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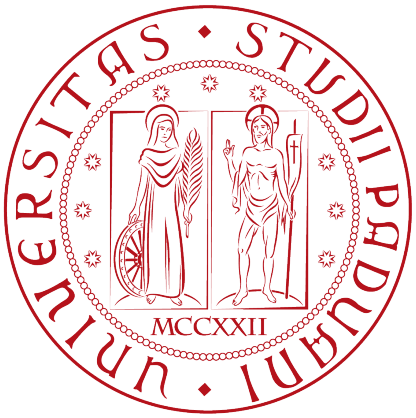

Elaborato di laurea

Interplay between autophagy and the NLRP3 inflammasome in microglia

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Abstract

Microglia are a type of glial cells that protect the nervous system from pathogens and toxic molecules, contributing to the maintenance of the brain's homeostasis. Microglia are part of our innate immune system.
Pathogen infection and misfolded protein aggregates can induce inflammasome activation in microglia. Inflammasomes are multi-protein complexes that assemble to promote the release of pro-inflammatory cytokines, such as IL-1β. Evidence from the literature suggests that inflammasome overactivation contributes to neuroinflammation, which is an established hallmark of neurodegenerative diseases.
Neuroinflammation is an inflammatory response, triggered by reactive microglia, aimed at restoring homeostasis in damaged nervous tissue. However, dysregulation of inflammasome signaling can result in a chronic neuroinflammatory state, with detrimental consequences for the neuronal tissue.
Since inflammasomes are mainly degraded through autophagy, which is known to be downregulated during aging and in neurodegenerative disease, an impairment of this process in microglia may result in a state of chronic neuroinflammation. Therefore, the project aims to investigate the interplay between autophagy and the NLRP3 inflammasome in microglia by monitoring the autophagic flux in the BV2 murine microglial cell line under basal and inflammatory conditions and assessing how impaired autophagy affects the degradation of the NLRP3 inflammasome and cytokines release.

Contents

1 INTRODUCTION

1.1 MICROGLIA

1.1.1 Origin and function

In the central nervous system (CNS) there are two main types of cells: neurons and glial cells. Glial cells in the CNS are divided into astrocytes, oligodendrocytes, and microglia cells. Microglia cells make up 12% of the total population of glial cells in our brain. [1] In the early 1900s, microglia were described for the first time by Pío del Río-Hortega as a new type of phagocytic brain cells with a mesodermal origin. Decades later, microglia were recognized as brain-resident macrophages. However, microglia maintain transcriptional and functional identities that distinguish them from other macrophages. [2] To date, microglia are considered to originate from a pool of macrophages produced during primitive hematopoiesis in the yolk sac. Microglia cells are part of our innate immune system, and thus, represent the first line of defense in our brain. They protect the nervous system from pathogens and toxic molecules, contributing to the maintenance of the brain's homeostasis. Microglia express a series of surface receptors that allow them to detect changes in their environment and react through the release of soluble factors and their phagocytic activity. [3]

1.1.2 Heterogeneity of microglia

Microglia cells are extremely plastic cells, exhibiting a distinct morphology depending on their location within the brain and the activity they are performing. Initially, microglia were considered to exist in two possible states: a "resting" state, characterized by complex ramifications, which is found in homeostatic conditions; and the "activated" microglia with "amoeboid" morphology, observed in diseased tissues. [2] Nowadays microglia are considered the most dynamic cells in the healthy mature brain. With the advancement of omics techniques, the concept of "active" and "resting" microglia became obsolete. These techniques have shown that microglia can exhibit a variety of reactive states.Different microglia states have been observed depending on intrinsic determinates such as species or sex, as well as the context where they inhabit, in particular the CNS region, aging, and pathophysiological context. [3] All these factors contribute to microglia's phenotype at different levels (i.e., epigenomic, transcriptomic, proteomic,) and determine microglial functional properties. For example, CNS development is associated with proliferative-associated microglia (PAM), while different neurodegenerative diseases can exhibit damage-associated microglia (DAMs). [3] These are just two examples, but it is possible to find more microglia reactive states in literature and new ones are continuously being discovered. For these reasons, the scientific community recently agreed to move from a dichotomous M1-M2 view (pro- and anti-inflammatory) to a new view in which multiple reactive states of microglia can coexist at the same time. [3]

1.1.3 Reactive microglia

The brain and spinal cord are considered immune-privileged organs because they are separated from the rest of the body by the blood-brain barrier, which is composed of the thigh junctions of the endothelial cells, the projections of astrocytic cells (called astrocytic peduncles) and pericytes. This barrier prevents peripheral infections to reach the central nervous system. If a toxic agent still manages to breach it, microglial cells quickly react, increasing the inflammatory response, eliminating the toxic agent through phagocytosis, and restoring homeostasis. There are many different factors to which microglia cells can respond, including trauma, cytotoxicity, microorganism infection, heavy metal-related toxicity, and drugs. [1] Aging seems to have a key influence on microglial homeostatic states, as it can lead to a dysregulation of the immune system. [3] Moreover, in aging and neurodegenerative disease, microglia seem to have a reduced ability to respond to perturbations of the brain homeostasis, contributing to CNS dysfunction and disease progression. [3]

1.2 NEUROINFLAMMATION AND THE NLRP3 INFLAMMASOME

1.2.1 Neuroinflammation

Neuroinflammation is defined by four primary features: microglial activation, increased cytokines and chemokines, and local tissue damage. [2] An alteration of homeostasis in the brain leads to subsequent activation of microglia, which is hallmarked by morphological changes, substantial increases in cytokine production, and upregulation of inflammatory pathways such as the NLRP3 inflammasome. [2] Neuroinflammation is an inflammatory response within the brain, triggered by reactive microglia, aimed at restoring homeostasis in damaged nervous tissue. However, dysregulation of inflammasome signaling can result in a chronic neuroinflammatory state, with detrimental consequences for the neuronal tissue. What seems to emerge is a double-edged sword response of neuroinflammation and microglia activation. In fact, acute neuroinflammation leads to the elimination of the stimulus that causes it and consequently, short-term microglia activation seems to be neuroprotective. On the other hand, chronic inflammation and consequent chronic microglial activation seem to be related to neurodegeneration. [2] Evidence from the literature suggests that inflammasome overactivation contributes to chronic neuroinflammation, which is an established hallmark of neurodegenerative diseases. [3]

1.2.2 Inflammasomes

Inflammasomes are multi-protein intracellular signaling complexes that function as intracellular sensors of environmental, metabolic, or cellular stress signals that orchestrate the inflammatory responses of immune cells. An inflammasome is composed by its sensor protein (a pattern recognition receptor, PRR), which oligomerizes to form a pro-caspase-1 activating platform in response to DAMPs or PAMPs. [4] Downstream their activation, the inflammasomes promote the release of pro-inflammatory cytokines, such as IL-1β. In fact, during neuroinflammation, the brain concentrations of IL-1β are much

higher than baseline, highlighting a key role of the inflammasomes in this process. [1]

1.2.3 The NLRP3 Inflammasome

The NLRP3 inflammasome is the most characterized inflammasome member and it is linked to the pathogenesis of inflammatory disease such as Parkinson's disease. [5] This inflammasome consists of the NLRP3 sensor, the ASC signaling adaptor and the effector protease caspase-1. [5] The NLRP3 sensor interact with the apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) to initiate the inflammasome assembly. [4] Then, the inflammasome recruits procaspase-1 through the CARD domain of ASC, and the autocatalytic activation of caspase-1 takes place. Finally, the active caspase-1 cleaves the pro-interleukin-1β (pro-IL-1 β) and pro-IL-18 into their mature and biologically active forms. [5] The activation process of the NLRP3 inflammasome is not completely understood. The widely accepted model involves two signals: priming (signal 1) and activation (signal 2). Microbial components (such as LPS) or endogenous cytokines provide the priming signal, which activates NF-kB, increasing NLRP3 and pro-IL-1β expression. Meanwhile, the activation signal is triggered by factors like ATP, toxins, viruses, or particles. This leads to cellular events such as ionic changes, mitochondrial issues, ROS generation, and lysosomal damage, ultimately activating the NLRP3 inflammasome. [4]

1.2.4 The NLRP3 degradation

The degradation of the NLRP3 inflammasome is an important regulatory mechanism to prevent chronic inflammation. Although the precise degradation mechanism is unknown, evidence in literature suggest that the most validated one is autophagy. [6]

1.3 THE AUTOPHAGIC FLUX

1.3.1 Autophagy

Autophagy is a regulated cellular self-degradation process. Through autophagy, the cell can degrade damaged organelles and misfolded proteins. [7] In addition, it sustains energetic homeostasis by recycling cytosolic components to compensate for the lack of nutrients during starvation. [7] The importance of the autophagy mechanism is underlined by the observation that knockout mice for the autophagy gene ATG-5 do not survive at birth. [7] The dynamic process of autophagy, defined as autophagic flux, is achieved through the generation of autophagosomes and its fusion with lysosomes forming autolysosomes. [7] The phagophores incorporate cytoplasmic cargo during their elongation to form double-membraned vesicles called autophagosomes. After this, the autophagosome fuse with the lysosomes to form acidic autolysosomes where the cargo is degraded. [7] 1.1 The correct function of the autophagy flux is essential for maintaining cellular homeostasis. [6]

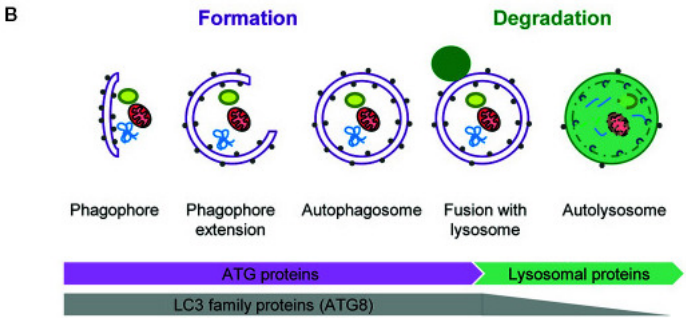

Figure 1.1: Schematic representation of the autophagic flux in microglia

1.3.2 The roles of autophagy in microglia

Autophagy is a very important regulatory process, especially in microglia, as it seems to play a crucial role in regulating their different reactive states. [8] Indeed, the transition from one phenotypic state to another is associated with rapid degradation of the previous proteome, leading to a cellular reprogramming. [8] An impairment of autophagy in microglia can negatively impact brain homeostasis and could contribute to the onset of neurodegenerative diseases, including Parkinson's disease. [6]

1.3.3 Interplay between the autophagic flux and the NLRP3 inflammasome in microglia

Recently, it has been shown that autophagy may be involved in the regulation of inflammatory responses in microglia. [6] Indeed, evidence shows that part of the NLRP3 inflammasome is degraded through the autophagic flux. [6] Moreover, autophagy inhibition in microglia has been shown to aggravate NLRP3-mediated inflammatory responses both in vitro and in vivo. [9] What seems to emerge is that dysregulation of autophagy in microglia may negatively impact innate immune activity and aggravate neuroinflammation. [6] However, it is not completely understood whether inflammasome activation inhibits microglial autophagy or triggers the initiation of autophagy as a negative feedback mechanism, as there are conflicting results in the literature.

1.4 AIM OF THE THESIS

The aim of this project is to investigate the interplay between autophagy and the NLRP3 inflammasome in microglia by monitoring the autophagic flux in the BV2 murine microglial cell line under basal and inflammatory conditions and assessing how inflammasome stimulation impacts the autophagic flux in microglia. Up to date is known that autophagy restricts the inflammasome activity but what could be interesting to understand is whether the activation of the inflammasome increases or inhibits autophagy. In order to do this, we relied on western blot to assess the protein level of the autophagic marker p62 in BV2 murine microglia cells treated with inflammatory stimulation. In the future, this protocol setting could be used on primary microglia with pathogenic mutations linked to autophagy dysfunctions, such as the Parkinson's disease-associated LRRK2-G2019S mutation.

2 MATERIALS AND METHODS

2.1 CELL CULTURE AND CULTURE MEDIUM

2.1.1 BV2 cell line

The murine microglia BV2 cell line is a type of microglia cells derived from the C57/BBL6 mouse strain. They are immortalized cells, meaning that they can divide and replicate indefinitely. These cell lines are commonly used as an alternative model system for primary microglia cultures. BV2 microglia were seeded in a T75 cell culture flask and incubated at 37°C, 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with Fetal Bovine Serum 10% (FBS, Gibco) and 1% penicillin and streptomycin.
Cell culture medium was replaced every 2-3 days and cells were passaged once they reached 80-90% confluence. Briefly, the culture medium was aspirated from the flask and the cell layer was gently washed with a sufficient volume of Phosphatase Buffer Saline (PBS).
Then, 3 mL of trypsin were added and cells were incubated for a couple of minutes at 37°C. Trypsin was inactivated with the addition of 7 mL of cell culture medium. Detached cells were collected, centrifuged for 5 minutes at 400 rcf, and resuspended in 2 mL of culture medium. Before seeding, cell counting was performed with LUNA-II™ Automated Cell Counter (Logos Biosystems). For western blot experiments, $2{,}5 \times 10^5$ cells were plated in each well of an uncoated 24-well plate and allowed to adhere overnight.

2.1.2 Cellular treatments

All cellular treatments were performed under a sterile laminar flow hood The cell culture medium was removed, and wells were rinsed with sterile PBS to remove residual FBS.
For autophagy induction, BV2 microglia were treated with 200nM Torin-1 for 2 h in FBS-free DMEM.
For canonical NLRP3 activation experiments, BV2 microglia were exposed to 100 ng/mL LPS in 245 uL of FBS-free cell culture medium for 3.5 hours. Then, 5 uL of microbial toxin nigericin were added to the medium to a final concentration of 5 µM for 30 minutes.
Control cells were treated with the solvent (vehicle) of LPS and nigericin, which were cell culture water and ethanol, respectively.
In order to understand the net effect on the autophagic flux in the different experimental conditions, all the treatments were performed either in the presence or absence of 50 mM chloroquine (lysosomal inhibitor).
2.1

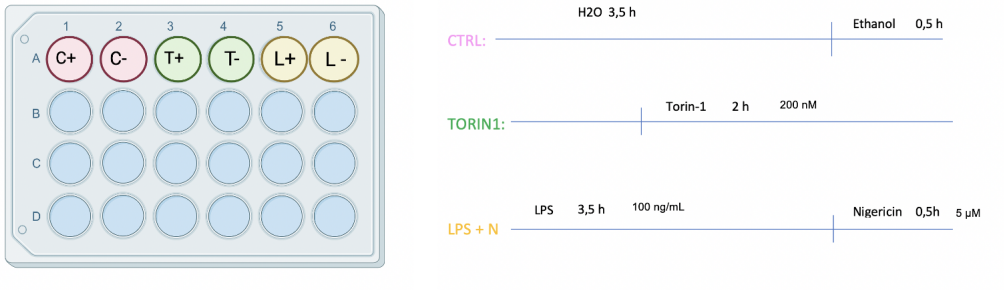

Figure 2.1: Schematic representation of cell treatments and timing.
Legend: **C+**: Control + chloroquine; **C-**: Control; **T+**: Torin-1 + chloroquine; **T-**: Torin-1; **L+**: LPS + Nigericin + chloroquine; **L-**: LPS + Nigericin

2.2 CELL LYSIS

Immediately after cell treatments (4h in total) BV2 microglia were lysed. The cell culture medium was removed, and cells were rinsed once with PBS.
An appropriate volume of RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% Sodium deoxycholate, 2.5 mM 30 sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, deionized water) enriched with 1X protease inhibitors cocktail (Sigma Aldrich), was added to each well, and lysis was performed using a cell scraper on the multi-well plate on ice.
The lysates were collected and transferred in 1.5 mL Eppendorf tubes and kept in ice for 30 minutes. Then, samples were centrifuged at max speed for 30 min at 4°C. The pellet containing remaining cell debris was discarded and supernatants were collected in a new tube and stored at -80°C until use.

2.3 PROTEIN QUANTIFICATION: BCA

Protein concentration quantification was performed using Pierce® BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Protein samples were diluted with RIPA buffer to the desired concentration and an appropriate amount of SDS sample buffer 4X (SB 4X, 200mM TRIS pH 6.8, 8% SDS, 400mM DDT, 0,4% bromophenol blue, 40% glycerol) was added to each sample.

2.4 IMMUNOBLOTTING (Western blot)

Soluble proteins from cell lysates were fractionated by SDS-PAGE. Several trials with different settings were performed and will be illustrated in the results section.
The best results were achieved using a pre-casted gel with a gradient concentration of 4-20% of acrylamide (GenScript), in combination with fresh Tris-MOPS running buffer (Genscript). Polyacrylamide Gels were subjected to

electrophoresis at 100V for the first 10 minutes and then at 150 V for 1 hour and 40 minutes, until the 10 kDa band was lost.
10 uL of Molecular weight markers (Biorad, #1610374), were loaded in at least one lane for each gel in order to infer the molecular weight of sample proteins. The use of fresh Tris-MOPS and a high amount of molecular weight markers were critical to stop the electrophoretic run at the right time and achieve optimal results.
Proteins were transferred to polyvinylidene fluoride (PVDF) membranes in semi-dry conditions using the Trans-Blot® TurboTM Transfer System (Bio-Rad) with the 1X Transfer Buffer (5X Bio-Rad Transfer Buffer, 20% ethanol, and deionized water), at 25V for 20 minutes (High Molecular Weight protocol). PVDF membranes were activated in methanol before use.
PVDF membranes were incubated for one hour in agitation in blocking solution (5% skimmed milk in Tris-Tween Buffered Saline, TBST).
Then, the membranes were cut and incubated overnight with the primary antibodies diluted in blocking solution to the appropriate concentration.
The following primary antibodies were used:

- Anti-p62 (1:1000, rabbit, abcam, ab109012);
- Anti-LC3B (1:1000, rabbit, Novus Biologicals, NB1002220);
- Anti-beta-actin (1:10000, mouse, sigma, A1978).

The following day, membranes were washed three times for a minimum of 30 minutes in TBST and incubated with appropriate HRP-conjugated secondary antibody for 1 hour. Membranes were washed three more times for a minimum 30 minutes in TBST to remove excess secondary antibodies.
Finally, immobilon® Forte Western HRP Substrate (Millipore) was applied to the membranes for one minute and HRP signal was visualized using VWR® Imager Chemi Premium.

2.5 DATA ANALYSIS

Densitometry analysis was performed using the Fiji software. Autophagic marker levels were normalized to the housekeeping actin protein levels. Graphs were generated using GraphPad Prism 8.0.2 software. Quantitative data are expressed as mean ± SEM (standard error of the mean).

3 RESULTS AND DISCUSSION

3.1 Inflammasome activation inhibits autophagic degradation of p62

In literature it is known that inflammasomes are mainly degraded through autophagy, but whether inflammatory stimulations also trigger autophagy as a self-regulating mechanism is currently debated. To understand this, we have treated BV2 microglia cells with the NLRP3 inflammasome activators (LPS + nigericin) and looked at autophagy markers, such as p62, to understand if the inflammasome activation inhibits or triggers autophagy in microglia.

Assessing autophagy is complicated and up-to-date guidelines recommend using complementary tools at the same time.
The gold standard method consists in the detection of LC3-II and p62 protein levels by western blot. [7] However, during this project, we faced a technical issue, as LC3-I and LC3-II bands separation was problematic in microglia cells. Therefore, we relied on the lysosomal inhibitor chloroquine for a better interpretation of the p62 results. Autophagy ends with the degradation of the autophagosome content in the lysosomes. Chloroquine inhibits the autophagic flux by preventing the fusion of autophagosomes with lysosomes. The result of using chloroquine is the accumulation of autophagosomes that would have progressed through the pathway during that period. [10] At the same time using chloroquine we excepted an accumulation of p62 that is not more degraded by the autophagic flux.

p62, is an autophagic cargo at the level of the fusion of autophagosome with the lysosome and it is considering a marker of autophagic flux at lysosomal degradation level. [10]
The role of p62 in autophagy is to deliver ubiquitinated cargos for autophagic degradation. Consequently, given it is a substrate during the autophagic degradation process, p62 undergoes degradation through autophagy. [7]
In literature, evidence show that activating autophagy leads to p62 degradation, meanwhile the pharmacological and genetic inhibition of autophagy increase p62 levels in various cell lines, tissues, and species. [7]

However, the expression of p62 alone is insufficient to comprehensively describe the direction of the autophagic flux, therefore our preliminary result should be evaluated carefully and validated using complementary readouts.

Firstly, we have treated BV2 microglia cells with inflammasome activators (LPS + nigericin) or appropriate vehicle as control and looked at the p62 autophagy marker to understand if the inflammasome activation inhibits or increase autophagy in microglia. Additionally, Torin1, a selective ATP-competitive inhibitor of mTORC1 was employed it as a positive control for autophagy in-

duction, as in literature it is shown to induce autophagy with great efficacy. [7] If inflammasome activation induces autophagy, a degradation of p62 is to be expected, as it is in the positive control with Torin-1.

Moreover, all three conditions (control, LPS+ Nigericin, Torin-1) were performed in the presence or absence of a lysosomal inhibitor: chloroquine. This because we find in literature that autophagosome degradation largely depends on lysosomal proteins and enzymes. [10] In the presence of chloroquine we have the inhibition of lysosomal degradation and the lysosomal cargo, such as p62, start to accumulate. This permits to have a better interpretation of the results.

What emerges from our experiment is that p62 protein levels are reduced with Torin-1 treatment and increased with LPS + Nigericin treatment compared to base condition. 3.1
Coherently with the literature, the presence of Torin-1 stimulated autophagy in BV2 microglia, as shown by the reduction of p62 (normalized to actin protein levels) compared to the control condition. 3.1
On the other hand, LPS + Nigericin (LPS+N) treatment resulted in an increase of p62 protein levels compared to control. 3.1
These preliminary results suggest that NLRP3 activation leads to the inhibition of the autophagic flux in microglia, as shown by the high level of p62 with LPS+N treatment.

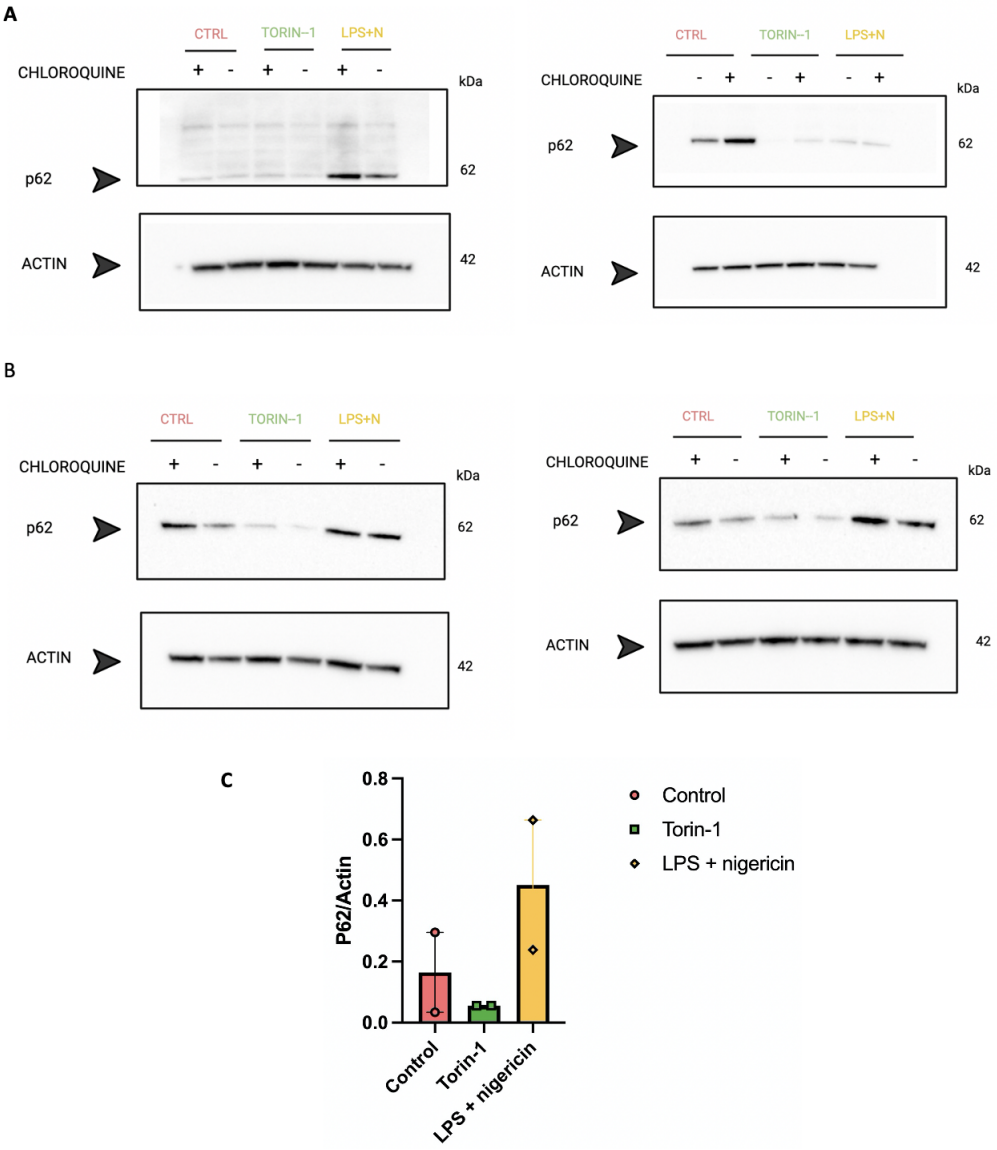

Figure 3.1: **(A)** Western blot of the first round of experiments. BV2 microglia were treated with either LPS + nigericin, their respective vehicles, or Torin1 both in presence and absence of chloroquine. Samples were loaded in 2 separate gels, analyzed by densitometric analysis and the results were averaged.
(B) Western blot of the second round of experiments. BV2 microglia were treated with either LPS + nigericin, their respective vehicles, or Torin1 both in presence and absence of chloroquine. Samples were loaded in 2 separate gels, analyzed by densitometric analysis and the results were normalized and averaged.
(C) Level of expression of p62/actin under three different conditions: Control, Torin-1 and LPS+ nigericin; each point in the graph represents the average of two technical replicates obtained in a total of two independent experiments (n=2). P62 expression, quantified by densitometric analysis, was normalized on actin protein levels.

3.2 Inhibition of lysosomal degradation increases p62 levels in basal condition and LPS+Nigericin-treated BV2 cells

Assessing autophagy is complicated and up to date guidelines recommend using complementary tools at the same time. [10]
Since the separation of LC3-II bands was incomplete, we have analysed the expressions of p62 in the presence/absence of chloroquine (CQ), a lysosomal inhibitor. [10]
Lysosomal inhibition affects autophagic flux by preventing the fusion of autophagosomes with lysosomes, resulting in the accumulation of autophagosomes that would have progressed through the pathway during that period. Considering that inflammasome activation condition (LPS + Nigericin), if the levels of p62 expression in absence of chloroquine are similar to the one of the control condition in presence of chloroquine we could deduce that inflammasome stimulation can suppress autophagy and lead to the accumulation of p62 in a similar way as the lysosomal inhibition.
However, this setting does not permit us to understand in which exact stage there is the impairment of the autophagic flux (i.e, autophagosome formation or fusion with lysosome). In future, this could be investigated analyzing the expression of LC3-II by Western blot.

What have seen is that in the control condition we have more p62 in the presence of chloroquine, as expected. 3.2
Interestingly, comparing the basal condition in presence of chloroquine (Control +CQ) with the LPS + Nigericin in absence of chloroquine (LPS+ nigericin CQ-), the values are similar, suggesting that LPS + nigericin could bring to an inhibition of the autophagic flux in a similar way as the lysosomal degradation inhibitor.

However, it is not clear why in the p62 level with Torin-1 treatment are similar in both the conditions (CQ+ and CQ-), as inhibition of lysosomal degradation in presence of an autophagy inducer should result in an accumulation of the p62 protein. Possibly, the autophagy inducer kinetics exceeded those of chloroquine, resulting in a fast p62 degradation before the lysosomal inhibition took place. Further experiments with chloroquine pre-treatment before Torin-1 administration should clarify this point.

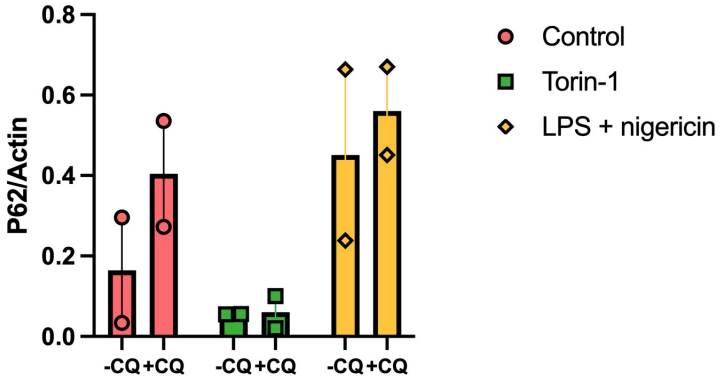

Figure 3.2: Levels of expression of p62/actin under six different conditions: Control, Torin-1 and LPS+ nigericin both the absence or presence of chloroquine. Each point in the graph represents the average of two technical replicates obtained in a total of two independent experiments (n=2).

These preliminary results suggest that NLRP3 activation leads to the inhibition of the autophagic flux in microglia, as shown by the high level of p62 with LPS+N treatment.
The inhibition of autophagy could serve to prevent premature degradation of the inflammasome in the presence of inflammatory stimuli. In fact, the acute inflammation is considered beneficial. In this case, autophagy may be disinhibited when the inflammatory stimulus is no longer present, thus allowing the degradation of the inflammasome and the resolution of the inflammatory response.
However, if autophagy is not disinhibited, failure in degrading the inflammasome could lead to a chronic state of inflammation.
Therefore, in the future, it would be interesting to assess whether the autophagic flux is disinhibited when the inflammatory stimulus is interrupted. To this aim, the cell lysis could be performed after different time point such as 6h, 12h, 24h. These results could better clarify interplay between the inflammasome and the autophagic flux.
In order to validate our interpretation of these results, other read out may be used. For example, monitoring p62 mRNA levels can be used to dissect whether p62 accumulation is truly caused by failure of its degradation or by an increased transcription and expression.
It would also be interesting to investigate the LC3-II expression in the experimental conditions that we have conducted.
LC3-II residing on the autophagosome inner membrane is degraded within the autolysosome. For this reason, the levels of LC3-II are correlated with the steady-state number of autophagosomes. [7]
The measurement of LC3-II levels by Western blot in the presence/absence of a lysosome inhibitor could permit also to underline in which stage of the autophagic flux there could be an impairment.

4 CONCLUSIONS

In conclusion, the results obtained suggest that LPS+ Nigericin condition might inhibit the autophagic flux. These preliminary results may be confirmed by further optimizing the protocol to assess the expression of LC3-II under the same conditions. This could permit also to understand in which exact step of the autophagic flux (i.e. autophagosome formation or lysosomal degradation) are inhibited during inflammatory conditions.

The more suitable interpretation of our results is that the inflammasome activation inhibits autophagy in order to prevent its premature degradation in the presence of the stimulation.
An important point to underline is that we performed the cell lysis immediately after the treatments (4h). Future experiments will aim to determine how long after the inflammatory stimulation, the autophagic flux is disinhibited to allow the degradation of NLRP3 and the resolution of the inflammatory response.
This could permit to better elucidate the discrepancies found in literature about the interplay between inflammation and autophagy.
Once the correct time point has been identified, it will be interesting to see whether mutations that hinder autophagy cause an impairment of NLRP3 degradation at this time point.

The complex interplay between inflammation and autophagy may be particularly relevant in the context of neurodegenerative diseases, as they are associated with both neuroinflammation and autophagy downregulation. For example, the Parkinson's disease-associated LRRK2-G2019S mutation correlated with both enhanced level of pro-inflammatory cytokines released from microglia and an impaired autophagy. The enhanced inflammatory response mediated by mutant LRRK2 may be related to a dysregulation of the autophagic flux. Thus, the experimental setup provided with this thesis may be useful to test this hypothesis in primary microglia harboring this pathogenic mutation and could highlight molecular pathway underlying Parkinson's disease.

Bibliography

[1] M. L. Block, L. Zecca, and J. S. Hong, "Microglia-mediated neurotoxicity: Uncovering the molecular mechanisms," pp. 57–69, 1 2007.

[2] S. C. Woodburn, J. L. Bollinger, and E. S. Wohleb, "The semantics of microglia activation: neuroinflammation, homeostasis, and stress," 12 2021.

[3] R. C. Paolicelli, A. Sierra, B. Stevens, M. E. Tremblay, A. Aguzzi, B. Ajami, I. Amit, E. Audinat, I. Bechmann, M. Bennett, F. Bennett, A. Bessis, K. Biber, S. Bilbo, M. Blurton-Jones, E. Boddeke, D. Brites, B. Brône, G. C. Brown, O. Butovsky, M. J. Carson, B. Castellano, M. Colonna, S. A. Cowley, C. Cunningham, D. Davalos, P. L. D. Jager, B. de Strooper, A. Denes, B. J. Eggen, U. Eyo, E. Galea, S. Garel, F. Ginhoux, C. K. Glass, O. Gokce, D. Gomez-Nicola, B. González, S. Gordon, M. B. Graeber, A. D. Greenhalgh, P. Gressens, M. Greter, D. H. Gutmann, C. Haass, M. T. Heneka, F. L. Heppner, S. Hong, D. A. Hume, S. Jung, H. Kettenmann, J. Kipnis, R. Koyama, G. Lemke, M. Lynch, A. Majewska, M. Malcangio, T. Malm, R. Mancuso, T. Masuda, M. Matteoli, B. W. McColl, V. E. Miron, A. V. Molofsky, M. Monje, E. Mracsko, A. Nadjar, J. J. Neher, U. Neniskyte, H. Neumann, M. Noda, B. Peng, F. Peri, V. H. Perry, P. G. Popovich, C. Pridans, J. Priller, M. Prinz, D. Ragozzino, R. M. Ransohoff, M. W. Salter, A. Schaefer, D. P. Schafer, M. Schwartz, M. Simons, C. J. Smith, W. J. Streit, T. L. Tay, L. H. Tsai, A. Verkhratsky, R. von Bernhardi, H. Wake, V. Wittamer, S. A. Wolf, L. J. Wu, and T. Wyss-Coray, "Microglia states and nomenclature: A field at its crossroads," pp. 3458–3483, 11 2022.

[4] N. Kelley, D. Jeltema, Y. Duan, and Y. He, "The nlrp3 inflammasome: An overview of mechanisms of activation and regulation," 7 2019.

[5] S. Jewell, A. M. Herath, and R. Gordon, "Inflammasome activation in parkinson's disease," pp. S113–S128, 7 2022.

[6] R. Zhu, Y. Luo, S. Li, and Z. Wang, "The role of microglial autophagy in parkinson's disease," 11 2022.

[7] S. Sarkar, "Regulation of autophagy by mtor-dependent and mtor-independent pathways: Autophagy dysfunction in neurodegenerative diseases and therapeutic application of autophagy enhancers," *Biochemical Society Transactions*, vol. 41, pp. 1103–1130, 10 2013.

[8] S. G. Zubova, I. I. Suvorova, and M. N. Karpenko, "Macrophage and microglia polarization: Focus on autophagy-dependent reprogramming," 2022.

[9] Y. Qin, J. Qiu, P. Wang, J. Liu, Y. Zhao, F. Jiang, and H. Lou, "Impaired autophagy in microglia aggravates dopaminergic neurodegeneration by regulating nlrp3 inflammasome activation in experimental models of parkinson's disease," *Brain, Behavior, and Immunity*, vol. 91, pp. 324–338, 1 2021.

[10] A. Plaza-Zabala, V. Sierra-Torre, and A. Sierra, "Assessing autophagy in microglia: A two-step model to determine autophagosome formation, degradation, and net turnover," *Frontiers in Immunology*, vol. 11, 1 2021.

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