral Particles at multiplicity of infection (MOI) 10

and allowed to incubate for 20 hours at 37°C, 5% CO_2. After 20 hours incubation, the medium containing the lentivirus particles was aspirated out, replaced with complete D1 medium, and allowed to rest for 48 hours at 37°C, 5% CO_2. After being allowed to rest, successfully transduced cells were selected 4 days by replacing the medium with D1 medium containing 0.5µg/mL of puromycin dihydrochloride (Calbiochem, Merck Millipore, Billerica, MA, USA), which was found to be the minimum concentration of puromycin to cause cell death in D1 cells. Cells remaining after the selection were then allowed to re-expand to reach suitable numbers for cryofreezing. For cryofreezing, cells were resuspended in freezing medium (90% v/v FBS, 10% v/v DMSO (Sigma Aldrich, St Louis, MO, USA)), and left overnight in a Mr. Frosty™ freezing container (Thermo Scientific, Waltham, MA, USA) at -80°C prior to long term storage in liquid nitrogen.

Cells were thawed and passaged two times in D1 medium containing 0.5µg/mL of puromycin dihydrochloride prior to stimulation.

3.8. Lysosome enrichment/isolation

Lysosomes were enriched from D1 cells as described by Graham (2001). Briefly, D1 cells were harvested from suspension plates and washed with PBS. The cell pellet was then resuspended in ice cold homogenization medium (0.25M sucrose, 1mM EDTA, 10mM HEPES, pH 7) and homogenized in a Wheaton type Dounce tissue grinder (Wheaton, Millville, NJ, USA) on ice until above 90% cell breakage was observed under the microscope by staining with PBS containing 0.04% v/v Tryphan blue (Sigma Aldrich, St Louis, MO, USA). The homogenate was then centrifuged at 800g for 10 minutes to pellet nuclei and cell debris. The resulting supernatant from the centrifuge was mixed with bovine serum albumin (final

proportion of 4% v/v) and Percoll (final proportion of 22% v/v). The mixture was then ultracentrifuged for 30 minutes at 36 000g without brake activation. After centrifugation, a visible band of the enriched lysosomes can be seen near the bottom of the tube. 400µL fractions, including that containing the enriched lysosomes were collected, and Igepal CA-630 (final proportion of 0.5% v/v) was used to solubilize the lysosome membranes. Solubilized fractions were centrifuged at 100 000g for 2 hours to pellet the Percoll, and the resulting supernatants were obtained for analysis by western blotting.

3.9. Cytokine detection

Cell culture medium was assayed for cytokine production by sandwich enzyme-linked immunosorbent assay (ELISA) for IL-2, IL-12/IL-23p40 and IL-23. The NFAT and NFκB pathway activation downstream of dectin-1 stimulation are known to result in the production of these cytokines (Dennehy et al., 2009; Rogers et al., 2005). While IL-2 is a cytokine more controlled by the NFAT pathway (Goodridge et al., 2007), IL-12/IL-23p40 and IL-23 are cytokines whose production is more influenced by the NFκB pathway (Romani, 2011), hence these cytokines post-stimulation were looked at to see which of these two pathways was more influenced by LRRK2.

IL-2 and IL-12/IL-23p40 were assayed using commercially available antibody pairs and standards from Biolegend (San Diego, CA, USA) and eBioscience (San Diego, CA, USA) respectively. Briefly, cytokine-specific capture antibody was coated in bicarbonate buffer overnight at 4°C in 96-well NUNC MaxiSorp® plates (eBioscience, San Diego, CA, USA). Wells were washed three times with PBS containing 0.05% v/v Tween-20 in between each of the following steps with a NUNC

12-channel Immuno Washer (Thermo Scientific, Waltham, MA, USA). After overnight incubation, well surfaces were blocked for 1 hour with PBS containing 5% w/v bovine serum albumin (Merck Millipore, Darmstadt, Germany). Following blocking, the appropriate cytokine standards and culture supernatants were diluted with the same buffer used for blocking as required and incubated in the wells for 2 hours. Following that, cytokine-specific biotin-labeled antibody was diluted in the blocking buffer and incubated in the wells for 1 hour, after which ExtrAvidin®-Peroxidase (Sigma Aldrich, St Louis, MO, USA) was diluted in blocking buffer and was incubated for 20 minutes. Finally, the ELISA signal was developed using 3,3',5,5'-Tetramethylbenzidine (eBioscience, San Diego, CA, USA) and the developing was stopped by adding 2N sulfuric acid at a 1:1 proportion. Measurements were detected using the TECAN infinite m200 together with the i-control™ software (Maennedorf, Switzerland; measurement wavelength 450nm, reference wavelength 570nm).

IL-23 was assayed using mouse IL-23 ELISA Ready-SET-Go!® (Second generation assay) (Affymetric eBioscience, San Diego, CA, USA) according to manufacturer's instructions. This assay is specific for IL-23 as it uses an IL-23-specific p19 capture antibody, and a detection antibody for the IL-23/IL-23p40 subunit.

In addition, selected supernatant samples were analyzed using the Milliplex Multi Analyte Panels (MAP) Mouse TH17 Magnetic Bead Panel Immunology Multiplex Assay (MTH17MAG-47K) in conjunction with the Luminex MAGPIX® system (Merck Millipore, Billerica, MA, USA). Cytokines in this kit included CD40 Ligand, TNFβ, TNF-α, MIP-3α/CCL20, IL-33, IL-31, IL-28B, IL-27, IL-23, IL-22,

IL-21, IL-17F, IL-17E/IL-25, IL-17A, IL-15, IL-13, IL-12 (p70), IL-10, IL-6, IL-5, IL-4, IL-2, IL-1β, IFN-γ and GM-CSF.

3.10. Western blot

3.10.1. General western blot protocol

A general western blot protocol was used to assay protein expression for the majority of proteins looked at. Briefly, whole cell lysates were obtained by lysing cells in radioimmunoprecipitation assay (RIPA) buffer containing 1x cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland), 1x PhosSTOP Phosphatase Inhibitor Cocktail (Roche, Basel, Switzerland), and 1mM phenylmethanesulfonylfluoride (PMSF). The protein concentration in the cell lysates were measured using the Pierce™ 660nm Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) and colormetric readings were obtained by measuring the wavelength at 660nm using the TECAN infinite m200 together with the i-control™ software (Maennedorf, Switzerland). Protein lysates were diluted with Laemmli buffer containing 2.5% v/v β-mercaptoethanol (Sigma Aldrich, St Louis, MO, USA) and heated at 95°C for 10 minutes prior to separation on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in Tris-glycine-SDS buffer, along side the Precision Plus Protein Dual Color standards protein ladder (Bio-Rad, Hercules, CA, USA). Up to 50μg of protein was loaded into each well for separation. The SDS-PAGE gel was run at 60V till all samples entered the resolving gel, before increasing the running voltage to 110V until the dye front had travelled to the end of the gel. The separated proteins were then transferred to polyvinylidene fluoride (PVDF) membrane in Tris-glycine

buffer containing 10% v/v methanol at either 240mA for 2 hours, or 200mA for 4 hours.

3.10.2 Western blot protocol for LRRK2

LRRK2 is a high molecular weight protein, and on western blot it is predicted to run in the range of 250kDa and above. As the general western blot protocol was found not optimal to assay protein levels of LRRK2 in whole cell lysates, a different approach was adopted. For whole cell lysates analyzed for LRRK2, lysates were first concentrated using the Vivaspin 500 molecular weight cut off (MWCO) 100 000 columns (Satorius, Goettingen, Germany), which enriches the lysates for proteins of higher molecular weight. Despite using these higher MWCO columns to concentrate the cell lysates, the highly expressed 37kDa protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) could still be detected downstream and was used as housekeeping control protein.

Protein concentration of the cell lysates were measured post-concentration using the Pierce™ 660nm Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) as described above. To assay for LRRK2, the NuPAGE® Large Protein Analysis System (Life Technologies, Carlsbad, CA, USA) was adopted. Protein lysates were diluted with NuPAGE® LDS Sample Buffer (Life Technologies, Carlsbad, CA, USA) and heated at 70°C for 10 minutes prior to separation on precast NuPAGE® Novex® 3-8% Tris-Acetate protein gels (Life Technologies, Carlsbad, CA, USA), in NuPAGE® Tris-Acetate SDS Running Buffer (Life Technologies, Carlsbad, CA, USA), alongside the HiMark™ Pre-stained Protein Ladder (Life Technologies,

Carlsbad, CA, USA). A maximum of 10μg of protein was loaded into the wells for separation. The gel was run at 150V until the dye front had travelled to the end of the gel. The separated proteins were then transferred to PVDF membrane in NuPAGE® Transfer Buffer (Life Technologies, Carlsbad, CA, USA) containing 10% v/v methanol at 15V for 18 hours.

PVDF membranes post-transfer were blocked in PBS or Tris-buffered saline containing 0.1% v/v Tween-20, and either 5% w/v non-fat milk or 5% w/v bovine serum albumin (Merck Millipore, Billerica, MA, USA). All antibodies were incubated with this same blocking solution. In between incubation steps, the membranes were washed with PBS or Tris-buffered saline containing 0.1% v/v Tween-20. Chemiluminescence signal was obtained by incubating the membranes for 2 minutes with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA), Western Lightning *Plus* ECL (PerkinElmer, Waltham, MA, USA), or SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA, USA). The luminescent signal was recorded by exposing the blots to Amersham Hyperfilm MP autoradiography film (GE Healthcare, Little Chalfont, UK). After exposure, the films were developed with Carestream® Kodak® autoradiography GBX developer and fixer solutions (Sigma Aldrich, St Louis, MO USA). Films were scanned at 300dpi and band pixel density analyzed from the film scans by ImageJ software (NIH, Bethesda, MD, USA).

Antibodies used for western blot include: LRRK2 Rabbit Monoclonal Antibody Clone: MJFF2 (Epitomics, Abcam, Cambridge, UK), LC3B antibody (Cell Signaling Technology, Danvers, MA, USA), Purified anti-mouse CD107a (LAMP-1) Clone: 1D4B (Biolegend, San Diego, CA, USA), anti-TATA binding protein (TBP)

Clone: 1TBP18 (Abcam, Cambridge, UK), Rab5 (C8B1) Rabbit mAb (Cell Signaling Technology, Danvers, MA, USA), JIP4/SPAG9 (D72F4) XP® Rabbit mAb (Cell Signaling Technology, Danvers, MA, USA), Anti-PPP2R1A antibody [6F9] (Abcam, Cambridge, UK), and GAPDH Antibody, Clone: 6C5 (Merck Millipore, Billerica, MA, USA).

3.11. RNA extraction, real-time quantitative PCR

Cells were lysed with 1mL of TRIzol® reagent (Ambion, Life Technologies, Carlsbad, CA, USA) and flash frozen on dry ice prior to storage at -80°C. To extract RNA, 200µL (20% v/v) of chloroform (Merck, Kenilworth, NJ, USA) was added to each sample. Each sample was then shaken vigorously, and then centrifuged at 15 000rpm, 4°C for 15 minutes. This separated the sample into a top organic phase containing the RNA, a thin interface containing DNA, and a bottom organic phase containing protein. The top supernatant was aspirated and transferred to a new tube. An equal volume of 70% v/v ethanol was added and mixed to the sample. RNA was extracted from this mixture using the RNeasy mini kit (Qiagen, Venlo, Limburg, Netherlands) according to the manufacturer's instructions.

Extracted RNA samples were then treated with DNase using the TURBO DNA-free™ Kit (Ambion, Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. The RNA content of each sample was then quantified using the Nanodrop™ 1000 (Thermo Scientific, Waltham, MA, USA). 2µg of RNA was retro-transcribed into cDNA using the High Capacity Reverse Transcription Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) according to manufacturer's instruction. The obtained cDNA was then used for real-time

quantitative PCR. The Brilliant SYBR® Green QPCR Mastermix and Reference Dye (Stratagene, Agilent Technologies, Santa Clara, CA, USA) was used to set up the real-time quantitative PCR reaction, and the samples were run in the MX3000P Instrument (Stratagene, Agilent Technologies, Santa Clara, CA, USA). Gene expression data was analyzed using the MxPro QPCR software (Stratagene, Agilent Technologies, Santa Clara, CA, USA).

Below is a table of primer sequences targeting mouse genes used in this study (Table 3):

Table 3: List of real-time quantitative PCR primers

Gene	Forward primer (5'-3')	Reverse Primer (5'-3')
CSE1L	GTGGGAAAGGACAGGAAACA	AAACCTTGGTGATCGTTTGC
GAPDH	TTGAGGTCAATGAAGGGGTC	TCGTCCCGTAGACAAAATGG
LRRK2	GATGTCAGTACGCCCCTGAT	CTGCCAGCGCTATGATGTTA
NRON	CAGTAAAGGAGCAGTAGTGGAAACAG	TGGGGGGAGCGAATGGCATCGGGAAC
PPP2R1A	CAACCTGGATTGGTGGAACG	GATCCACTAGCCAGGCCATA
SPAG9	AAACCTCAGGGACTCCAGGT	CCCCACCACTGCTACTTTGT

3.12. Immunofluorescence confocal microscopy

D1 cells were seeded and stimulated in μ-slide 8-well chambers (Ibidi, Martinsried, Germany). Post-stimulation, cells were fixed in 2% paraformaldehyde (Sigma Aldrich, St Louis, Missouri, USA), and permeabilized in PBS containing 0.1% Saponin (Sigma Aldrich, St Louis, MO, USA), 0.2% gelatin (Fluka Analytical, Sigma Aldrich, St Louis, MO, USA) and 5% bovine serum albumin (Merck Millipore, Darmstadt, Germany). Cells were stained and washed using PBS containing 0.01% Saponin and 0.2% gelatin.

In order to understand whether the autophagy induced by *A. fumigatus* was macroautophagy (marked by intracellular LC3 punctae (Mizushima et al., 2010)) or non-canonical autophagy, cells were stained with a primary LC3B antibody (Cell Signaling Technology, Danvers, MA, USA) and a secondary goat α-rabbit IgG

antibody conjugated to Alexa Fluor® 488 (Invitrogen, Life Technologies, Carlsbad, CA, USA). In addition, 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Life Technologies, Carlsbad, CA, USA) was used to stain the cell nuclei. Images were acquired on an Olympus FV1000 confocal microscope in conjunction with the Fluorview FV1000 software (Olympus, Tokyo, Japan).

3.13. Transmission electron microscopy

To visualize subcellular structures formed in response to stimuli, D1 cells were prepared for transmission electron microscopy post-stimulation.

Stimulated D1 cells were harvested from the suspension plate with 2mM EDTA in PBS, and washed with 0.2M sodium cacodylate buffer (pH 7.4). Cells were then fixed in cacodylate fixative buffer (0.1M sodium cacodylate, 2% paraformaldehyde, 3% gluteraldehyde) overnight at 4°C. The cells were then washed with 0.2M sodium cacodylate buffer and dehydrated on an alcohol series (30%, 50%, 70%, 80%, 90% and 100%) for 15 minutes each. Specimens were then embedded into acrylic resin. Ultrafine sections were obtained by cutting into the resin specimens with a glass blade on an ultramicrotome, and mounted on nickel grids. The grids were then washed with PBS, and then stained with antibodies LRRK2 Rabbit Monoclonal Antibody Clone: MJFF2 (Epitomics, Abcam, Cambridge, UK) and Purified anti-mouse CD107a (LAMP-1) Clone: 1D4B (Biolegend, San Diego, CA, USA), followed by secondary antibodies that have been conjugated with either 5nm or 15nm gold particles (Cytodiagnostics, Burlington, ON, Canada). All antibody incubations were done in PBS containing 1% bovine serum albumin. After antibody staining, grids were post-fixed with cacodylate fixative buffer for 15 minutes, and then stained with

2% uranyl acetate. Micrographs were taken with an EM 208 transmission electron microscope (Phillips, Amsterdam, Netherlands).

3.14. Graphs and Statistics

Statistical significance of experiments was determined either by Student's *t*-test one-way ANOVA as indicated. For statistics generated by one-way ANOVA, the differences between individual groups were compared using the Bonferroni's Multiple Comparison post-test. All graphs and statistics were generated using the Graphpad Prism® software version 6.0 (Graphpad Software, La Jolla, CA, USA).

Chapter 4. Results

4.1. *Aspergillus fumigatus* activates the Ca^{2+}-NFAT-IL-2 response in DCs

It has been reported that DCs (D1 cells) exposed to particulate β-glucan, a polysaccharide found in fungal cell walls, show an activation of the Ca^{2+}-NFAT-IL-2 pathway (Fric et al., 2014). The fungus, *A. fumigatus*, also contains β-glucan in its cell wall. However in resting *A. fumigatus* conidia, this β-glucan exposure on the fungal surface is limited as due to the presence an outer crystalline rodlet layer. It is only when the fungus germinates that this rodlet layer is lost, resulting in increased β-glucan exposed on the fungal surface (Dague et al., 2008). As a result, *A. fumigatus* show increasing amounts of β-glucan on their surface as they germinate from conidia, to swollen conidia and lastly to hyphae stage. In line with this, the lab has also shown that NFAT-IL-2 pathway activation is proportionate to the amount of β-glucan by using these different germination stages of *A. fumigatus* (Zelante et al., 2015).

Here, D1 cells were stimulated with *A. fumigatus* swollen conidia. DCs show cytosolic Ca^{2+} influx (Figure 7A), increased NFAT translocation (Figure 7B, 7C) and IL-2 cytokine production (Figure 7D) in response to *Aspergillus* swollen conidia. Calcineurin is a Ca^{2+}-sensitive Ser/Thr phosphatase that functions in the NFAT pathway to activate NFAT for nuclear translocation (Wu et al., 2007). The use of calcineurin inhibitors, Cyclosporin-A and FK506, resulted in a decrease of IL-2 production from D1 cells in response to *Aspergillus* swollen conidia (Figure 7E), showing that this response is indeed dependent on the Ca^{2+}-NFAT-IL-2 pathway.

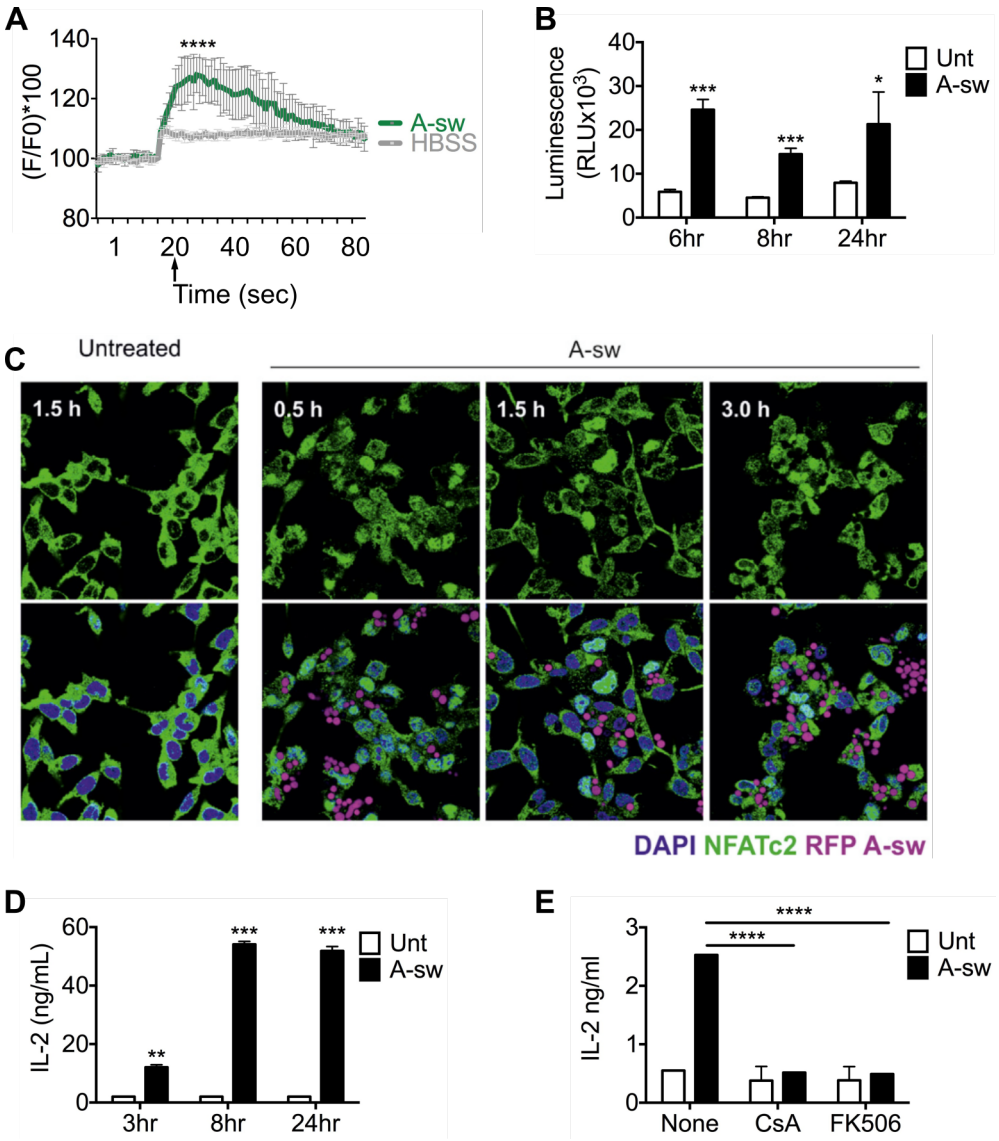


Figure 7: *Aspergillus fumigatus* activates the Ca^{2+}-NFAT-IL-2 response in DCs. (A) Ca^{2+} measurements of D1 cells in response to *A. fumigatus* swollen conidia in comparison to the HBSS control over time. Data is displayed as the mean±SEM of five biological replicates and statistical significance determined by one-way ANOVA with Bonferonni's post-test. **(B)** NFAT translocation as measured by luminescence signal in fungal-stimulated NFAT-luciferase reporter D1 cells. Data is displayed as the mean luminescence signal±SEM of three biological replicates and statistical significance determined by Student's *t*-test. **(C)** Immunofluorescent staining of NFATc2 (green) in D1 cells treated with RFP-expressing *A. fumigatus* swollen conidia as published in Zelante et al. (2015). Magnification is at 100x and is representative of three independent experiments. **(D)** IL-2 production from D1 cells stimulated with *A. fumigatus* swollen conidia. Data is displayed as the mean cytokine concentration±SEM of two biological replicates. **(E)** IL-2 production from D1 cells stimulated for 8 hours with *A. fumigatus* swollen conidia in the presence or absence of calcineurin inhibitors, Cyclosporin A (1µg/mL) and FK506 (0.1µg/mL). Cells were treated with Cyclosporin A and FK506 for 1 hour prior to stimulation. Statistical significance was determined by one-way ANOVA with Bonferroni's post-test. Data is displayed as the mean cytokine concentration±SEM of two biological replicates. Differences found to be statistically significant are indicated (* - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$; **** - $p<0.0001$). Abbreviations used: *A. fumigatus* swollen conidia (A-sw); Cyclosporin A (CsA); Hank's Balanced Salt Solution (HBSS); Untreated (Unt).

4.2. LRRK2 is expressed in DCs and is degraded in response to *Aspergillus fumigatus*

LRRK2 is known to regulate NFAT translocation in response to TLR ligands (Liu et al., 2011). In addition, it has been reported that LRRK2 is expressed in the human $CD11b^+$ DC population (Gardet et al., 2010), as well as in BMDCs (Liu et al., 2011), hence the expression and role of LRRK2 in DCs exposed to *Aspergillus* swollen conidia was studied here in order to better understand any cross-regulation between NFAT-IL-2 axis and LRRK2.

LRRK2 expression at the mRNA and protein level were investigated in D1 cells at resting state as well as in D1 cells stimulated with swollen conidia. It was found that at resting state, DCs expressed LRRK2 on both the mRNA (Figure 8A) and the protein level (Figure 8B). In response to stimulation with swollen conidia, LRRK2 levels in DCs were found to be downregulated on both the mRNA as well as the protein level. This downregulation started to be observed 3 hours post-stimulation on both the mRNA and protein level, however on the mRNA level it was not found to be statistically significant. At 8 hours post-stimulation, downregulation of LRRK2 on the gene level was found to be statistically significant, while LRRK2 protein levels decreased further. The downregulation of LRRK2 was further maintained on protein level at 18 hours, and on gene expression level 24 hours post-stimulation, showing that *Aspergillus* is able to influence LRRK2 gene expression on both transcriptional and translational level.

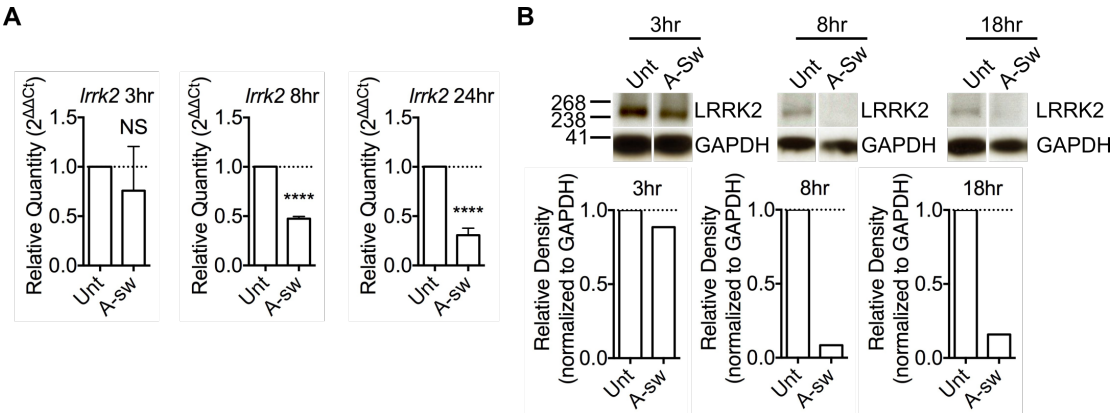


Figure 8: LRRK2 is expressed in DCs and is degraded in response to *Aspergillus fumigatus*. (A) Expression of level of LRRK2 mRNA in D1 cells post-stimulation with *A. fumigatus* swollen conidia. Data is displayed as the mean gene expression±SEM of two biological replicates and normalized to the housekeeping protein, GAPDH. Differences found to be statistically significant by one-way ANOVA with Bonferonni's Multiple Comparison post-test are indicated (NS – non-significant; **** - $p<0.0001$). **(B)** Protein expression level of LRRK2 in whole cell lysates of D1 cells stimulated with *A. fumigatus* swollen conidia. For densitometry analysis, band densities for LRRK2 were normalized to the band density of the housekeeping protein, GAPDH, after which the density LRRK2 band of the treated sample was compared with that of the untreated sample. Data is representative of two independent experiments. Abbreviations used: Untreated (Unt); *A. fumigatus* swollen conidia (A-sw).

4.3. LRRK2 and NFAT are localized on lysosomes and endosomes of DCs

It has been reported that LRRK2 is degraded in lysosomes (Orenstein et al., 2013). In order to understand better the subcellular localization of LRRK2 in DCs until now never described, lysosomes and early endosomes were enriched from D1 cells by gradient centrifugation protocol (adapted from Graham (2001)), and the protein content of the fractions enriched with these organelles were analyzed by western blot. Fractions analyzed were negative for the nuclear marker, TBP, indicating that there was no contamination with nuclear material.

It was found that LRRK2 was present in fractions that also expressed lysosomal-associated membrane protein 1 (LAMP-1) and Rab5, which are markers for lysosomes and early endosomes respectively. This is in line with literature that has reported LRRK2 localization to endosomes and their related structures (Alegre-

Abarrategui et al., 2009; Schreij et al., 2015). NFAT1 was also found in the same fractions as well (Figure 9A).

To investigate the intracellular localization of these proteins in a more specific manner, electron microscopy along with gold-conjugated secondary antibodies were used to visualize their location in DCs. LAMP-1 staining was used to better identify and recognize the morphology and structure of DC lysosomes. By electron microscopy, lysosomes appeared LAMP-1 positive, spherical, enclosed by one membrane, with a diameter of 70-150 nm, homogenous and electron-dense interior. Endosomes appears LAMP-1 negative and poorly electron-dense (Figure 9B). Interestingly, both LRRK2 (Figure 9C) and NFAT1 (Figure 9D) were found localized on endosomic structures.

Interpreting the results from both methodology used, lysosome enrichment showed that LRRK2 and NFAT localize to endosomes and to lysosomes in steady state DCs, while electron microscopy showed that they localize to endosomes. The finding of LRRK2 in lysosomes is consistent with a previous publication that LRRK2 can be taken up into these cellular organelles (Orenstein et al., 2013). In addition, as endosomes have been proposed to be signaling hubs where components of signaling pathways can localize and interact (Pálfy et al., 2012), the finding of LRRK2 and NFAT on endosomes could imply that the endosomes serve to bring these two components of the NFAT signaling pathway together for interaction. Therefore, the compartmentalization in endosomes likely has an important function to regulate the phosphorylation of NFAT and the subsequent translocation and gene transcription in the nucleus.

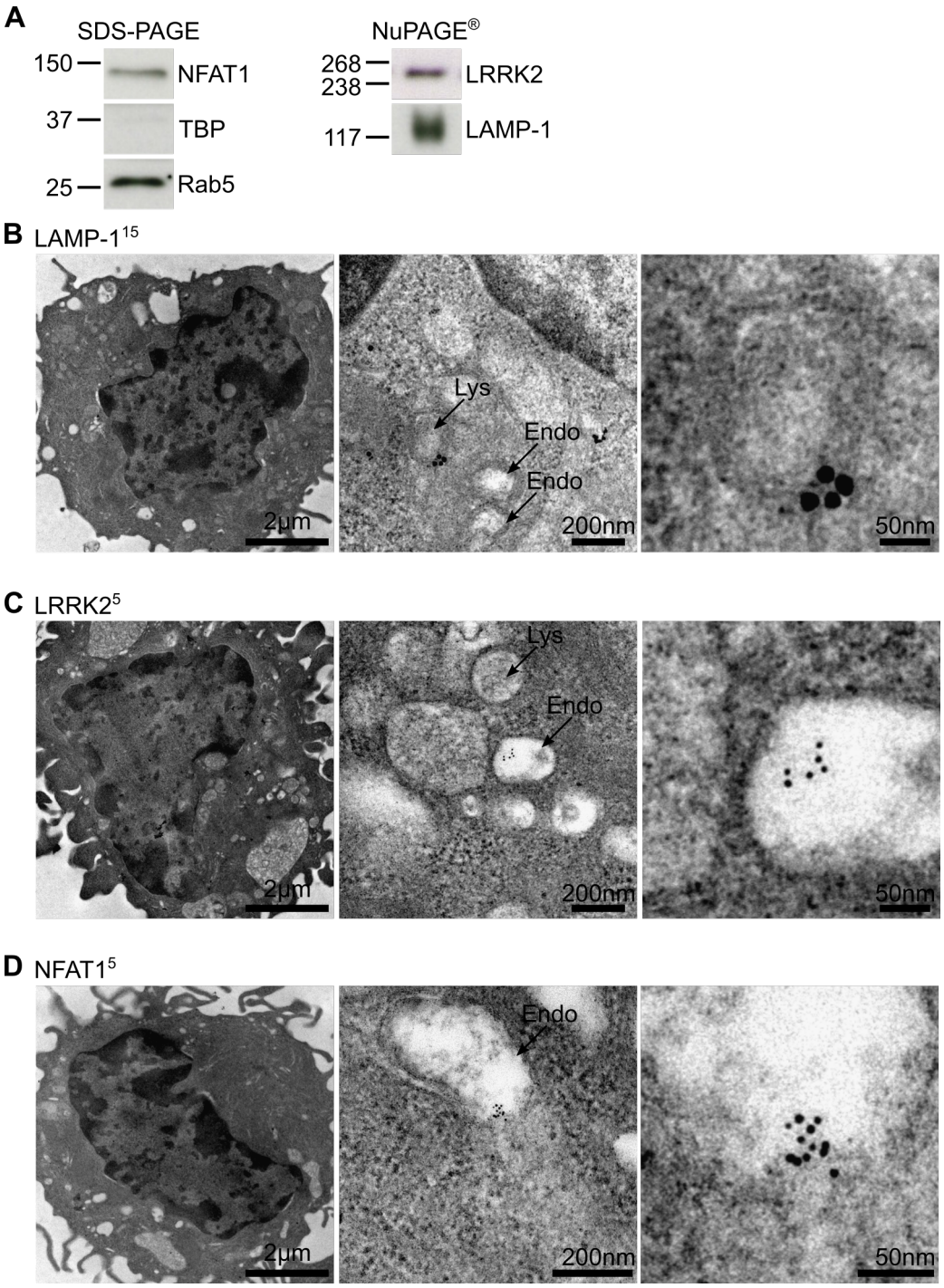


Figure 9: LRRK2 and NFAT are localized on lysosomes and endosomes. (A) Western blot analysis of presence of NFAT, TBP, Rab5, LRRK2 and LAMP-1 in a representative protein fraction obtained from the enrichment of lysosomes from D1 cells. Lysate from the protein fraction was run on both SDS-PAGE and NuPAGE® to probe for the proteins indicated. Data is representative of 3 independent experiments **(B-D)** D1 cells were processed for immune-electron microscopy, stained for LAMP-1, LRRK2 or NFAT1, and labeled with appropriate secondary antibodies conjugated with 5nm or 15nm gold particles (size of particles indicated in superscript). Lysosomic (Lys) and endosomic (Endo) structures are as indicated. Electron micrographs of D1 cells show LAMP-1 positive lysosome **(B)**, as well as endosomic structures positive for LRRK2 **(C)**, NFAT1 **(D)**. Size of scale bars are as indicated.

4.4. *Aspergillus fumigatus* swollen conidia activate the non-canonical autophagic pathway in DCs

Lysosomes are known to be involved in the maturation of autophagic vesicles. Given that autophagy is activated by β-glucan (Ma et al., 2012) and *A. fumigatus* (De Luca et al., 2012), autophagy was investigated in D1 cells stimulated with *Aspergillus* swollen conidia in order to establish a possible role of the autophagic response in the regulation of the NFAT translocation.

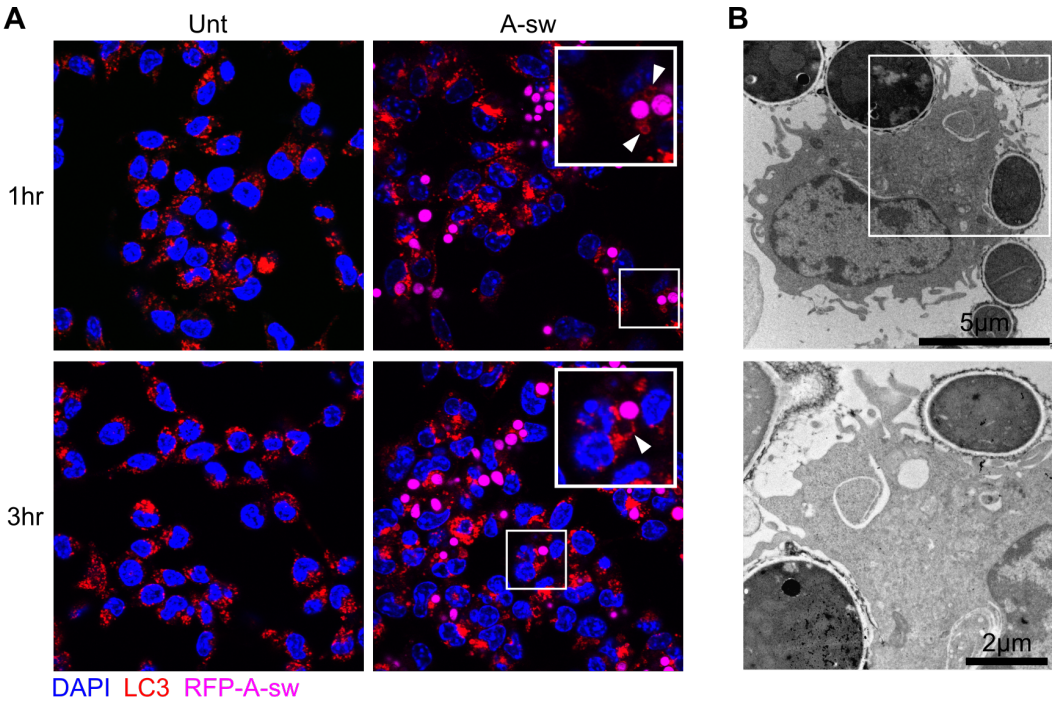
D1 cells stimulated with swollen conidia showed the formation of LC3-positive phagosomes (Figure 10A) similar to that induced by β-glucan (Ma et al., 2012) and *A. fumigatus* (Kyrmizi et al., 2013) in other studies. By electron microscopy, it was determined that the resulting phagosomes were not formed by the double-membranes characteristic of classical autophagy (Figure 10B), which is in line with the findings of Kyrmizi et al. (2013). Taken together, the observations from immunofluorescent staining of LC3 and electron microscopy indicate that the autophagic response induced by *A. fumigatus* in DCs is non-canonical.

To further confirm the finding of *A. fumigatus*-induced non-canonical autophagy, molecular methods were used. During classical autophagy, LC3 conversion from LC3-I to LC3-II, LC3-II turnover, and p62 degradation is observed. This LC3 conversion, LC3-II turnover and p62 degradation can be monitored by western blot (Mizushima et al., 2010). In experiments, *A. fumigatus*-stimulated DCs were observed to have LC3-I to LC3-II conversion and higher LC3-II turnover (Figure 10C). For p62, two protein bands were detected by western blot, of which the higher band corresponded to its expected molecular weight of 60kD and thus was used for densitometry analysis. The lower band ran at around 50kD, and while no other report was found to show this smaller lower band, it might be corresponding the

second smaller isoform of p62 that is missing 38 amino acids as found in the UniProt database (UniProt ID: Q64337). Nevertheless, both bands of p62 were not degraded (Figure 10C). This indicated that while swollen conidia did induce autophagy in DCs, it was not classical autophagy.

In addition, it was found that there was an increase of subcellular multivesicular structures present in DCs stimulated with swollen conidia. By electron microscopy, these structures expressed LAMP-1 as well as LRRK2 and comprised of several multilamellar vesicles, some of which are enclosed within other vesicles (Figure 10D). These structures strongly resemble multilamellar bodies, which are lysosomal organelles comprising of multiple concentric layers of membrane, that have been shown to require autophagy for its formation (Hariri et al., 2000).

Therefore taking into account all observations described thus far, the germinated form of *Aspergillus* is able to trigger both the non-canonical autophagic response in D1 cells, as well as the activation of the NFAT-IL-2 axis. In addition, LRRK2 and NFAT were found on lysosomes and endosomes where LRRK2 could plays the important role of regulating NFAT translocation in to the nucleus.


A
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A-sw
1hr
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DAPI LC3 RFP-A-sw
B
5μm
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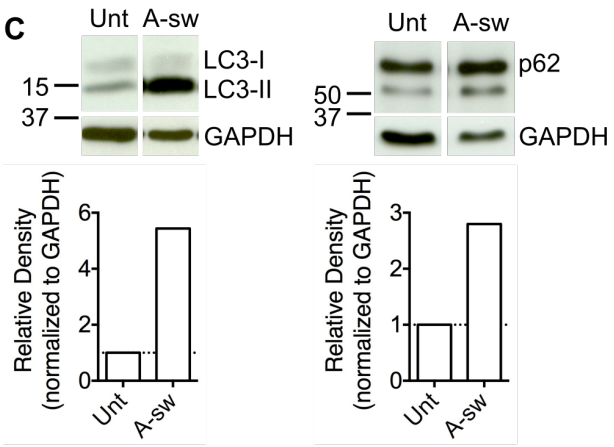
C
Unt A-sw
LC3-I
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GAPDH
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p62
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GAPDH
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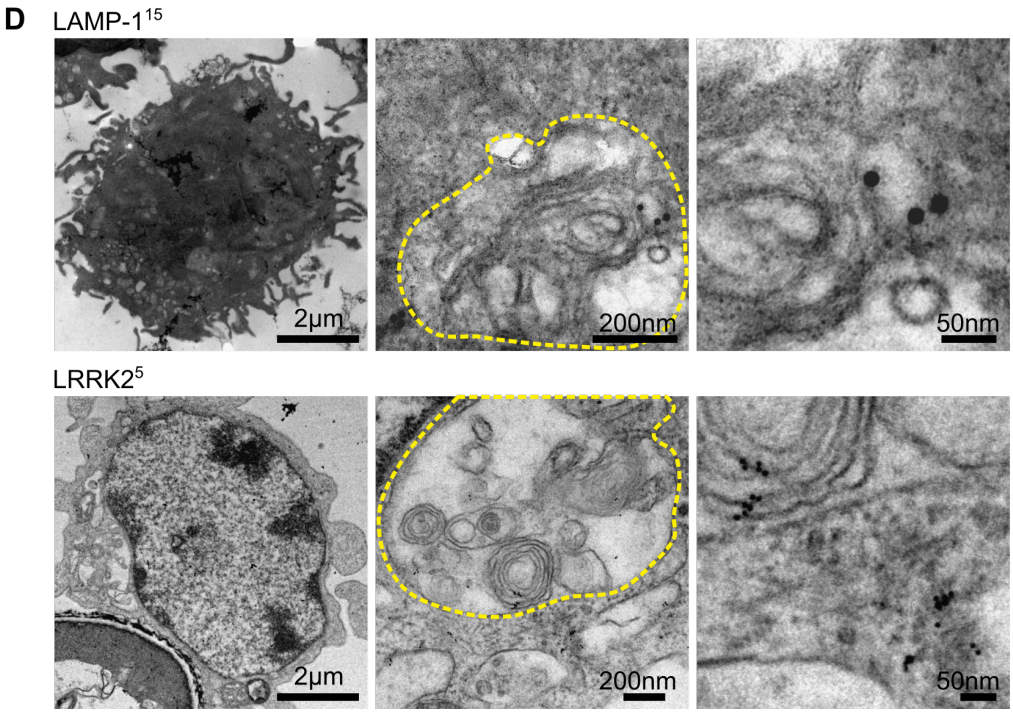

D
LAMP-1[15]
2μm
200nm
50nm
LRRK2[5]
2μm
200nm
50nm

Figure 10: *Aspergillus fumigatus* swollen conidia activate non-canonical autophagy in DCs. (A) Immunofluorescent staining of LC3 (red) in *A. fumigatus* swollen conidia-stimulated D1 cells. Data is representative of two independent experiments. **(B)** Electron micrograph of phagosome formation by DCs incubated for 3 hours with *A. fumigatus* swollen conidia. The bottom panel is zoomed in from the area indicated in the top panel. Data is representative of two independent experiments. **(C)** Expression of LC3 and p62 proteins by western blot in whole cell lysates of D1 cells stimulated for 24 hours with *A. fumigatus* swollen conidia. Densitometry for LC3 was performed on the LC3-II band to measure LC3-II turnover, while densitometry analysis of p62 was done on the upper band corresponding roughly to its expected molecular weight of 60kD. Data is representative of two biological replicates **(D)** Multilamellar bodies (encircled in yellow dashed lines) observed by electron microscopy in *A. fumigatus* swollen conidia-stimulated D1 cells and labeled for LAMP-1 or LRRK2 with 5nm or 15nm gold particles (size of particles indicated in superscript). Cells were incubated with the fungi for 3 hours. Size of scale bars are as indicated. Abbreviations used: Untreated (Unt); *A. fumigatus* swollen conidia (A-sw).

4.5. The Ca^{2+}-NFAT-IL-2 axis is influenced by early autophagic events, phagocytosis and lysosomal maturation, but not by late autophagic events

Based on the previous findings that DCs were able to activate the NFAT-IL-2 axis in response to *Aspergillus* swollen conidia, two types of autophagy inhibitors, 3-methyladenine (3-MA) and Bafilomycin-A, were used to investigate whether this axis is mediated by autophagic response. 3-MA is a PI3 kinase inhibitor. As the initiation of autophagosome formation requires Class III PI3 kinase activity, 3-MA functions by inhibiting autophagy in the early stages of its initiation. Bafilomycin-A, on the other hand, inhibits the last step of autophagosome cargo degradation by preventing the autophagosome-lysosome fusion as well as lysosome acidification (Mizushima et al., 2010). When these two drugs were used in experiments, it was found that the NFAT translocation and IL-2 cytokine production in DCs stimulated with *Aspergillus* swollen conidia was significantly decreased upon inhibition of the early stages of autophagy by 3-MA, but not with an inhibitor of the late stage of autophagy, Bafilomycin (Figure 11A).

In addition, the NFAT pathway in stimulated DCs was also significantly decreased when cells were treated with the phagocytosis inhibitor, Cytochalasin D (Figure 11B), showing that the induction of the NFAT pathway downstream of dectin

is dependent on phagocytosis. DCs were also treated with a combination of Leupeptin and ammonium chloride to inhibit lysosomal maturation by preventing acidification, and it was observed that NFAT translocation increased, while IL-2 production decreased in drug-exposed DCs in response to fungi (Figure 11C).

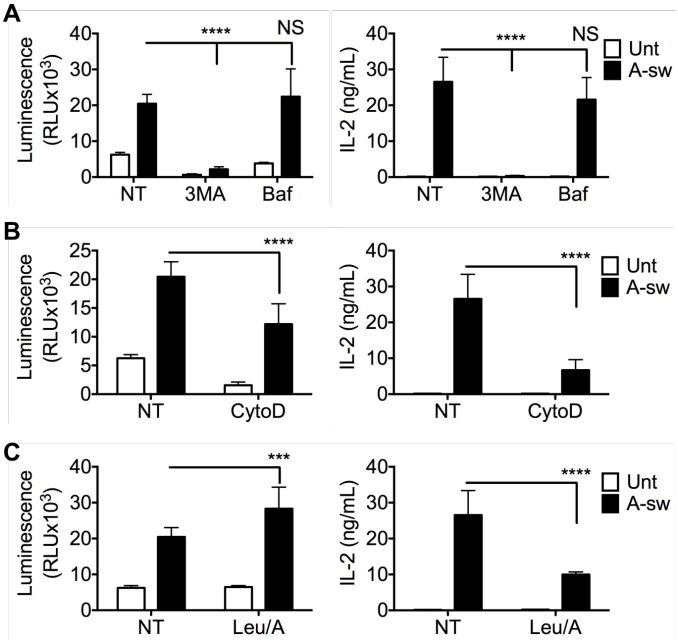


Figure 11: The Ca^{2+}-NFAT-IL-2 axis is influenced by early autophagic events, phagocytosis and lysosomal maturation, but not by late autophagic events. NFAT translocation and IL-2 production of NFAT-luciferase reporter D1 cells that have been stimulated with *A. fumigatus* swollen conidia for 6 hours in the presence and absence of drugs inhibiting specific cellular processes. **(A)** Fungus-stimulated D1 cells in the presence and absence of the type III Phosphatidylinositol 3-kinase inhibitor, 3-methyladenine (10mM), or the vacuolar H+ ATPase inhibitor, Bafilomycin (50nM), which inhibits the early and late stage of autophagy respectively. **(B)** Fungus-stimulated D1 cells in the presence or absence of the phagocytosis inhibitor, Cytochalasin D (2µg/mL). **(C)** D1 cells stimulated with fungi in the presence or absence of the lysosomal acidification inhibiting combination of Leupeptin (100µM) and ammonium chloride (20mM) (Leu/A). Data is displayed as the mean±SEM of five (in the case of Leu/A) or eight biological replicates. Differences found to be statistically significant by one-way ANOVA with Bonferonni's Multiple Comparison post-test are indicated (NS – non-significant; *** - $p<0.001$; **** - $p<0.0001$). Abbreviations used: Untreated (Unt); *A. fumigatus* swollen conidia (A-sw); Vehicle non-treated control (NT); 3-methyladenine (3MA); Bafilomycin (Baf); Cytochalasin D (CytoD), combination of Leupeptin and ammonium chloride (Leu/A).

Together, the results show that early events that occur after fungal stimulation, such as phagocytosis and the early part of the autophagy pathway, are important in the activation of the NFAT pathway, but not late-stage autophagy. The findings that the NFAT-IL-2 pathway is influenced by autophagy are in line with what has been

described here thus far. The induction of non-canonical autophagy as well as the downregulation of LRRK2 expression in *A. fumigatus*-stimulated DCs raises the possibility of autophagy being responsible for the degradation of LRRK2, resulting in the activation of NFAT. This is further supported by the presence of LRRK2 in lysosomic-endosomic structures and multilamellar bodies. Therefore, in the early stages of the non-canonical autophagic response, LRRK2 may probably undergo progressive degradation, and regulate NFAT translocation from the endosomes to the nucleus.

4.6. The absence of LRRK2 in DCs leads to increased IL-2 production in response to *Aspergillus fumigatus*

Results so far show that LRRK2 is expressed in lysosomes and endosomes in DCs, and that the expression was downregulated with *Aspergillus* treatment. Hence whether the absence of LRRK2 could be involved in the regulation of the NFAT-IL-2 response was investigated, since Liu et al. (2011) has reported LRRK2 as a negative regulator of the NFAT pathway. In line with these results, $LRRK2^{-/-}$ BMDCs (Figure 12A) showed a significant increase in IL-2 production when stimulated with *Aspergillus* swollen conidia, while cytokines that were influenced more by the NFκB pathway, IL-12/IL-23p40 and IL-23 (Romani, 2011), did not show significant changes (Figure 12B), showing that LRRK2 regulates the NFAT pathway in response to the fungus *Aspergillus*. Therefore, LRRK2 deficiency may disentangle a possible dysregulation of the NFAT-IL-2 cascade in response to the germinated fungus *Aspergillus*.

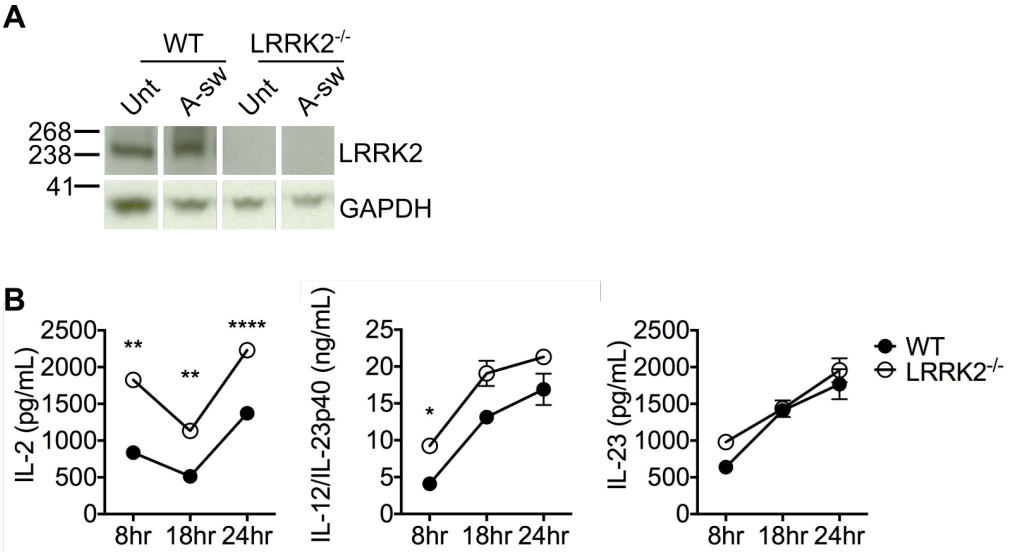


Figure 12: The absence of LRRK2 in DCs leads to increased IL-2 production in response to *Aspergillus fumigatus*. (A) Western blot of LRRK2 of wild type (closed circles) and LRRK2$^{-/-}$ (open circles) BMDCs in response to 3 hours of fungal stimulation with *A. fumigatus* swollen conidia. **(B)** IL-2, IL-12/IL-23p40, and IL-23 cytokine production of wild type (closed circles) and LRRK2$^{-/-}$ (open circles) BMDCs in response to fungal stimulation with *A. fumigatus* swollen conidia. Data is displayed as the mean cytokine concentration±SEM of two biological replicates. Differences found to be statistically significant by one-way ANOVA with Bonferonni's Multiple Comparison post-test are indicated (* - $p<0.05$; ** - $p<0.01$; **** - $p<0.0001$). Abbreviations used: Untreated (Unt); *A. fumigatus* swollen condia (A-sw).

4.7. Expression of NRON complex components in *Aspergillus*-stimulated DCs

Liu et al. (2011) also reported that LRRK2 physically associated with certain components of the NRON complex, and that NRON was important for mediating the regulation of NFAT nuclear translocation by LRRK2, hence the expression of four members of the NRON complex - NRON, PPP2R1A, CSE1L and SPAG9 – were looked at in D1 cells in response to *Aspergillus* swollen conidia. Gene expression analysis shows that components of the NRON complex are expressed in DCs, and could be negatively influenced by fungal exposure (Figure 13A). CSE1L and NRON in particular were found to be consistently downregulated on the gene expression level in DCs exposed to fungi. Expression of PPP2R1A also seemed to be downregulated in DCs upon exposure to *Aspergillus*, but to a lesser magnitude

compared to CSE1L or NRON. On the other hand, SPAG9 gene expression did not show much change in DCs treated with *Aspergillus* with respect to the untreated cells (Figure 13A)**.**

On the protein level, the expression of the NRON complex components, PPP2R1A, CSE1L and SPAG9, could also be detected at basal level. Interestingly, *Aspergillus*-stimulated D1 cells showed marked downregulation of the PPP2R1A protein. This was not seen in the case of CSE1L and SPAG9 (Figure 13B). NRON was not looked at by western blot as it is not a protein. Altogether, this shows that *Aspergillus* by inducing phagocytosis and autophagy may also influence the expression of other components of the NRON complex as LRRK2 in DCs.

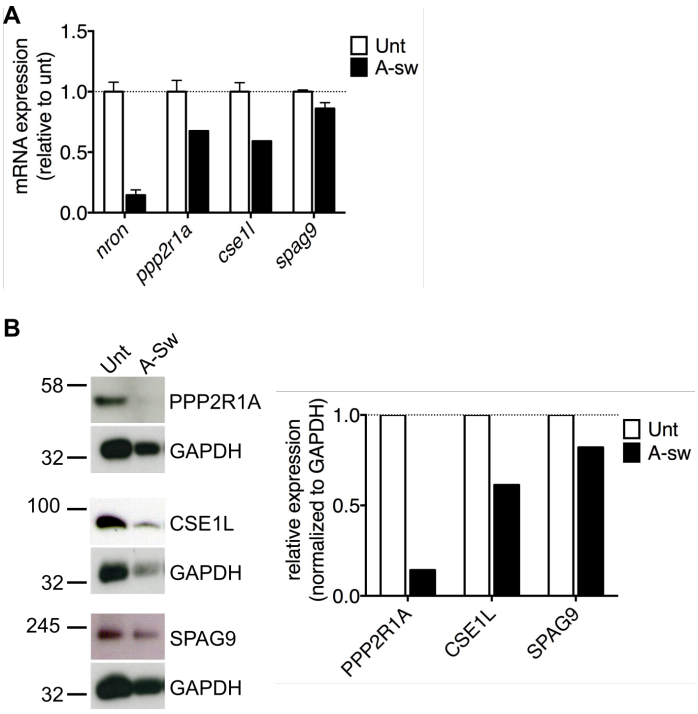


Figure 13: Expression of NRON complex components in *Aspergillus*-stimulated DCs. (A) Gene expression of NRON, PPP2R1A, CSE1L and SPAG9 in D1 cells in response to 8 hour stimulation with *A. fumigatus* swollen conidia. Data is expressed as the mean relative expression±SEM of two technical replicates and normalized to the GAPDH gene expression level. **(B)** Protein expression of PPP2R1A, CSE1L and SPAG9 by western blot in fungal-stimulated D1 cells. Abbreviations used: Untreated (Unt); *A. fumigatus* swollen conidia (A-sw).

4.8. Knock down of components of the NRON complex in DCs

Liu et al. (2011) reported that in addition to the NRON regulatory RNA, LRRK2 was found bound to 5 proteins of the NRON complex (IQGAP, CSE1L, PPP2RA, TNPO1 and PSMD11). In order to investigate the yet unexplored role of the NRON complex in DCs, D1 cells were knocked down for NRON, IQGAP, CSE1L, PPP2R1A (a subunit of the PPP2RA protein), or SPAG9 using shRNA-containing lentiviral particles. The shRNA vector in the lentiviral particles also encode for the mammalian gene for puromycin resistance, hence cells that are successfully transduced will be able to survive when puromycin is used as a selection factor in culture medium. D1 cells were thus selected with puromycin post-tranduction.

Post-silencing, it was observed that the vast majority of D1 cells were sensitive to puromycin, which meant that they are very resistant to the gene silencing by shRNA-containing lentiviral particles and did not transduce successfully. However a small number of cells did survive the puromycin selection. These cells were presumed to be successfully transduced, and thus were expanded in culture. The success of knock down was then assessed for the degree of gene knock down on the mRNA and protein level (Figure 14).

Silencing on the gene level was assessed by qPCR (Figure 14A). From qPCR, it was hard to judge if the knock down of PPP2R1A, CSE1L and SPAG9 were successful as there was little or no change in gene expression levels compared to the cells transduced with control shRNA. For NRON silencing, the last replicate was judged to have the best knock down efficiency, with a knock down of 57% of gene expression level compared with the control transduced cells.

Silencing on the protein expression level was judged by western blot for PPP2R1A, CSE1L and SPAG9 (Figure 14B). NRON silencing cannot be assessed by

western blot as it is a regulatory RNA, not a protein. For PPP2R1A, CSE1L and SPAG9, the last replicates for each gene silencing was judged to have the lowest protein expression compared with the control transduced cells with a knock down of expression between 32-63%, and hence they were selected for further cytokine analysis.

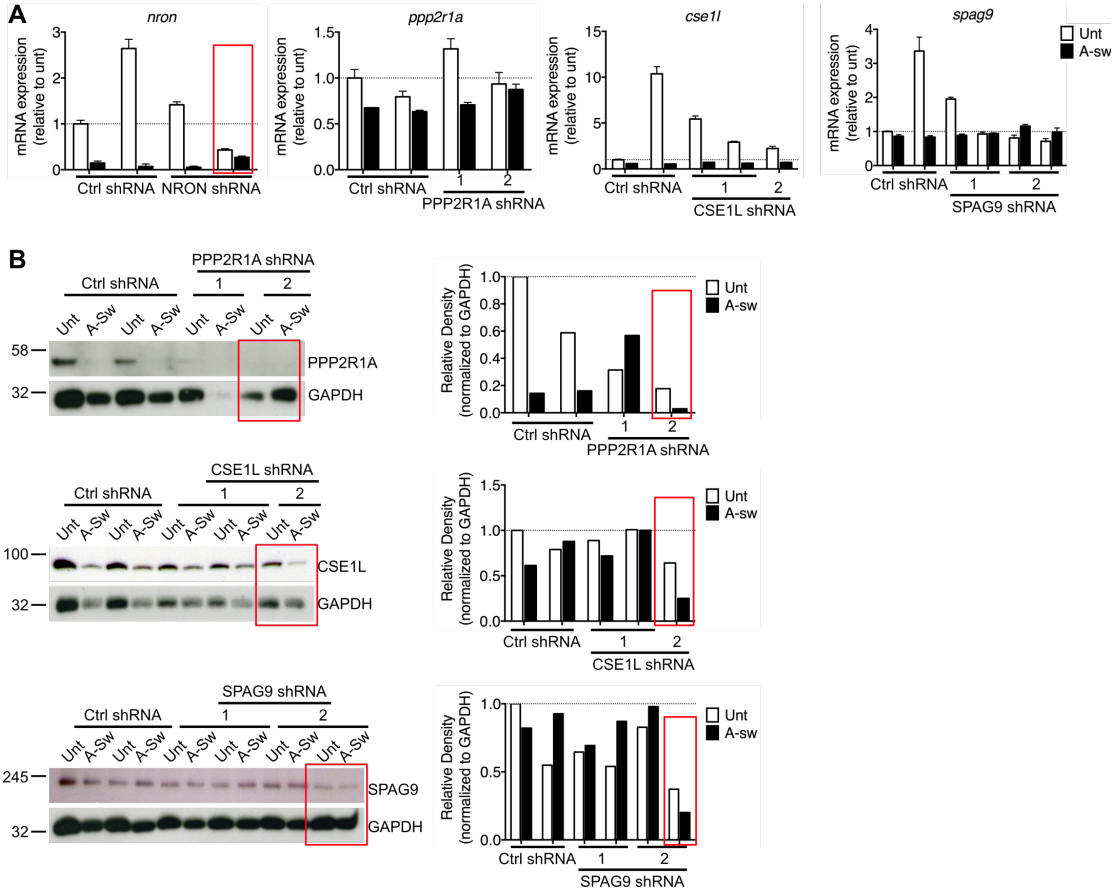


Figure 14: Knock down of components of the NRON complex in DCs. Knock down of NRON, PPP2R1A, CSE1L and SPAG9 in D1 cells were assessed by qPCR and western blot post-selection with 0.5µg/mL of puromycin. **(A)** mRNA expression of NRON, PPP2R1A, CSE1L and SPAG9 in D1 cells tranduced with lentivirus particles containing shRNA targeting the gene indicated, and treated with *A. fumigatus* swollen conidia for 8 hours. Data is expressed as the mean relative expression±SEM of two technical replicates and normalized to the GAPDH gene expression level. **(B)** Protein expression of PPP2R1A, CSE1L and SPAG9 in *A. fumigatus* swollen conidia-treated D1 cells tranduced with lentiviruses particles containing shRNA targeting the gene indicated. One experiment of silencing for each gene was done, and all replicates of the silencing are displayed. Replicates judged to have the most degree of knock down were selected for further analysis (highlighted in red). Arabic numerals refer to the two different shRNA sequences used for the silencing. Abbreviations used: *A. fumigatus* swollen conidia (A-sw); D1 cells transduced with non-targeting control shRNA (Ctrl).

One observation of the data presented in Figure 14 was that the GAPDH band intensity decreased with *A. fumigatus* stimulation compared to the untreated sample despite loading the same amount of protein in the well for western blot. This was not observed in prior experiments that do not involve shRNA silencing, hence the shRNA silencing could have been a causative factor for this observation. Despite this observation, the intensity of the GAPDH band was used to normalize the band intensity of the protein of interest of the same sample.

4.9. Knock down of other components of the NRON complex does not control the NFAT-IL-2 axis as LRRK2 in DCs

The cells assessed to have the most level of knock down through qPCR and western blot were selected for further analysis. Since *Aspergillus*, as shown in Figure 13 and 14, led to a decrease in NRON complex expression, silencing of the NRON components was expected to lead to a dysregulation of the NFAT-IL-2 cascade in response to the fungus *Aspergillus*. Surprisingly, no increases in IL-2 release in response to *Aspergillus* stimulation from silenced D1 cells were observed. Only knocking down of SPAG9 led to a decrease of IL-2 production by DCs, underlining a more prominent role of LRRK2 in regulating the NFAT translocation from the endocytic vesicles to the nucleus (Figure 15). This also may suggest that some components of the NRON complex are indeed positively regulating the NFAT pathway in response to *Aspergillus* in contrast to the negative regulation for LRRK2.

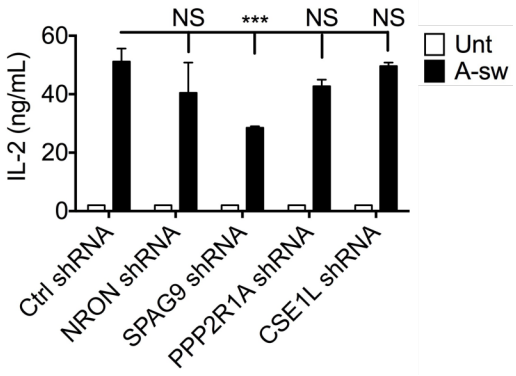


Figure 15: Knock down of other components of the NRON complex does not control the NFAT-IL-2 axis as LRRK2 in DCs. IL-2 cytokine production at 8 hours post-exposure from *A. fumigatus* swollen conidia-stimulated D1 cells that were knocked down for NRON, SPAG9, PPP2R1A or CSE1L through the use of shRNA-lentiviral particles. Data is displayed as the mean cytokine concentration±SEM of two biological replicates. Differences between *Aspergillus*-stimulated cells found to be statistically significant by one-way ANOVA with Bonferonni's Multiple Comparison post-test are indicated (NS – non-significant; *** - $p<0.001$). Abbreviations used: Untreated (Unt); *A. fumigatus* swollen condia (A-sw); D1 cells transduced with non-targeting control shRNA (Ctrl).

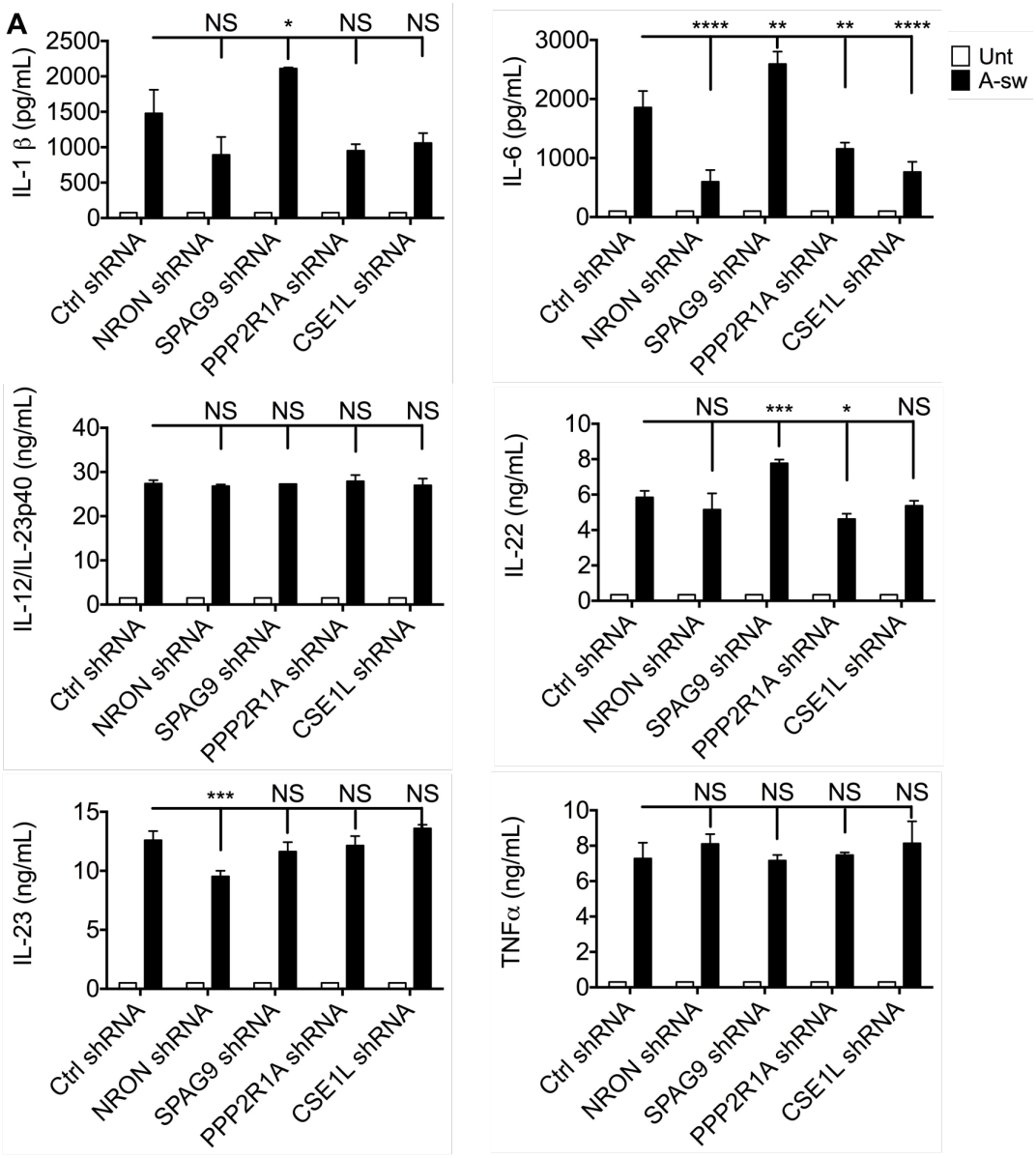
4.10. Individual NRON complex components regulate the cytokine response in *Aspergillus*-treated DCs

The analysis of how the NRON complex components could affect the DC response to fungi was further extended to other cytokines as IL-1β, IL-6, IL12/IL-23p40, IL-22, IL-23 and TNFα (Figure 16).

Of these cytokines, it was found that the production of IL-12/IL-23p40 and TNFα were not affected by the knock down of any of the four NRON complex components. The other cytokines were affected differently depending on which component was knocked down. DCs knocked down for NRON had significantly decreased production of IL-6, and IL-23, but not IL-1β or IL-22. DCs knocked down for SPAG9 had significantly increased IL-1β, IL-6 and IL-22 production. The knock down of PPP2R1A significantly decreased IL-6 production by around 50%, but only slightly decreased IL-22 production. The knock down of CSE1L only led to a significant decrease in IL-6 production, but not other cytokines (Figure 16A). Figure

16B summarizes the main observations of how cytokines produced by *Aspergillus*-stimulated DCs were affected by the knock down of NRON, SPAG9 or PPP2R1A.

This multiple cytokine regulation may underline a possible role of the NRON complex in regulating multiple transcriptional events.



B

	NRON	SPAG9	PPP2R1A
IL-2	-	↓	-
IL-1β	-	↑	-
IL-6	↓	↑	↓
IL-22	-	↑	↓
IL-23	↓	-	-

Figure 16: Individual NRON complex components regulate the cytokine response in *Aspergillus*-treated DCs. (A) Cytokine production at 8 hours post-exposure from *A. fumigatus* swollen conidia-stimulated D1 cells that were knocked down for NRON, SPAG9, PPP2R1A or CSE1L through the use of shRNA-lentiviral particles. Data is displayed as the mean cytokine expression±SEM of two biological replicates. Differences between *Aspergillus*-stimulated cells found to be statistically significant by one-way ANOVA with Bonferonni's Multiple Comparison post-test are indicated (* - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$; **** - $p<0.0001$). **(B)** Table summarizing the main observations of cytokine production in response to fungal stimulation as a result of knocking down the stated genes in D1 in comparison with that of control-shRNA transduced control cells. Abbreviations used: Untreated (Unt); *A. fumigatus* swollen condia (A-sw); D1 cells transduced with non-targeting control shRNA (Ctrl).

Chapter 5. Discussion

LRRK2 was first discovered in 2002 in association with Parkinson's Disease (Funayama et al., 2002), and since then it has been shown to be involved in various signaling pathways and cellular processes. It is also evident that this protein has a role in the immune system. In this respect, it has been shown to be genetically associated with other inflammatory diseases (Barrett et al., 2008; Liu et al., 2011; Törkvist et al., 2010), expressed in various immune cell populations and is modulated in cells in response to inflammation and PAMPs. Literature has shown that LRRK2 is expressed in DCs (Gardet et al., 2010; Liu et al., 2011). Also, LRRK2 was reported to be a negative regulator of the NFAT pathway in BMDMs (Liu et al., 2011), a pathway that is activated downstream of the dectin 1 (Goodridge et al., 2007). However, as of now the role of the LRRK2 protein in DCs and in fungal immunity has not been explored.

In support of observations of Liu et al. (2011), the study here demonstrates that LRRK2 is indeed involved as a negative regulator of the NFAT pathway in the DC response to fungi. Unlike pure β-glucan, fungi are able to bind to both CLRs and TLRs, hence they are capable of inducing both the NFAT and the NFκB pathway. As with the study of Liu et al. (2011) in BMDMs, this study shows that LRRK2 specifically controls the NFAT pathway in DCs, as the absence of LRRK2 only affected IL-2 and not cytokines IL-12/IL-23p40 and IL-23. While this finding is contrary to a previous report that found LRRK2 to be able to activate the NFκB pathway (Gardet et al., 2010), the cells used in this particular study were human embryonic kidney (HEK293T) cells, hence the difference in experimental context could likely account for this contradiction.

The kinetics of LRRK2 expression in *Aspergillus*-stimulated DCs was also looked at, and it was interesting that *Aspergillus* could induce and maintain a decrease

of LRRK2 expression on both the gene and protein level in DCs for as long as 18 hours or 24 hours post-stimulation. The mechanism of this maintained downregulation in the DC response to *Aspergillus* is not explored in this study, however literature offers some clues to explain this observation on the protein level.

Autophagy could be the mechanism responsible for degrading the LRRK2 protein as it has been shown that LRRK2 localizes to lysosomes and can be degraded by CMA in neurons (Orenstein et al., 2013). In a similar way, the present study here shows that LRRK2 is localized to endosomes and lysosomes in steady state DCs, and that *Aspergillus* is able to induce a non-canonical type of autophagy in DCs that is reminiscent of LC3-positive phagosomes, previously reported by Ma et al. (2012), Kyrmizi et al. (2013) and Nicola et al. (2012). Ma et al. (2012) also reports that the recruitment of LC3II to phagosomes was important for fungal antigen processing and presentation, however whether this form of non-canonical autophagy is also responsible for LRRK2 degradation will need to be investigated in future studies. Future possible experiments to delineate if autophagy has an impact on cellular levels could include the use of cells that are knocked out or overexpressing LC3 in experiments to see if this has an influence on LRRK2 levels in the cell.

The use of transmission electron microscopy allowed the observation of LRRK2-positive multilamellar body formation in *Aspergillus*-stimulated DCs, further strengthening the relationship of LRRK2 with lysosomes and autophagy. Multilamellar bodies are reported to be part of the lysosomal pathway and its formation is dependent on autophagy and lysosomal degradation (Hariri et al., 2000), and the localization of LRRK2 to these structures is supported by what has been previously reported in cultured human cells (Alegre-Abarrategui et al., 2009).

Also studied here is the relationship of the endosome-lysosome network with the Ca^{2+}-NFAT-IL-2 pathway. Up till now in literature, the subcellular localization of NFAT in resting cells has only been reported as the cytoplasm. Here in this study, it is demonstrated for the first time that NFAT can also be localized to the endosomes and lysosomes in steady state cells. Endosomes have recently been proposed to be hubs where the interaction between signaling pathways occurs due to the localization of numerous signaling components, including signaling enzymes and transcription factors, as well as scaffolds that can bind and mediate crosstalks between different pathways on endosomal membranes. Interestingly, GSK3-β, a protein involved in phosphorylating NFAT, has also been shown to localize to the endosomal membrane and this is thought to serve to isolate it from interaction with other signaling components (Pálfy et al., 2012). In addition, LRRK2 interaction with various proteins involved in the endocytic network have been recently demonstrated (Gómez-Suaga et al., 2014; Schreij et al., 2015; Yun et al., 2015). Liu et al. (2011) has also reported LRRK2 to be bound to 5 of the 11 proteins of the NRON complex. In view of these reports, the localization of LRRK2 and possibly also other NRON complex components to endosomes and lysosomes at steady state could serve to regulate NFAT, and this is possibly accomplished by keeping NFAT from translocating to the nucleus through sequestering it at the endosomal and lysosomal membrane. Future studies utilizing the electron microscopy in conjunction with antibodies specific for NRON complex components are needed to determine these proteins are able to localize to these cellular structures just as LRRK2 and NFAT.

How the NFAT pathway is influenced by the events following the engagement of dectin 1 by *Aspergillus* in DCs was also investigated here. The dependency of phagocytosis on IL-2 production in response to particulate β-glucan in DCs has been

previously demonstrated (Fric et al., 2014), hence in this study the finding that the NFAT-IL-2 axis in response to *Aspergillus* is also dependent on this cellular process was expected. What was interesting was that early, but not late autophagic events are also required to activate the NFAT-IL-2 pathway. When taken together with previous findings that NFAT nuclear translocation in response to fungi is an early event occurring 0.5 to 3 hours post-stimulation (Zelante et al., 2015), a probable explanation for these findings is that NFAT could be sequestered on early endosomic membranes after dectin 1 engagement, and that by late stages of autophagy that NFAT would have already dissociated from endosomic/lysosomic membranes and hence is not influenced by this phase of the autophagy process. This hypothesis of NFAT association and subsequent dissociation from autophagic membranes should be investigated in future studies perhaps by demonstrating co-localization of NFAT with LC3 at the phagosomes containing fungi with time by the use of confocal microscopy, or by electron microscopy.

Four components, NRON, PPP2R1A, CSE1L and SPAG9, of the NRON complex were also looked at, and it was found that expression levels of NRON and PPP2R1A in particular were downregulated in DCs in response to *Aspergillus* stimulation, just as LRRK2. This is in line with what has been reported that the NRON complex, together with LRRK2, mediates NFAT translocation regulation (Liu et al., 2011) since a downregulation of NRON complex components would likely lead to a dissociation of this regulatory complex. A knock down of these components was carried out to investigate whether other than LRRK2 other components may affect NFAT nuclear translocation in DCs. IL-2 production from *Aspergillus*-stimulated DCs knocked down for any of these components was found not to be significantly affected, or was decreased as in the case when SPAG9 was knocked down. This

indicates that perhaps NRON, PPP2R1A and CSE1L alone are not sufficient to regulate the NFAT-IL-2 axis. SPAG9, on the other hand, individually could be positively regulating the NFAT-IL-2 axis, rather than inhibiting it.

More interestingly, the individual components were regulating DC cytokine response differently from one another, implying that they may not work in concert. While looking at cytokine response alone is not sufficient to conclude specifically, results also indicate that their regulation of fungal immunity in DCs is not confined only to the NFAT pathway. For instance, NRON seems to also positively regulate IL-6 and IL-23 as well, which are cytokines that are produced downstream of the NFκB pathway, contrary to literature indicating that this regulatory RNA does not control this pathway (Willingham et al., 2005). While it is challenging to knock down genes in DCs, it would be beneficial to have cells that are double or triple knocked out for NRON complex components to further delineate the role of the NRON complex in its interaction with LRRK2 and its regulation of the NFAT-IL-2 pathway in response to fungal stimulus.

For SPAG9, CSE1L and PPP2R1A, besides being known to be part of the NRON complex (Liu et al., 2011; Willingham et al., 2005), their reported functions in literature were not originally immunological. SPAG9 has been proposed as a biomarker for diagnosis in carcinoma of the breast (Kanojia et al., 2009), endometrium (Yu et al., 2012), cervix (Garg, Kanojia, Salhan, et al., 2009), thyroid (Garg, Kanojia, Suri, et al., 2009) and colon (Kanojia et al., 2011), and has been proposed to be involved in the tumorogenesis and growth. CSE1L is a nuclear exportin protein that is involved in the cell cycle, and like SPAG9 has also been associated with various carcinomas (as reviewed in Behrens et al. (2003)). PPP2R1A is a subunit of protein phosphatase 2A (PP2A). PP2A has been implicated in meiosis

and mitosis in numerous studies (Hu et al., 2014; Porter et al., 2013) and is currently being explored as a treatment target for pancreatic cancer (Chien et al., 2015). More related to the context of this study, PP2A proteins have been shown to interact with signaling pathways such as the TLR-TRIF signaling (Woo et al., 2012), Ras signaling (Ory et al., 2003) as well as Ca^{2+}/Calmodulin-dependent protein kinase B/Akt (PKB) activation (Fedida-Metula et al., 2012). In relation to neurological disorders PP2A has been suggested as a possible treatment target for neurological disorders Alzheimer's Disease (Sontag & Sontag, 2014) and has been recently implicated in Tau pathology of Parkinson's Disease (Arif et al., 2014). This study hints that the function of SPAG9, CSE1L and PPP2R1A may also be related to the immune response to pathogens. Future focused studies will be required to dissect their role in immunological signaling pathways. Also, while this study was only confined to these components of the NRON complex, the analysis should be expanded to the other members of this complex in future work as it is likely that the other members could also be regulated, and also be involved in controlling the response to *Aspergillus* in DCs.

Lastly, while this study was focused on DCs, macrophages too play a role in the immune response to fungi. Alveolar macrophages present in the lung are the first line of defense against inhaled fungal spores. Like DCs, macrophages are also capable of sensing fungal PAMPs through the presence of PRRs on their surface. In response to dectin engagement, macrophages are capable of coordinating the immune response to fungi by the production of cytokines IL-12, IL-10, TNFα, and macrophage inflammatory protein type 2 (MIP-2) (Segal, 2007). Macrophages also express NFAT, and NFAT has been shown to activate downstream of dectin activation (Goodridge et al., 2007). Given that Liu et al. (2011) have also demonstrated through the use of

LRRK2-deficient mice that the production of IL-6 and IL-12 from macrophages stimulated with zymosan, an extract of the cells wall of yeast that activates both dectin 1 and TLR2, is NFAT-dependent, the role of LRRK2 in the immune response of macrophages to fungi should also be explored.

Autophagy also occurs in macrophages, and in this respect Ma et al. (2012) and Kyrmizi et al. (2013) have both demonstrated that the formation of LC3-positive phagosomes also occur in macrophages incubated with fungi, and that the recruitment of LC3 is important for macrophage signaling and function in response to fungi. In particular, Kyrmizi et al. (2013) show that the recruitment of LC3 is needed for macrophage ROS production and killing of *Aspergillus* spores. It would thus also be interesting to investigate further the role that LRRK2 and autophagy play in macrophage fungal defense.

Chapter 6. Conclusion

To conclude, this study has shown that DCs do indeed express LRRK2 and that it negatively regulates the NFAT pathway activated in response to *Aspergillus*. At resting state in DCs, LRRK2 together with NFAT was observed to localize to endosomes and lysosomes. Upon *Aspergillus* binding to dectin 1 in DCs, non-canonical autophagy as well as multilamellar body formation is triggered. Taking into account the findings of previously published reports, the sequestration of LRRK2 in the multilamellar bodies could lead to the dissociation of the NRON complex. This allows calcineurin, now activated by the Ca^{2+} flux induced by dectin 1 engagement, to dephosphorylate NFAT, allowing NFAT to translocate to the nucleus and to activate gene transcription. At 8 hours post-stimulation, LRRK2 expression in DCs was observed to be reduced, and a possible mechanism for this is through autophagic protein degradation (Figure 17).

Therefore, the role of the NRON complex in immune response of DCs to *Aspergillus* has been shown to be more complex than previously thought, and their interaction with other signaling pathways activated in the immune response will add a new dimension to their currently known cellular functions. Given that DCs have an important role in the immune system as antigen presenting cells and initiating the appropriate adaptive immune response, future *in vivo* studies could elucidate better the immunological function of LRRK2 and the NRON complex eventually in T cell priming. The knowledge obtained from such studies not only sheds light on the control of the NFAT pathway, but also could have implications in other diseases associated with LRRK2 such as Crohn's Disease, IBD and Parkinson's Disease.

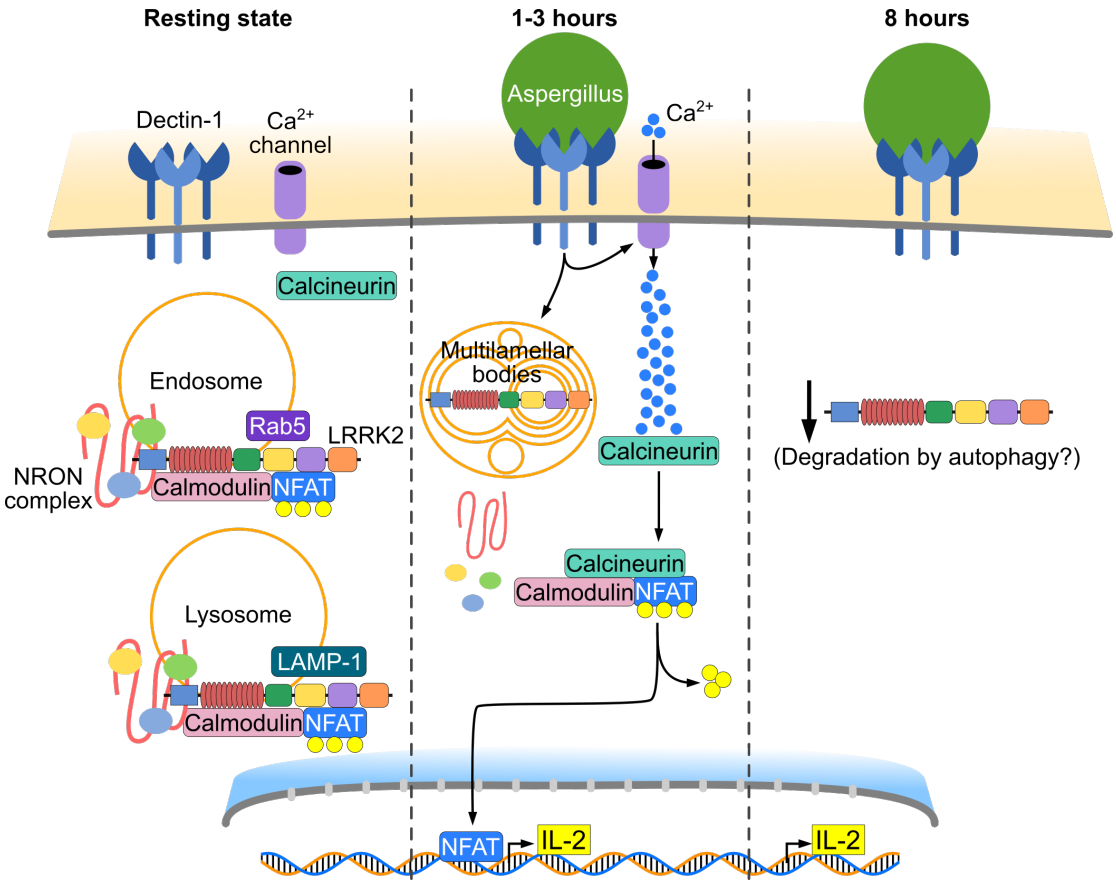


Figure 17: The regulation of the NFAT pathway in DCs is likely accomplished by cellular sequestration and autophagy. This schematic describes a hypothesis to explain the observations of this work. At resting state in DCs, NFAT is localized on endosomes and lysosomes along with LRRK2 and the NRON complex (left panel). When *Aspergillus* engages dectin 1, autophagy and the formation of multilamellar bodies is triggered, sequestering LRRK2 there, and likely inducing a disassembly of the NRON complex. Calcineurin, activated by the Ca^{2+} flux triggered as well by dectin 1 binding to *Aspergillus*, is then able to mediate the dephosphorylation and activation of NFAT (middle panel). After 8 hours post-stimulation, LRRK2 levels in the cell was observed to be decreased, and this possibly occurs through autophagic degradation (right panel).

Chapter 7. Bibliography

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Appendix I – Scientific Publications

Wong A. Y. W., Teresa Zelante, Jan Fric, Tay Hock Soon, and Paola Ricciardi-Castagnoli . (Unpublished manuscript). Control of the Ca^{2+}-NFAT-IL-2 pathway by leucine-rich repeat kinase 2 in *Aspergillus*-stimulated dendritic cells. *Frontiers in Immunology*.

Fric J., Zelante T., Wong A. Y. W., Mencarelli A., Lee B., Poidinger M., & Ricciardi-Castagnoli P. (Submitted, undergoing revisions). Impaired Calcineurin Signaling in Dendritic Cells and Macrophages Increases Susceptibility to Aspergillosis Through Pentraxin-3 Downregulation. *Mucosal Immunology*.

Zelante T., Wong A. Y., Ping T. J., Chen J., Sumatoh H. R., Viganò E., Hong Bing Y., Lee B., Zolezzi F., Fric J., Newell E. W., Mortellaro A., Poidinger M., Puccetti P., & Ricciardi-Castagnoli P. (2015). CD103(+) Dendritic Cells Control Th17 Cell Function in the Lung. *Cell Reports*.

Zelante, T., Fric, J., Wong, A. Y., & Ricciardi-Castagnoli, P. (2012). Interleukin-2 production by dendritic cells and its immuno-regulatory functions. *Frontiers in Immunology*,*3*, 161. doi:10.3389/fimmu.2012.00161

Fric, J., Zelante, T., Wong, A. Y., Mertes, A., Yu, H.-B. B., & Ricciardi-Castagnoli, P. (2012). NFAT control of innate immunity. *Blood*, *120*(7), 1380–9. doi:10.1182/blood-2012-02-404475

Claser C., Malleret B., Gun S., Wong A., Chang Z., Teo P., See P., Howland S., Ginhoux F., & Rénia L. (2011). CD8+ T Cells and IFN-γ Mediate the Time-Dependent Accumulation of Infected Red Blood Cells in Deep Organs during Experimental Cerebral Malaria. *PLoS ONE, 6*(4).

Appendix II - Local and International Scientific Posters and Oral Presentations

18th – 25th January 2015
Gordon Research Seminar (GRS) and Gordon Research Conference (GRC) on Immunology of Fungal Infections (Texas, USA)
Presented a poster and oral presentation entitled:
Control of the Ca^{2+}/Calcineurin/NFAT Pathway by Fungi Through LRRK2 Protein Degradation
Wong Yoke Wei Alicia, Teresa Zelante, Jan Fric, Tay Hock Soon, and Paola Ricciardi-Castagnoli

25th January 2013
NGS L.O.R. (Singapore)
Presented a poster entitled:
Modulation of the LRRK2/NFAT Pathway In Response to *Aspergillus fumigatus* Morphotypes
Wong Yoke Wei Alicia, Teresa Zelante, Jan Fric, Tay Hock Soon, and Paola Ricciardi-Castagnoli

12th-18th January 2013
Gordon Research Seminar (GRS) and Gordon Research Conference (GRC) on Immunology of Fungal Infections (Texas, USA)
Presented a poster entitled:
Modulation of the Immune System in Response to Fungi through NFAT Pathway
Wong Yoke Wei Alicia, Teresa Zelante, Jan Fric, Tay Hock Soon, and Paola Ricciardi-Castagnoli

15th – 22nd April 2012
7th ENII EFIS/EJI Spring School of Advanced Immunology (Sardinia, Italy)
Presented a poster entitled:
IL-2 production by Dendritic Cells: a novel innate cytokine in antimicrobial defense
Teresa Zelante, Jan Fric, Wong Yoke Wei Alicia, Tay Hock Soon, and Paola Ricciardi-Castagnoli.