Master Thesis

Identification of the effect of IFNα on the mitophagy pathway and the autoreactive phenotype of Peripheral Blood Monocytes in the context of SLE

Διερεύνηση της επίδρασης της Ιντερφερόνης-α στο μονοπάτι μιτοφαγίας και στον αυτοδραστικό φαινότυπο των Μονοκυττάρων Περιφερικού Αίματος στο πλαίσιο του ΣΛΕ

Graduate Program: The Molecular Basis of Human Disease
Laboratory of Autoimmunity and Inflammation
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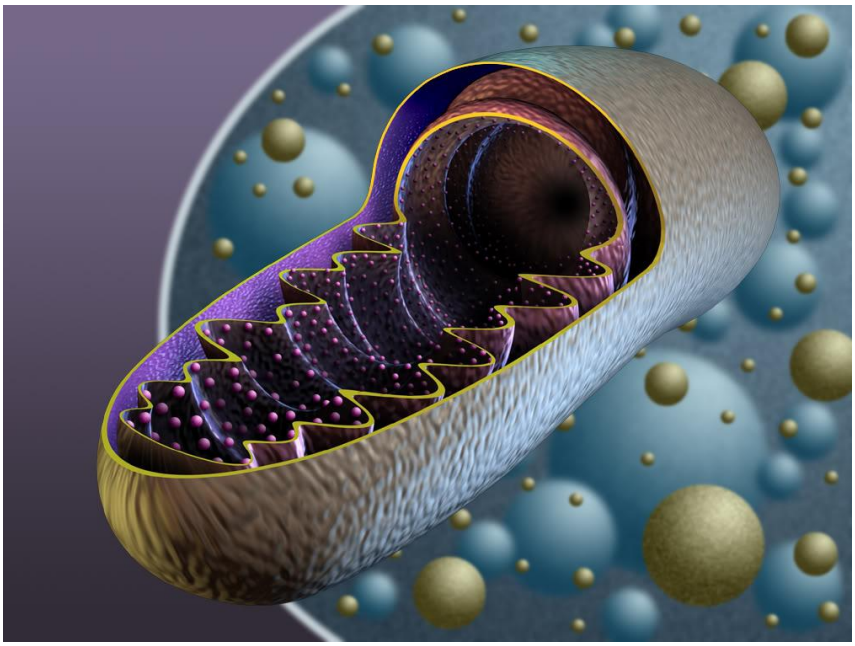

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Ι) Περίληψη

Ο Συστηματικός Ερυθηματώδης Λύκος (ΣΛΕ) είναι μία χρόνια αυτοάνοση διαταραχή, η οποία επηρεάζει κατά κύριο λόγο τα θηλυκά άτομα και στην οποία η απώλεια ανοχής σε νουκλεϊκά οξέα (όπως και στις πρωτεΐνες που αλληλεπιδρούν με αυτά) οδηγεί στην παραγωγή παθογονικών αυτό-αντισωμάτων που κατ' επέκταση προκαλούν φλεγμονή και ιστική βλάβη. Ο παθογονικός ρόλος των ιντερφερονών τύπου Ι στον ΣΛΕ υποστηρίζεται από μία 'υπογραφή' γονιδίων επαγόμενα από ιντερφερόνη-α στα κύτταρα του ανοσοποιητικού συστήματος που κυκλοφορούν στο περιφερικό αίμα των ενεργών ασθενών με ΣΛΕ, καθώς επίσης και από τα υψηλά επίπεδα ιντερφερόνης-α στον ορό ασθενών, γεγονός που αντικατοπτρίζει τόσο την ενεργότητα όσο και τη σοβαρότητα της ασθένειας. Τα αυτό-αντιδραστικά μονοκύτταρα στο ΣΛΕ (η πρωτότυπη αυτοάνοση ασθένεια στον άνθρωπο) χαρακτηρίζονται από τον επαγόμενο από ιντερφερόνη-α φαινότυπο δενδριτικών κυττάρων κατά τη διαφοροποίησής τους. Επιπρόσθετα, έχει δειχτεί ότι η αντιγονοπαρουσίαση αυτοαντιγόνων από πρωτεΐνες του κύριου συμπλόκου ιστοσυμβατότητας τύπου ΙΙ εξαρτάται από την κυτταρική μακρο-αυτοφαγία. Πιο συγκεκριμένα, η αυτοφαγία είναι μία κυτταρική καταβολική διαδικασία που περιλαμβάνει τη διάσπαση περιττών ή μη λειτουργικών συστατικών του κυττάρου και βασίζεται στη συνεργασία των αυτοφαγοσωμάτων με τα λυσοσώματα. Επιπρόσθετα, η μιτοφαγία είναι μία ειδική κατηγορία της αυτοφαγίας, υπεύθυνη για την επιλεκτική απομάκρυνση των περιττών ή ελαττωματικών μιτοχονδρίων του κυττάρου. Κατά τη διάρκεια αυτής της μελέτης θα εξεταστεί η πιθανή σύνδεση μεταξύ της επαγόμενης από ιντερφερόνη-α σηματοδότησης και ρύθμισης της μιτοφαγίας στα μονοκύτταρα του ΣΛΕ και πρόκειται να διερευνηθεί η πιθανή επιρροή αυτής στον αυτοδραστικό φαινότυπο των μονοκυττάρων και στη παρεμπόδιση της απομάκρυνσης/καθαρισμού συστατικών του εαυτού. Ειδικότερα, θα πραγματοποιηθεί προσπάθεια ανίχνευσης της προέλευσης των αυτοαντιγόνων δίκλωνου DNA –χαρακτηριστικό του ΣΛΕ στον άνθρωπο- και παράλληλα διάσπασης ενός από τους μηχανισμούς που πιθανόν να οδηγούν στη δημιουργία μιας δεξαμενής ανοσογονικών αυτοαντιγόνων αλλά και στην επιτυχημένη παρουσίασή τους. Συνεπώς, η παρούσα μελέτη αποσκοπεί στην εστίαση για πρώτη φορά σε ένα συμβιωτικό αλλά ταυτόχρονα και ανοσογονικό προκαρυωτικό νουκλεικό οξύ, το μιτοχονδριακό DNA.

II) Abstract

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder, predominantly affecting females in which loss of tolerance to nucleic acids (as well as their interacting proteins) results in the production of pathogenic autoantibodies that cause inflammation and tissue damage. The pathogenic role of type I IFNs in SLE is supported by a signature of IFNa-induced genes in circulating immune cells of the peripheral blood of active SLE patients and from high IFNa serum levels in these patients, which reflects to disease activity and severity. Autoreactive monocytes in SLE, the prototype autoimmune disease in humans, are characterized by IFNa-DC phenotype and their differentiation and self-antigen presentation by major histocompatibility class (MHC-) II proteins has been shown to depend on cellular macroautophagy. Autophagy is a cellular catabolic process that involves degradation of unnecessary or dysfunctional cellular components and relies on the cooperation of autophagosomes with lysosomes. Furthermore, mitophagy is a selective form of autophagy, responsible for the removal of excess or defective mitochondria of the cell. During this project we will examine a possible link between IFNa signaling and mitophagy regulation in SLE monocytes and investigate its potent impact on the autoreactive phenotype of monocytes and the disturbance of self content clearance. Specifically, we will try to find the origin of dsDNA autoantigens –characteristic of human SLE- and simultaneously dissect one of the mechanisms that may result in the generation of an immunogenic pool of self antigens and their successful presentation. Therefore, the present study aims to focus for the very first time on a symbiotic but at the same time immunogenic prokaryotic nucleic acid, mitochondrial DNA.

III) Introduction

A) Innate and adaptive immunity

Consisting of both humoral and cellular components, the immune system is a remarkably adaptive biological system having evolved in vertebrates to provide protection against evading pathogens. In order to be effective, it is divided into two major domains that act in a highly interactive and cooperative way, the innate immune system (innate immunity) and the adaptive immune system (adaptive immunity) [**1**].

The innate immune system refers to the non-specific defense of vertebrates and consists of anatomic and physiologic barriers (skin, mucous membranes, temperature, pH and chemical mediators) as well as phagocytic cells (blood monocytes, neutrophils and tissue macrophages), which conduct the ingestion of pathogens. This process is of utmost importance and provides the first line of defense immediately after a pathogen enters the host's organism. Normally, most of the pathogens are cleared by this system before they activate the adaptive immune system. However, if the pathogen manages to escape from these disease-resistant mechanisms, the specific response of the adaptive immune system is mounted. In this case, lymphocytes (B and T cells) are activated by antigen-presenting cells, which display parts of the antigens on MHC class (Major Histocompatibility Complex) II molecules on their cell surface. After a crosstalk among activated lymphocytes, specific antibodies are produced by differentiated B cells (plasma cells) and specific cytotoxic responses from T lymphocytes begin with the ultimate aim to eliminate the foreign invader. Except for these responses, memory B cells are generated to sustain immunological memory for this pathogen [**1**] [**Fig.A1].**

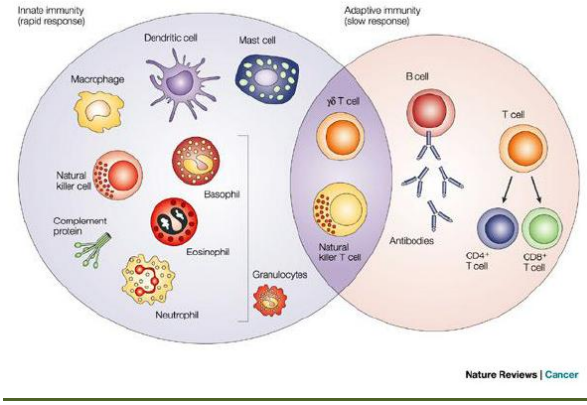


Figure A1: The innate and adaptive immune response. Dranoff et al., 2004

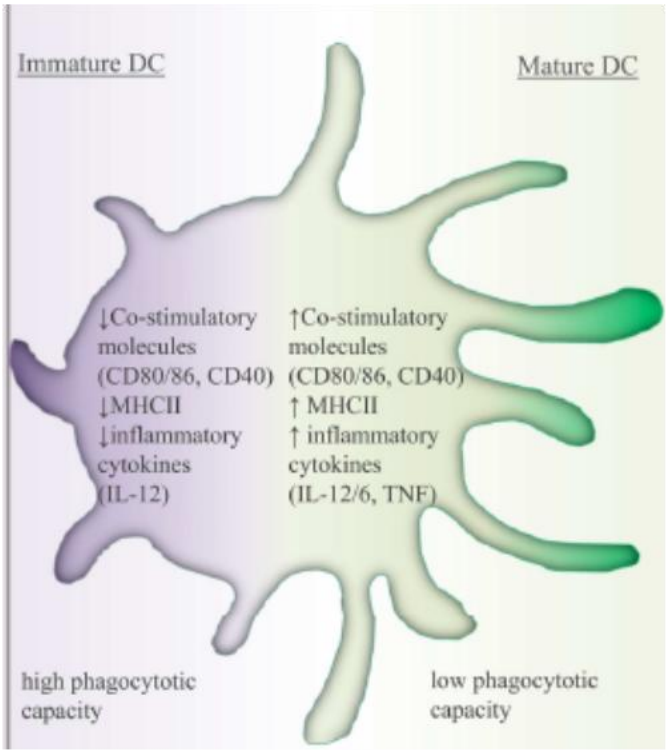
B) Antigen presenting cells (APCs)

Monocytes develop in the bone marrow and enter the blood stream until they are recruited to extravascular compartments not only during inflammatory processes but also under steady-state conditions, to maintain the homeostasis of the monocytic cell system. Monocytes were originally described as key elements of the mononuclear phagocytic system, due to their capacity to differentiate into different subsets of tissue macrophages with specific functions [**2**]. Moreover, monocytes can also give rise to a subset of DCs during infection or inflammation, when high levels of proinflammatory mediators such as TNF-a, IL-1β and IFN-a are produced [**3**]. This discovery has led to the concept that monocyte-derived DCs differentiated locally in inflammatory foci could play an important role to the induction and regulation of immune responses against pathogens but also in the development of inflammatory and autoimmune diseases. Monocyte-derived DCs have the capacity to induce Th1-polarized $CD4^+$ T-cell responses [**4**], crossprime antigen-specific $CD8^+$ T cells [**5**], exert a microbicidal action by producing tumor-necrosis factor-a (TNF-a) and iNOS [**6**] and regulate IgA production by B cells [**7**]. Recent studies have also revealed that monocytes are the precursors for some important DC subsets found in the steady state, such as Langerhans cells [**8**] and DC subsets present in the intestinal and respiratory mucosas [**9**].

Dendritic cells (DCs) are highly specialized antigen presenting cells (APCs) with the unique capacity to establish and control primary immune responses. DCs reside in peripheral tissues in an immature state where they are capable of recognizing pathogens and various danger signals capture and process antigens for presentation in the context of MHC molecules [**10**]. Additionally, via communicating with various immune cells [e.g., natural killer cells (NKs)] DCs bridge the innate and adaptive arm of the immune response [**11**].

Ligation of receptors for inflammatory chemokines recruits immature DCs and their blood precursors to sites of inflammation or infection [**12**]. Upon encounter with microbial, proinflammatory, or T cell-derived stimuli, characteristic phenotypic and functional changes are induced, a process referred to as maturation of DCs. Mature DCs exhibit reduced phagocytic activity and increased expression of MHC and co-stimulatory molecules and secrete large amounts of immunostimulatory cytokines [**13**] [**Fig.B1**].

Mature DCs also change their pattern of chemokine receptor expression, being sensitive to lymphoid chemokines. Thereby, mature DCs acquire the capacity to migrate to the T cell areas of draining secondary lymphoid organs, where they encounter naive T cells and initiate an adaptive immune response [**14**].



Front. Immunol., 2014

Figure B1: Two different states of DCs: immature and mature DCs. Kornek et al., 2014

C) Autophagy

The term autophagy refers to a collection of diverse processes — including macroautophagy, microautophagy, chaperone-mediated autophagy [**15**] and non-canonical autophagy [**16**] — that enable cells to digest their cytoplasmic contents in lysosomes. Macroautophagy (hereafter referred to as ‘‘autophagy’’) initiates with the sequestration of organelles or portions of the cytoplasm within double-membraned vesicles, called autophagosomes. Autophagosomes then fuse with lysosomes to generate autolysosomes which content is degraded [**16**].

C1) Autophagic machinery- key players

Autophagy involves 3 morphological stages: initiation (formation of phagophores), elongation and closure (increase in the size of the phagophore and its closure into a completed autophagosome), and maturation (conversion of autophagosomes into degradative organelles, termed autophagolysosomes, by fusion with late endosomal and lysosomal organelles). The serine/ threonine kinase mammalian target of rapamycin (mTOR) plays a major role in the regulation of autophagy. The rapamycin-sensitive mTORC1 complex promotes mRNA translation and inhibits autophagy by integrating nutrient signals that are generated by amino acids, growth factors, energy and various stressors including hypoxia and DNA damage [**17**].

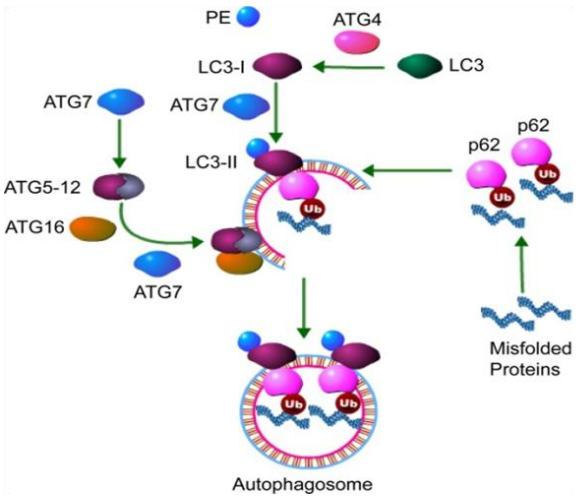
In mammals, the core autophagic pathway starts with the formation of an isolation membrane (also known as a phagophore), most often at contact sites between mitochondria and the endoplasmic reticulum (ER) [**18**]. The phagophore’s membrane then expands forming the so called autophagosome.

The journey into the molecular realm of autophagy began with the identification of the AuTophaGy-related (ATG) genes. Among these ATG genes, one subset of genes is required for autophagosome formation. Autophagosome formation requires two ubiquitin-like conjugation systems, the Atg12 and Atg8 system, which are tightly associated with the expansion of the autophagosomal membrane [**19**]. Atg12 is conjugated to Atg5, forming the irreversible Atg12-Atg5 conjugate. During autophagosome formation the Atg12-Atg5 conjugate complexes with Atg16L; this complex dimerizes and associates with the exterior membrane of the phagophore. Upon

completion of autophagosome formation, the Atg12–Atg5–Atg16 complex is released into the cytosol [**20**].

Another protein that decorates the autophagosome's membrane is the microtubule-associated protein 1A/1B-light chain 3 (LC3). LC3 is a mammalian homolog of the yeast ATG8 protein, a ubiquitin-like protein that becomes lipidated and tightly associated with the autophagosomal membranes [**21**]. A cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine (PE) to form LC3-PE conjugate (LC3-II), which is recruited to autophagosomal membranes. In the autophagosome, LC3-II is distributed to both the exterior and the lumen of the vesicle. Superficial LC3-II is removed by cleavage of the PE by Atg4, while the luminal LC3-II is digested along with the cargo. LC3-II is essential for autophagosome biosynthesis and may be involved in membrane closure [**22**]. The final stages of the biogenesis of the autophagosome include the closure to form a double membrane vesicle.

LC3-II recruits the cargo adaptor protein p62. The p62 protein, also called sequestosome 1 (SQSTM1), is commonly found in inclusion bodies containing polyubiquitinated protein aggregates. In neurodegenerative diseases p62 is detected in ubiquitinated protein aggregates, including Lewy bodies in Parkinson disease, neurofibrillary tangles in Alzheimer disease, and Huntingtin aggregates in Huntington disease [**23**]. p62/SQSTM1 has been shown to bind directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy [**24**] [**Fig.C1.1**].



Physiological Genomics, 2013

Figure C1.1: Schematic presentation of select genes in autophagosome formation. Larsson et al., 2013

Autophagosomes subsequently fuse with lysosomes, forming the mature autophagolysosome, exposing the inner compartment to lysosomal hydrolases which digest proteins, lipids, and nucleic acids in an acidic microenvironment [**25**]. Eventually the inner membrane of the autophagosome, together with the enclosed cargo, LC3-II and p62 proteins, is degraded and the resulting macromolecules are released into the cytosol through lysosomal membrane permeases for recycling [**19**] [**Fig.C1.2**].

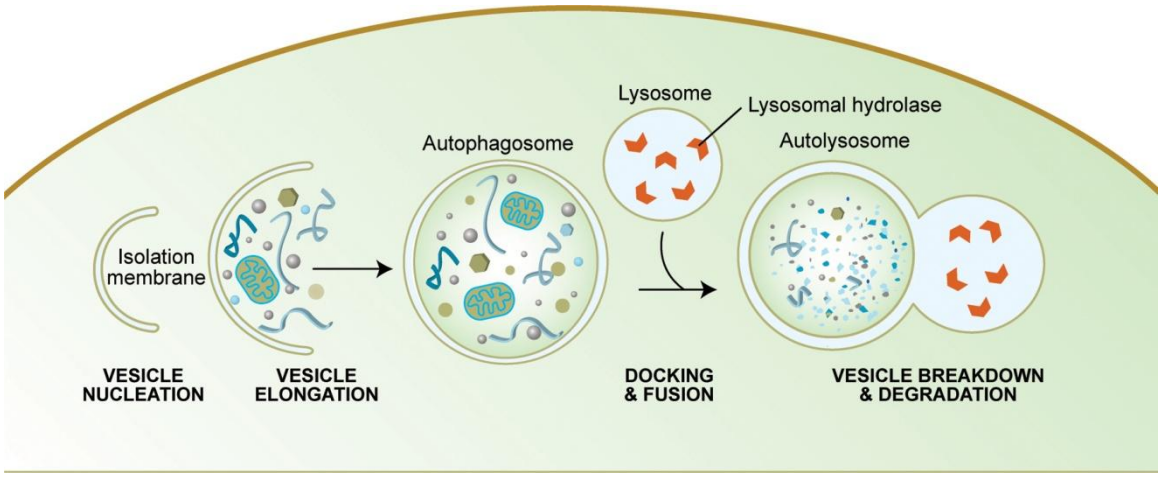


Figure C1.2: Schematic diagram of the steps of autophagy. Melendez and Levine, 2009

Luminal LC3-II can be preserved by inhibiting its lysosomal degradation with protease inhibitors, the proton pump inhibitor, bafilomycin A1 or the lysosomotropic agent chloroquine (CQ) that prevents endosomal acidification. LC3-II/I ratio is often used as a marker for the index of autophagy [**26**].

Autophagy not only preserves cellular homeostasis in conditions of endogenous distress [**27**] but also plays a primordial role in controlling intracellular pathogens in evolutionarily distant species, ranging from unicellular organisms to humans [**28**]. Among the many functions of autophagy are cellular homeostasis [**29**], anti-aging [**30, 31**] and development [**32**]. Recent evidence indicates that autophagic responses in antigen-donor cells affect the release of several cytokines and ‘‘danger signals’’. Thus, especially when it precedes cell death, autophagy alerts innate immune effectors to elicit cognate immune responses. Autophagy is also important for the differentiation, survival, and activation of myeloid and lymphoid cells. Accordingly, inherited mutations in autophagy-relevant

genes are associated with immune diseases, whereas oncogenesis-associated autophagic defects promote the escape of developing tumors from immunosurveillance [**33**].

There are multiple mechanisms through which the cell-intrinsic regulation of autophagy is connected to cell-extrinsic stress response pathways. This is highlighted by the fact that although autophagy constitutes a cell-autonomous mechanism for the control of noninfectious stress and microbial pathogens is stimulated or inhibited by multiple soluble factors. In addition, autophagy can modulate the production of various cytokines [**33**]. One primordial response to viral infection is the secretion of type I interferons (IFNs). Type I IFNs in turn, have been shown to stimulate autophagic responses in several human cancer cell lines [**34**]. Other soluble mediators that promote autophagy include the Th1 cytokines, tumor necrosis factor a (TNF-a), IFN-g, the pro-inflammatory interleukin (IL)-1b and a large panel of DAMPs, such as histone-DNA complexes. These signals are perceived by specific cytokine receptors or by a series of extracellular or intracellular PRRs including TLRs and AIM2 [**35**]. Contrariwise, Th2 cytokines, including IL-4 and IL-13, as well as the anti-inflammatory mediator IL-10, inhibit autophagy [**36**]. In addition, autophagy is upregulated when cells are confronted with potentially dangerous environmental cues, like physical (thermal stress, irradiation) [**37**], chemical (changes in pH, osmolarity) [**38**], or metabolic (shortage in nutrients or oxygen) [**39**].

C2) Autophagy in antigen presenting cells

Autophagy is also important for antigen presentation since autophagic activity has been shown to be elevated in DCs. Furthermore, autophagy's role is extended to other APCs including B cells and macrophages [**33**]. For instance, both macrophages and B cells treated with inhibitors of autophagy are impaired in their ability to present antigens on MHC class II molecules [**40**]. Moreover, autophagy induction is essential for macrophage differentiation of human monocytes [**41**]. Finally, autophagy is required for cellular "housekeeping". Under sterile conditions, autophagy clears the cytoplasm of debris, protein aggregates and defective organelles that can function as endogenous inflammasome agonists such as mitochondria. Finally, some studies suggest that basal levels of autophagy control the set point for inflammasome activation. If autophagy is blocked, this leads to an accumulation of depolarized mitochondria that leak endogenous inflammasome agonists, such as mitochondrial DNA and ROS, which can activate the NLRP3 inflammasome [**42, 43**].

D) Mitochondria

It is well-known that mitochondria are maternally inherited double membrane- bound organellesfound in most eukaryotic cells. In 1890, Altman described them for the first time as ''bioblasts'' and later on, in 1898, Benda managed to observe their heterogeneous morphologic features. More specifically, the fact that they are sometimes ball-shaped and other times linear, led to the inspiration of the name mitochondrion, coming from the Greek words mitos (meaning thread) and chondrion (meaning granule) [**44**].

It was Merezhkovsky in 1905 and then Margulis in 1967 who proposed independently that mitochondria are derived from eubacterial endosymbionts and are capable of aerobic respiration [**45**]. To date, we know that the aerobic alpha-proteobacteria are the precursors of mitochondria [**46**]. Moreover, the observations by Lewis in 1914 established the field of mitochondrial dynamics, since they form a dynamic interconnected intracellular network, moving through the use of cytoskeletal motors and undergoing continuously the process of mitochondrial fission and fusion (even when the cells are resting). As it was noted: ''Any one type of mitochondria such as a granule, rod or thread may at times change into any other type or may fuse with another mitochondrion, or it may divide into one or several mitochondria'' [**47**].

Mitochondria range from 0.5 to 1.0 μm in diameter and obtain several characteristics that make them unique. First of all, the number of mitochondria inside a cell can vary widely depending on the organism, tissue and cell type. For instance, red blood cells lack mitochondria, whereas in liver cells more than 2000 mitochondria can be identified. Furthermore, mitochondria possess their own genome, transcriptome and proteome [**48**]. The human mitochondrial genome is a circular DNA molecule consisting of about 16 kilobases and by which 37 genes are encoded: 13 for subunits of complexes I, III, IV and V of the respiratory Electron Transfer Chain (ETC), 22 for mitochondrial tRNA (for the 20 standard amino acids, plus an extra gene for leucine and serine), and 2 for rRNA [**49**]. One mitochondrion can contain two to ten copies of it's DNA [**50**].

Regarding their function, mitochondria are considered the main intracellular producers of energy (heat and ATP). Every single mitochondrion is able to carry out oxidative phosphorylation (OXPHOS) with the use of it's Electron Transfer Chain. During this process, the metabolic products generated from the Krebs cycle drive the generation of a proton gradient at the inner mitochondrial membrane (IMM), providing by this way the energy required for ATP generation. In addition to ATP

production, mitochondria have the ability to sense oxygen, calcium and fuel (such as carbohydrates and fatty acids), they are manufacturers of several metabolites as well as Reactive Oxygen Species (ROS) and also effective inducers of programmed cell death (apoptosis) [**44**]. Finally, it has been shown that they can sense danger signals and subsequently induce inflammation by activating and controlling the immune system [**51**].

D1) Mitochondrial Dynamics- Mitochondrial Fission and Fusion

Undoubtedly, mitochondrial dynamics plays a pivotal role regarding cell growth and survival. It has been shown that disorders of mitochondrial dynamics can contribute to the pathogenesis of complex diseases that are not classically considered to involve mitochondria, such as cancer, cardiovascular disease as well as neurodegenerative diseases.

The balance between the rates of mitochondrial fission and fusion determines the length of mitochondria as well as mitochondrial redistribution inside the cell. It should be mentioned that mitochondria often exist as solitary units. However, they do occasionally fuse with other mitochondria so that they can form a healthy closed network that enhances communication with the Endoplasmic Reticulum (ER) [**52**]. Furthermore, fusion contributes to the diffusion of matrix content among mitochondria, diluting by this way the accumulated mitochondrial DNA mutations [**53**] and oxidized proteins. On the other hand, mitochondria can exit from this network by the process of fission, which gives rise to smaller, more discrete mitochondria with different membrane potential. Subsequently, the depolarized mitochondria (with persistently low membrane potential) are eliminated via mitophagy, whereas those ones that recover are capable of rejoining the mitochondrial network by fusion [**Fig.D1.1**].

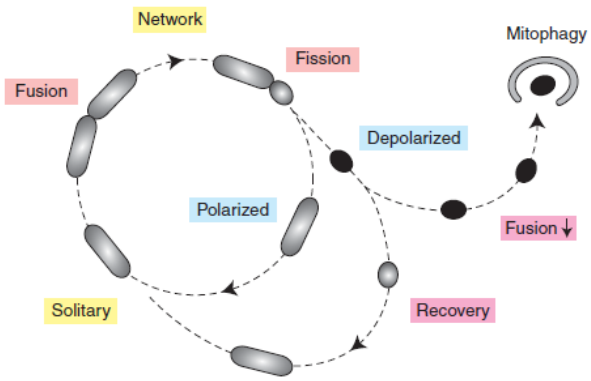


Figure D1.1: Mitochondrial life cycle. Twig et al., 2008

Both mitochondrial fission and fusion are mediated by a small number of highly conserved guanosine triphosphates (GTPases) that belong to the Dynamin family. Mitofusins (Mfn1, Mfn2) and Optic Atrophy 1 (OPA1) are proteins responsible for fusion; Dynamin-Related Protein 1 (DRP1) is involved in fission [**54**]. Mfn1 and Mfn2 are located in the outer mitochondrial membrane and tether adjacent mitochondria. They fulfill partially redundant function, since it has been observed that both of them are capable of supporting mitochondrial fusion by themselves. OPA1 is a GTPase located in the inner mitochondrial membrane and mediates fusion of this membrane. Finally, DRP1 is a cytosolic GTPase that, once activated, translocates to the outer mitochondrial membrane. Following that, DRP1 multimerizes and creates a ringlike structure that constricts and divides the organelle [**Fig.D1.2**].

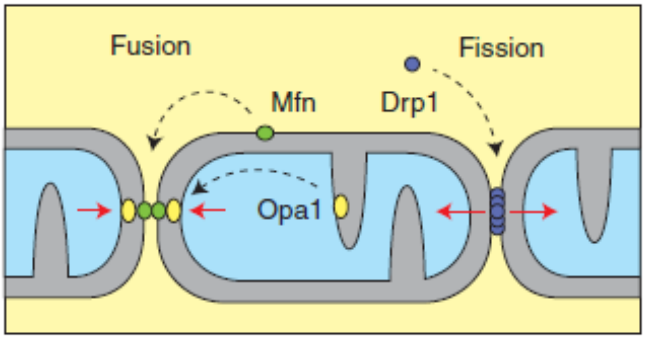


Figure D1.2: Functions of the mitochondrial Dynamin family members. Kawajiri et al., 2013

A number of non-GTPase receptor proteins activate DRP1 and target it to the outer mitochondrial membrane. These are the following ones: Mitochondrial fission 1 protein (Fis1), Mitochondrial fission factor (MFF), and Mitochondrial elongation factor 1. Interestingly, the fission apparatus is assebled with the assistance of Endoplasmic Reticulum (ER) which is in contact with the mitochondria [**55**].

Moreover, mitochondrial outer membrane fusion occurs in most of the cases with concomitant inner membrane fusion. However, outer membrane fusion can take place without being coordinated with the inner membrane fusion when the latter one is blocked due to mutations or loss of membrane potential [**56**]. Generally, mitochondrial fusion mediators are regulated by proteolysis and ubiquitination, whereas mitochondrial fission proteins are regulated by several protein modifications, such as phosphorylation, ubiquitination, sumoylation and nitrosylation [**54**].

D2) Electron Transfer Chain (ETC): ATP generation by mitochondria

Mitochondria are traditionally known as the energy generating centers of cells and the largest proportion of ATP (the most commonly used 'energy currency' of cells from most organisms) synthesized in these organelles is the result of oxidative phosphorylation conducted via the Electron Transfer Chain (ETC). In this case, ATP is generated from products (NADH, succinate) of the citric acid cycle (or Kreb's cycle), fatty oxidation and amino acid oxidation.

ETC is an organized sequence of compounds located in the inner mitochondrial membrane (IMM), which is impermeable to low-molecular weight solutes. More specifically, ETC consists of four membrane-bound protein complexes (complex I-IV) [**57**]. According to the chemiosmotic hypothesis proposed by Mitchell in 1961, electrons are transferred through these complexes from electron donors to electron acceptors via redox reactions and by this way ETC couples the electron transfer with the transfer of protons (H^+ ions) across a membrane. This creates an electrochemical proton gradient (exergonic reaction) which drives ATP synthesis by an evolutionary conserved enzyme that is called ATP synthase (complex V) and is located in IMM as well [**58**].

So, due to this electrochemical energy (found in the form of a proton-motive force), the IMM is hyperpolarized, extruding newly synthesized ATP into the cytoplasm. The final acceptor of electrons in the ETC is molecular oxygen. At the same time, protons flow passively back into the mitochondrial matrix through a proton pore that is associated with ATP synthase [**Fig.D2**].

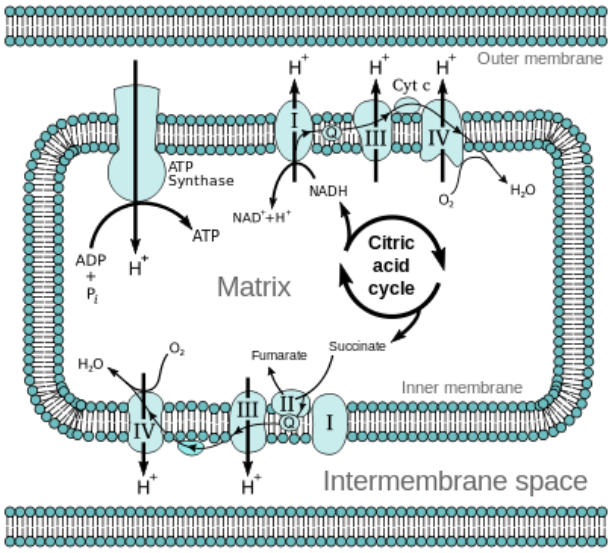


Figure D2: Electron Transfer Chain (ETC)

D3) Electron Transfer Chain (ETC): ROS generation by mitochondria

In mammalian cells Reactive Oxygen Species (ROS) can be generated in different cellular compartments such as membranes, cytoplasm, mitochondria, endoplasmic reticulum (ER), lysosomes and peroxisomes [**59**]. ROS production is at least partially associated with the occurrence of a number of chronic diseases including adiposity, atherosclerosis, type II diabetes and cancer [**60-64**]. Recently, the association of ROS production and autoimmunity has been proposed too [**65**]. However, the whole view on ROS has been changed the last few years, since they are considered not only harmful byproducts but also fundamental for the maintenance of cellular communication and homeostasis in different organisms ranging from bacteria to mammals. Some of the physiological processes that they modulate are related to the regulation of growth factor signaling, the hypoxic response, inflammation and the immune response. So, the balance between ROS generation and ROS neutralization by cellular antioxidants seems to be of vital importance, so that the cells are able to exert their functions properly and survive.

As a major site of ROS generation, mitochondria have drawn considerable interest. In 1966 came into light the first report supporting the idea that the respiratory chain produces ROS [**66**]. After that, Chance and colleagues showed that isolated mitochondria produce hydrogen peroxide (H_2O_2) [**67, 68**]. Remarkably, mitochondria in females produce less ROS with respect to those in males, revealing a potential association between mitochondrial ROS generation and estrogens [**69**].

ROS are alternatively called 'free radicals', since the majority of them is characterized by at least one unpaired electron in their outer orbitals [**Fig.D3.1**]. Peroxides like hydrogen peroxide are also able to give rise to the formation of oxygen radicals and thus they are considered ROS too. So, the incomplete reduction of oxygen by one electron (producing superoxide anion) is the initial step for the formation of several other ROS [**70**]. Therefore, it is the kinetic and thermodynamic factors underlying the interaction of potential one-electron donors with O_2 that control mitochondrial ROS production. To date, it is known that the proportion of oxygen that is utilized for ROS generation by mitochondria is variable and ranges from 0.15% to 4% [**71**].

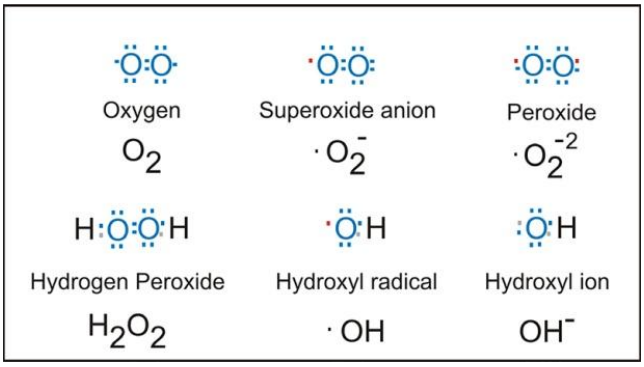


Figure D3.1: Types of Reactive Oxygen Species (ROS). Paul Held, 2014

As it was mentioned above, mitochondrial ROS are created at the ETC during the process of oxidative phosphorylation (OXPHOS). Actually, there are three leak events: complex I leaks superoxide anion towards the mitochondrial matrix, while complex III leaks superoxide anion towards both the intermembrane space and mitochondrial matrix [**72**]. Following that, a dismutation event takes place during which superoxide anion is very quickly dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase 1 (SOD1) in the mitochondrial intermembrane space and by superoxide dismutase 2 (SOD2) in mitochondrial matrix [**73**]. Afterwards, H_2O_2 is fully reduced to to water by the enzyme glutathione peroxidase (GPX) [**Fig.D3.2**].

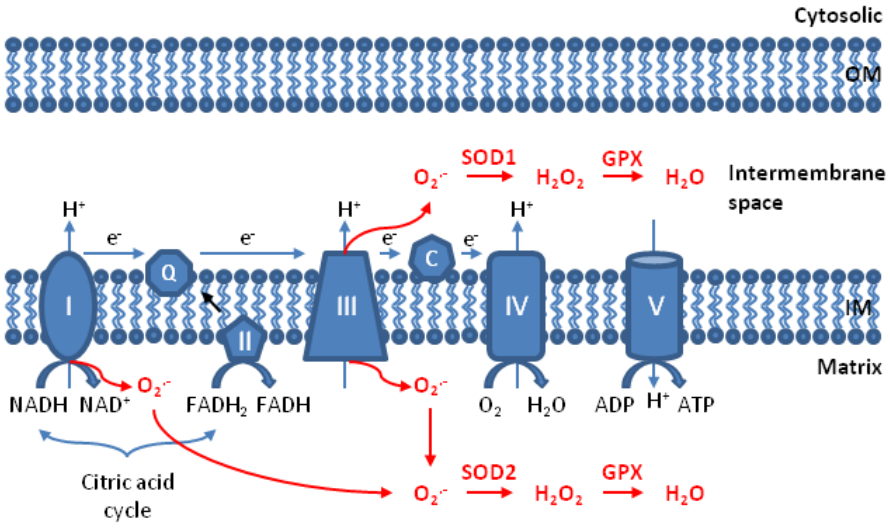


Figure D3.2: Generation and disposal of mtROS. Li et al., 2013

In spite of the fact that both superoxide anion and hydrogen peroxide are both considered as mitochondrial ROS, their fate differs to a great extent [**72**]. Due t to it's short half-life and the electrophilic properties that characterize it, superoxide anion is not capable of passing through the outer mitochondrial membrane (OMM) and for this reason it is unlikely to participate in subsequent signal transduction pathways in the cell. Instead, it can react with nitric oxide (NO) via a radical-radical reaction and form peroxynitrite within mitochondria. This is a detrimental oxidant that disrupts mitochondrial integrity, induces severe mitochondrial DNA damage and irreversible modifications of mitochondrial proteins. On the contrary, superoxide anion (H_2O_2) is more stable and electrophobic. So, it is considered as an ideal candidate for the participation in subsequent signaling pathways inside the cell [**74**].

ROS production by mitochondria is regulated by a number of different factors such as mitochondrial membrane potential ($\Delta\psi_m$), mitochondrial metabolic state and O2 concentration [**75-77**]. Moreover, it has been shown that ROS generated by other cellular compartments can significantly enhance mitochondrial ROS production in a process that is well-known as 'ROS-induced ROS'. This occurs through the action of ROS-producing enzymes such as NADPH oxidase [**78**], xanthine oxidase [**79**] and uncoupled eNOS [**80**]. Meanwhile, the transcription factor STAT3 contributes to the suppression of mitochondrial ROS production in a mode of action that is independent of it's nuclear factor activity [**81**] [**Fig.D3.3**].

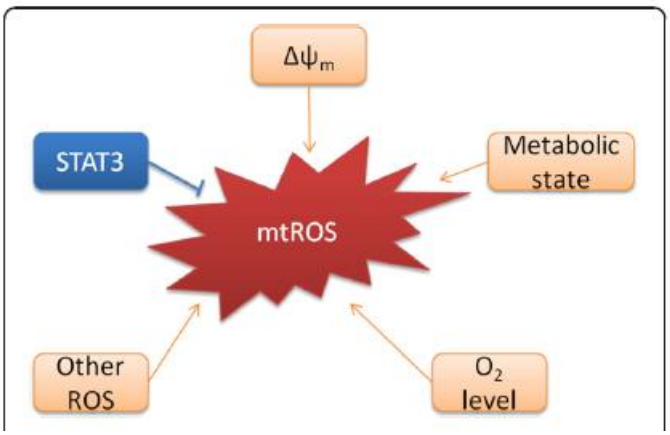


Figure D3.3: Regulation of mtROS production. Li et al., 2013

The unbalanced production of ROS by mitochondria can lead to a pro-oxidant state that is commonly referred to as oxidative stress. Oxidative stress is strongly associated with a variety of harmful effects due to the primary chemical reactions of ROS with mitochondrial DNA, lipids and proteins [**82, 83**]. The oxidative damage of these mitochondrial macromolecules impairs the ability of mitochondria to produce ATP and to exert the wide range of their metabolic functions. In addition, mitochondrial oxidative damage is able to augment mitochondrial outer membrane permeabilization (MOMP), resulting in the release of intermembrane space proteins such cytochrome c (cyt c) to the cytoplasm and to the subsequent activation of the cell's apoptotic machinery. Finally, mitochondrial ROS are inducers of the mitochondrial permeability transition pore (PTP), which in turn renders the inner membrane permeable to small molecules in circumstances such as ischemia/perfusion injury [**84**] [**Fig.D3.4**].

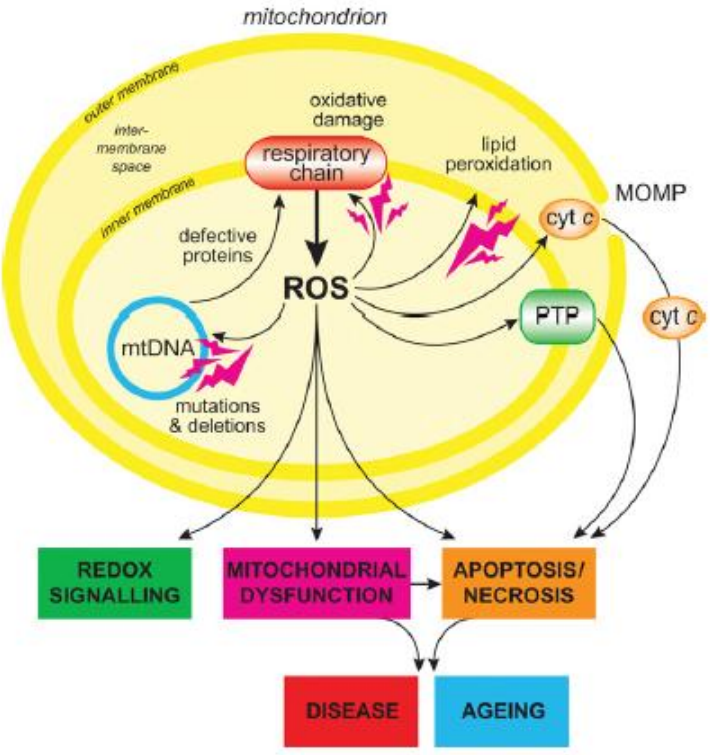


Figure D3.4: Overview of mtROS oxidative damage. Murphy, 2009

Mitochondrial ROS play also a fundamental role in signaling pathways inside the cell. More specifically, mtROS promote redox signaling via the oxidation of certain reactive cysteine residues of proteins [**85**], but this is property is dependent on the concentration of mitochondrial ROS.

It has been shown that mitochondrial ROS at low levels regulate the stability of Hypoxia Inducible Factor 1a (HIF-1a); thus they participate in the process of hypoxia adaptation. Moderate levels of mtROS are associated with the regulation of inflammatory processes and more specifically with the production of proinflammatory cytokines. This occurs because they are direct activators of the inflammasome and the Mitogen-Activated Protein Kinase (MAPK). On the contrary, high levels of mtROS have the ability to induce apoptosis as well as autophagy by oxidation of the mitochondrial pores and Autophagy-specific Gene 4 (ATG4) respectively [**84**] [**Fig.D3.5**].

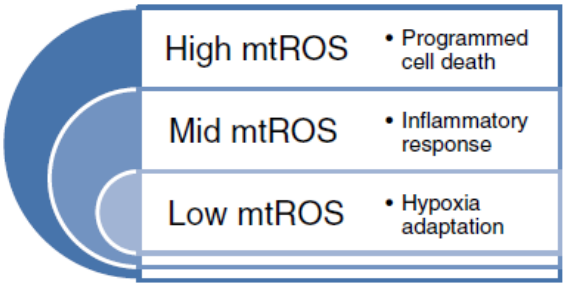


Figure D3.5: Mitochondrial ROS signaling. Li et al., 2013

Because of the high reactivity and toxicity of mitochondrial ROS, mammalian cells have evolved a variety of anti-oxidant enzyme systems so that they can scavenge mitochondrial ROS almost immediately after their production. It is important to mention that all these anti-oxidant enzymes are encoded by the nuclear genome and that they are targeted to mitochondria after their protein translation. Some examples of these are the following ones: superoxide dismutases (SODS) [**86**], catalase [**87**], glutathione peroxidase (GPX) [**81**], peroxiredoxins [**88**] and thioredoxins (Trx) [**84**]. Interestingly, vitamin E has been shown to decrease mitochondrial ROS too, but only in low concentrations. This is because some antioxidants including vitamin E, vitamin C and quercetin can act as prooxidants at high concentrations [**89**].

However, the effectiveness of the aforementioned natural anti-oxidants is in many cases limited, since they are not always capable of accumulating within mitochondria or passing through all the biological membranes, such as the Blood Brain Barrier (BBB) [**90**]. So, in order this serious issue to be addressed, several synthetic mitochondrial ROS scavengers have been developed. Representative examples of these scavengers are MitoTEMPO [**91**], Mito Vit-E [**92**] and MitoQ10 [**93**].

Finally, a number of fluorescence probes have been developed and are currenlty used for the characterization and detection of mitochondrial ROS. These fluorescent dyes include dichlorodihydrofluorescein (DCF) and dihydroethidium (DHE). Some of them are specific for the detection of superoxide anion (MitoSOX), others for hydrogen peroxide (MitoPY1), whereas MitoAR and MitoHR are able to detect hydroxyl radicals [**84**]. However, there is still a clear need to develop improved methods for the measurement of mitochondrial superoxide and hydrogen peroxide formation in vivo, as uncertainty about these values hampers studies on the role of mitochondrial ROS in pathological oxidative damage and redox signaling.

D4) Mitochondrial Quality Control

Besides the critical metabolic functions of mitochondria (including fatty acid oxidation, the Kreb's cycle and oxidative phosphorylation), these organelles can potentially damage cells (via mitochondrial ROS production). Thus, certain quality control mechanisms have evolved for the preservation of a healthy mitochondrial population inside mammalian cells [**Fig.D4**].

First of all, mitochondria acquire their own proteolytic system, consisting of two AAA proteases (membrane-embedded ATP-dependent proteolytic complexes) in the inner mitochondrial membrane (IMM) [**94**]. The exact function of these complexes is to remove through degradation the short-lived and the unfolded membrane proteins, in spite of the fact that cytosolic proteasomes are also able to degrade some of the defective proteins of the inner and outer mitochondrial membrane [**95**].

Secondly, there is recent evidence pointing to a lysosomal pathway for the control of mitochondrial quality. During this process, vesicles budding from the mitochondrial tubules sequester selected mitochondrial cargos, and subsequently deliver those mitochondrial components to lysosomes for degradation. It is important to mention that this pathway is active under steady-state conditions and is further stimulated by oxidative stress. So, it is proposed that these mitochondrially-derived vesicles may represent a mechanism for the selective degradation of oxidized mitochondrial proteins, while the whole organelle remains intact [**96**].

The aforementioned pathways are responsible for the removal of only a subset of defective mitochondrial proteins. However, an alternative mechanism for bulk degradation of mitochondria

exists, since entire mitochondrial organelles have been observed inside mammalian lysosomes in electron microscopy studies [**97**]. This mechanism is termed mitophagy and will be analyzed in the following section.

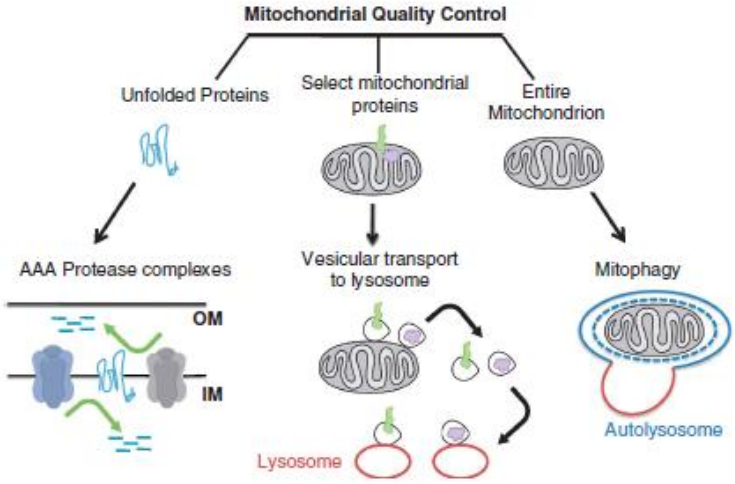


Figure D4: Three major pathways of mitochondrial quality control. Ashrafi and Schwarz, 2013

E) Overview of Mitophagy

It is well-known that the bulk degradation of cellular components occurs through a highly regulated process named autophagy. Additionally, mitophagy is considered as a selective form of autophagy that is chiefly responsible for the digestion and subsequent elimination of damaged or superfluous mitochondria. The term 'mitophagy' was coined by Lemasters in 2005 [**98**]. In this process, mitochondria are sequestered in double-membrane vesicles and delivered to lysosomes for degradation via the action of lysosomal hydrolytic enzymes [**Fig.D4**].

Although the turnover and clearance of dysfunctional/depolarized (low $\Delta\psi_m$) mitochondria and/or mitochondria producing the highest levels of ROS may represent the primary functions of mitophagy, some other specialized cases of mitophagy for the regulation of organelle number in response to developmental or physiological cues have been identified as well. One of these cases is the complete removal of mitochondria during erythrocyte maturation that occurs through the action of a specific mitochondrial protein named Nix [**99**] [**Fig.E1**]. Moreover, the selective

destruction of sperm-derived mitochondria after oocyte fertilization takes place also via the process of mitophagy [**100**] [**Fig.E2**].

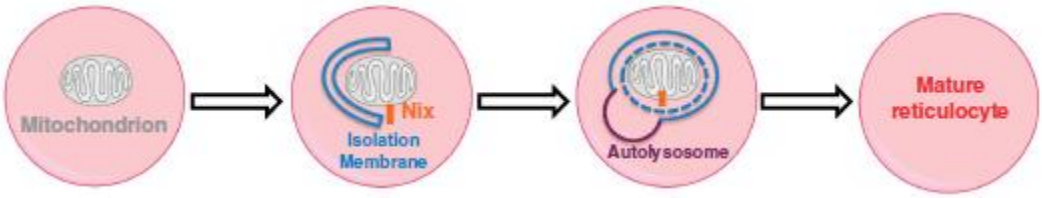


Figure E1: Mitophagy in reticulocyte development. Ashrafi and Schwarz, 2013

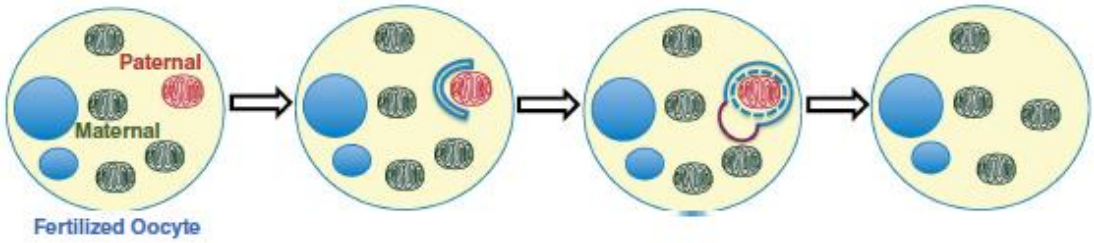


Figure E2: Mitophagy of paternal mitochondria in fertilized oocyte. Ashrafi and Schwarz, 2013

However, an obvious question is raised due to the extensive similarity of the pathways for general autophagy and mitophagy. How could an autophagosome be directed selectively to mitochondria and also how could this process be triggered without the activation of bulk autophagy?

E1) Mitophagy-specific effectors

This question led to the identification of certain factors that might account for the selectivity of mitophagy. More specifically, two independent groups performed screens for yeast mutants defective in mitophagy [**101, 102**]. Accordingly, they found approximately 40 genes that were distinct from known ATGs and required for mitophagy but not bulk autophagy, including UTH1, YmeI, AUP1, mdm38/Mkh1 and ATG32. Unfortunately, most of these genes do not have any identifiable homologs in higher eukaryotes [**103**]. However, it seems that mammals do obtain certain mitophagy-specific effectors as well, the most important of which are PINK1, Parkin, NIX and BNIP3 [**104**].

E2) The PINK1/Parkin pathway of Mitophagy

A recently identified pathway that has emerged as a paradigm for mammalian mitophagy is mediated by the effectors PINK1 and Parkin. The genes encoding these proteins have been found mutated in certain forms of autosomal recessive Parkinson's disease (PD), also termed Parkinsonism [**105, 106**]. Accumulation of dysfunctional mitochondria in the brains of PD patients in combination with the clinical similarity between patients with PINK1 or Parkin mutations suggest that these molecules might function in a common pathway regarding the regulation of mitochondrial quality control. Genetic studies in Drosophila have further proposed a role for PINK1 and Parkin in the regulation of mitochondrial integrity [**107**]. However, Parkin and PINK1 may exert independent functions in some other cellular pathways.

PINK1 is a serine/threonine protein kinase that is targeted to mitochondria due to the expression of a mitochondrial targeting sequence (MTS) [**108**]. Like most other mitochondrial proteins, PINK1 is encoded by the nuclear genome and subsequently synthesized in the cytoplasm before it is imported in mitochondria via the TOM (translocase of outer membrane)/TIM23 (translocase of inner membrane 23) complexes [**109**]. Moreover, PINK1 requires the electrical component of the inner mitochondrial membrane potential ($\Delta\psi$) for it's import [**110**].

Interestingly, imported PINK1 is constitutively cleaved into an unstable product by an inner mitochondrial membrane rhomboid protease called PARL as well as by other mitochondrial proteases [**111**]; thus PINK1 is present at very low levels in those mitochondria that are healthy enough to maintain the $\Delta\psi$-dependent import pathway. This is the explanation for the fact that PINK1 cannot be detected in proteomics studies very often, is spite of it's relatively large size. In addition, PINK1 contains a second weaker signal sequence that targets it to the outer mitochondrial membrane (OMM) in the case that $\Delta\psi$ has collapsed and consequently the TOM/TIM import pathway is blocked [**112**].

So, PINK1 is responsible for the assessment of the internal state of all mitochondria inside the cell due to the aforementioned features. Therefore, the healthy mitochondria acquiring a strong inner membrane potential (high $\Delta\psi$) quickly dispose of PINK1 via degradation, whereas failing mitochondria (low $\Delta\psi$) are unable to import and degrade the kinase, accumulating it on their surface and thus displaying their inner dysfunction on their outer surface. This mechanism is very elegant and fine-tuned, since PINK1 constitutively associates with the TOM complex (once it is on

the outer mitochondrial membrane), so that it can be re-imported and degraded if mitochondrial membrane potential is restored [**113**]. This is a quick on/off signal of mitochondrial dysfunction [**Fig.E2.1**].

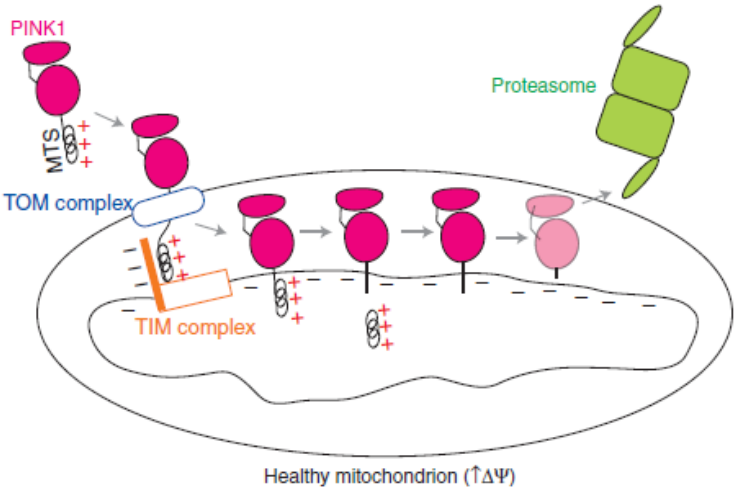


Figure E3.1: Regulation of PINK1 import in mitochondria. Youle et al., 2012

Mammalian Parkin is an E3 ubiquitin ligase that under basal conditions resides in the cytoplasm (likely inert). This E3 ligase is the most recent candidate for ATG5-dependant mitophagy in mammals [**114**]. Upon mitochondrial damage (low Δψ), PINK1 is exposed to the cytosolic surface and thereby activates and directs Parkin specifically to the failing mitochondrion. Afterwards, Parkin ubiquitinates several outer mitochondrial membrane proteins of that dysfunctional organelle, leading to either their degradation by the proteasome or to the recruitment of ubiquitin-binding adaptors (such as p62) to remove the damaged mitochondrion by selective autophagy (mitophagy) [**Fig.E2.2**].

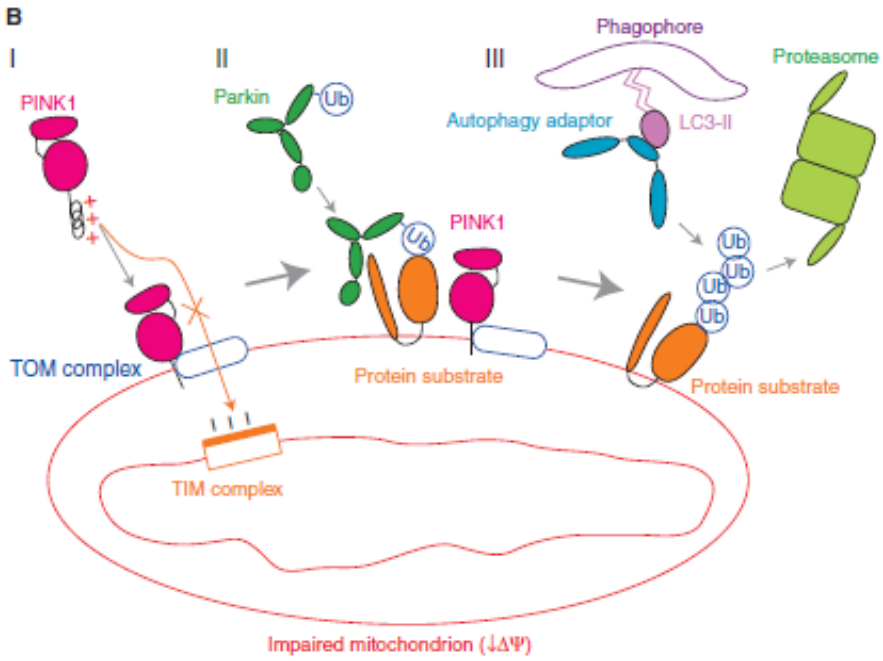


Figure E2.2: Regulation of the PINK1-Parkin mitochondrial quality control pathway. Youle et al., 2012

However, the exact mechanism for the recruitment of Parkin selectively to mitochondria, on the surface of which PINK1 accumulates, has not been deciphered so far. There are three major hypotheses. Firstly, it is suggested that PINK1 and Parkin can directly bind each other, since PINK1-Parkin complexes of different sizes have been observed by size-exclusion chromatography and native PAGE electrophoresis [**112**]. Secondly, there is mixed evidence that PINK1 directly phosphorylates Parkin on residues Thr175 and Thr217 at the linker region of Parkin [**115**]. Finally, an alternative but not mutually exclusive hypothesis includes the priming through phosphorylation of particular substrates of Parkin on the outer mitochondrial membrane (OMM) by PINK1. The latter hypothesis is known as the 'shared-substrate model', supporting the existence of a motif that is recognized by PINK1 in it's unphosphorylated form and by Parkin in it's phosphorylated one [**112**].

To date, it is known that Parkin mediates directly or indirectly the ubiquitination of mitochondrial outer membrane proteins with various ubiquitin linkages. The target proteins with predominantly K48-linked ubiquitin chains are removed by proteasomal degradation, whereas those with K63-linked ubiquitin chains are able to recruit adaptor proteins from the cytoplasm [**116**]. There are many substrates that have been identified to be ubiquitinated by Parkin, but which one of them is

ubiquitinated more efficiently in vivo and which is of greatest physiological importance is still less clear. Remarkably, these substrates differ also in the number of transmembrane domains they possess, their size and their association with other proteins [**112**]. Conclusively, the substrates towards which Parkin has high activity display significant diversity. Representative examples of these are Mitofusins (Mfn1/2), Miro1, VDAC and TOM70 [**Fig.E2.3**].

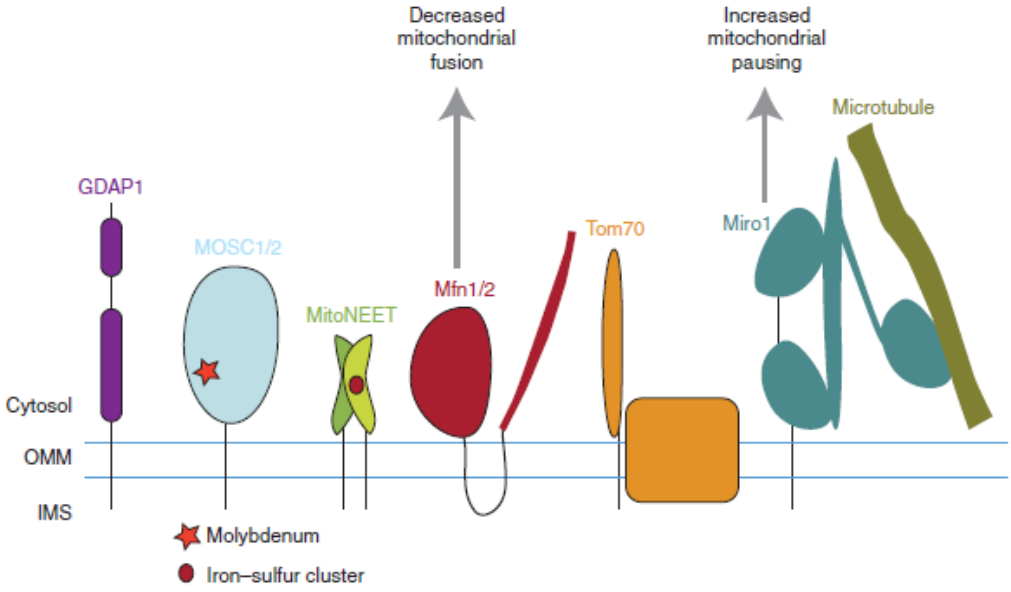


Figure E2.3: Proposed substrates of Parkin for ubiquitination. Youle et al., 2012

Finally, besides ubiquitination, Parkin can interact with other proteins such as Ambra1 (Autophagy/Beclin1 regulator 1). This interaction leads to the activation of PI3K complex around damaged mitochondria. Since this complex is essential for the formation of new phagophores, it can subsequently facilitate selective mitophagy [**117**]. So, the interaction of Parkin with Ambra1 can be considered as a key mechanism for the induction of the final clearance step of Parkin-mediated mitophagy [**118**] [**Fig.E3**].

E3) Parkin-independent pathway of Mitophagy

A second pathway of mitophagy that is mostly induced under hypoxic conditions and that is independent of the action of Parkin has been proposed too [**104**]. During this process, dysfunctional mitochondria exhibit elevated expression of the mitochondrial proteins BNIP3, NIX and FUNDC1, which in turn are able to recruit autophagosomes to mitochondria by direct interaction with LC3 (through their LIR domains). Upon mitochondrial depolarization after CCCP treatment of cells (mitochondrial uncoupler), the mitochondrial protein Smurf1 has been also found to promote mitophagy, most likely via ubiquitination of a number of mitochondrial proteins. Furthermore, the complex Hsp90-Cdc37 leads to the stabilization and activation of Ulk1, which is responsible for the phosphorylation of ATG13. Once phosphorylated, ATG13 is targeted to damaged mitochondria and induces the mitophagic flux. Both the Parkin-dependent and the Parkin-independent pathways are considered as ATG-dependent mechanisms due to the participation of ATG proteins in these processes. It should be mentioned that in the ATG-dependent mechanisms ROS and ATP depletion are considered as significant inducers of mitophagy by repressing mTOR complex. However, an ATG-independent mechanism has been described as well but is less understood so far [**104**]. In this particular mechanism, 15-lipoxygenase promotes mitochondrial degradation. In addition, direct lysosomal invagination or interaction with damaged mitochondria (microautophagy) might also play a pivotal role in the clearance of the defective organelles [**Fig.E3**].

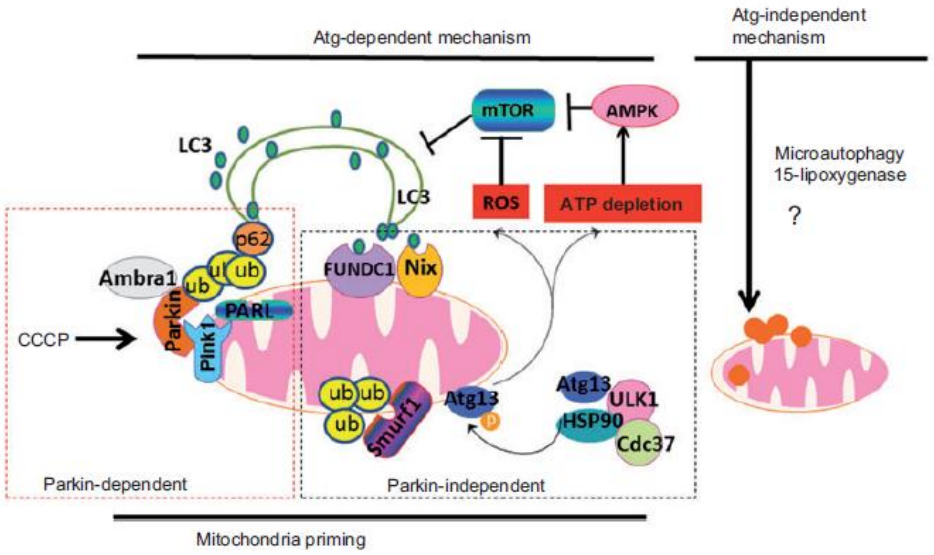


Figure E3: Proposed models for mitophagy in mammalian cells. Ding and Yin, 2012

F) Rapamycin- Induction of Autophagy

As it was mentioned in section C1, the mTOR (mammalian Target of Rapamycin) complex is a well-known inhibitor of the autophagy pathway in cells ranging from yeast to human. It is ubiquitously expressed in various cell types and functionally is a serine/threonine protein kinase that regulates important cellular processes, including growth, proliferation, motility, survival, protein synthesis and transcription [**119**]. However, once it is blocked by rapamycin, the autophagic flux is induced.

Rapamycin is a macrolide fungicide characterized by immunosuppressant properties that bear molecular and structural similarities to the calcineurin inhibitor, tacrolimus [**120**]. Interestingly, the mechanism of action of rapamycin is distinct from that of calcineurin inhibitors, such as cyclosporine and tacrolimus. More specifically, rapamycin binds to its intracellular receptor, the immunophilin 12-kDa FK506-binding protein (FKBP-12) and then the rapamycin-FKBP-12 complex binds to and inhibits the mammalian target of rapamycin (mTOR) [**121**]. Inhibition of mTOR leads to arrest of the cell cycle at the G1 to S phase and thus, blockade of growth-factor-driven proliferation of not only activated T cells, which constitute the basis of its immunosuppressive action, but also of other hematopoietic and non-hematopoietic cells [**122**].

Additionally, when target of rapamycin complex 1 (TORC1) is inactivated following nutrient depletion or rapamycin treatment, autophagy-related 13 (Atg13) is dephosphorylated. This allows the association of Atg1 subfamily proteins with Atg13, followed by the upregulation of the Atg1 kinase activity and recruitment of other core Atg proteins to the pre-autophagosomal structure (PAS) to initiate autophagosome formation [**123**]. These events are immediately reversed on the addition of nutrients [**Fig.F**].

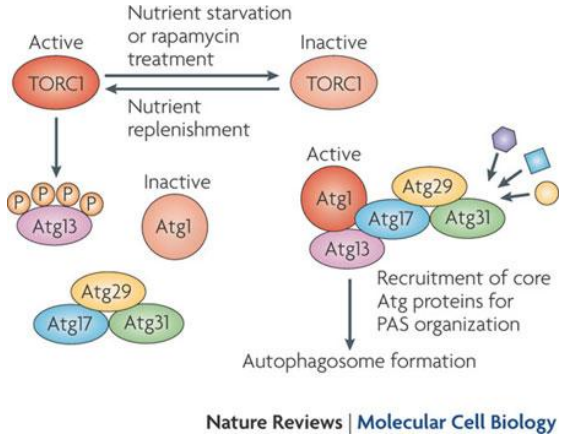


Figure F: Dynamics and diversity in autophagy mechanisms: lessons from yeast. Ohsumi et al., 2009

G) Type I Interferons

Signals generated after the recognition of infectious agents by cells of the innate immune system are thought to be important in triggering the antigen-specific adaptive immune response [**124**]. In this regard, cytokines expressed in response to pathogen encounter may play a key role because of their potential to modify both the magnitude and the quality of the immune response elicited. Type I interferons (IFNs), the major species of which are IFN-a and IFN-b, are expressed at low levels under normal physiological conditions but are induced to high levels by a number of stimuli, including viral or bacterial infection and exposure to double-stranded DNA [**125**]. Sources of type I IFNs include fibroblasts, NK cells, T cells, dendritic cells and a group of specialized leukocytes, the plasmacytoid monocytes [**126**]. Plasmacytoid monocytes (which have a plasma-cell-like morphology) constitute a rare cell type that differs from monocytes and monocyte derived dendritic cells by the absence of myeloid markers (e.g. CD11c, CD13 and CD33) and the production of high amounts of type I IFNs in human peripheral blood upon infection with viruses or bacteria [**127**].

The action of type I IFNs on target cells such as fibroblasts, T cells, macrophages or dendritic cells is mediated by the type I IFN receptor (a member of the class II helical cytokine receptor family) that consists of two subunits, the a-chain (IFNAR-1) and the β-chain (IFNAR-2). The latter has long (βL) and short (βS) forms. Mutational analyses and studies in gene-deficient mice revealed that for the induction of certain interferon response genes and/or for full (antiviral) activity of IFN-a or IFN-b, both receptor subunits, the Janus kinases Jak1 and Tyk2, signal transducer and activator of transcription 1 (Stat1) and to some extent the interferon-regulatory factor 1 (IRF-1) were required [**128**]. The potent activity of IFN-a/b against viral infections is based firstly on the expression of IFN-inducible protective genes that confer cellular resistance, inhibit viral replication and impede viral dissemination and secondly on certain immunomodulatory effects. Various other functions of IFN-a/b in the immune system— such as the modulation of antibody production, the enhancement of T cell and NK cell cytotoxicity, the inhibition of lymphocyte proliferation, the inhibition of suppressor T cells and the preferential differentiation of T helper cells into Th1 cells have been recognized [**125**].

H) Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder, predominantly affecting females in which loss of tolerance to nucleic acids and their interacting proteins results in the production of pathogenic autoantibodies that cause inflammation and tissue damage [**129**]. SLE in humans manifests with a diverse array of clinical symptoms that potentially involve multiple organ systems. This heterogeneity reflects direct autoantibody mediated tissue injury as well as blood vessel inflammation (termed vasculitis) caused by the deposition of complement-fixing immune complexes. Approximately, one-half of lupus patients will manifest the more severe complications of the disease, which can include nephritis, central nervous system vasculitis, pulmonary hypertension, interstitial lung disease, and stroke [**130**]. The diagnosis of SLE is complicated by these extensive variations in clinical symptoms. Additionally, the development of targeted therapies that specifically address disease pathogenesis and progression has lagged, resulting in a limited therapeutic armamentarium of broad-spectrum immunosuppressive agents that have substantial toxicities and are not always adequate to control symptoms or prevent disease flare [**129**].

H1) The pathogenic role of type I IFNs in SLE

The pathogenic role of type I IFNs in SLE is supported by a signature of IFN-induced genes in the peripheral blood of patients [**130**], an association with risk alleles involved in TLR and IFN pathways [**131**], disease acceleration by exogenous IFN-α in several lupus models [**132**] and disease amelioration in some lupus-prone mouse strains that have been rendered deficient for the type I IFN receptor [**133**]. Further inference of the role of type I IFN in SLE came from the presence of circulating inducers of IFN-a in SLE blood and the induction of autoimmunity during IFN-a therapy [**134**]. Finally, IFNa not only induces the development of mature DCs from monocytes in vitro but high serum levels of IFN-a have been shown to be associated with the acquisition of DC characteristics by monocytes isolated from peripheral blood in patients with systemic lupus erythematosus [**135**]. As a result, a key pathogenic event in SLE might be a break in peripheral tolerance mechanisms after activation of myeloid dendritic cells (mDCs) in response to an excess of IFN-a [**135**].

H2) SLE monocytes act as DCs

$CD14^+$ blood cells are normally immunologically quiescent monocytes that are unable to mount the so-called lymphocyte reaction (MLR). However, in SLE these cells are able to induce the proliferation of alloreactive T cells, a property characteristic of DCs [**135**]. Incubation of monocytes from healthy individuals with serum from active SLE patients results in the generation of CD14 cells with certain characteristics of DCs including phenotype and ability to induce an MLR. Neutralizing anti-IFN-a/b has demonstrated IFN-a/b to be the key SLE serum factor responsible for the differentiation monocytes into DCs. Furthermore, normal serum spiked with recombinant IFN-a/b can also induce the differentiation of monocytes into DCs [**136, 137**].

H3) Autoantibodies in SLE

Immune reaction against self-antigens is primarily prevented within the thymus in a process called central tolerance. Despite the rigorous screening of the evolving T-cell repertoire, some autoreactive T cells escape from the thymus [**138**]. To avoid autoimmunity, multiple operations ensure the control of the "escaped" T-cell repertoire at the periphery such as induction of anergy, deletion of autoreactive T cells, and activation or induction of regulatory T cells (Tregs) [**138**]. The presentation of self-antigens at the periphery, similarly to the thymus, is carried out by multiple antigen presenting cells (APCs) such as stromal cells and dendritic cells (DCs) [**139**].

Dendritic cells acquire antigens via phagocytosis, receptor mediated endocytosis, and macropinocytosis that lead to the presentation of these antigens to T cells. Autoimmune diseases are associated with multiple auto antigens against which the tolerance is broken [**140**]. Firstly, the mechanism of antigen capture can influence the outcome of the response induced by DCs. Indeed, apoptotic cells (unlike necrotic cells) or soluble proteins, as major sources of self-antigen presentation at the periphery, resulted in DC activation [**141**]. Secondly, in several autoimmune disorders multiple post-translational protein modifications have been observed resulting in alteration of self-antigens formation against which the immune system has not been exposed and tolerized. Multiple autoimmune disorders were dependent on the presence of such post-translational modifications of autoantigens [**142**]. Acetylation of myelin basic protein was required for the development of EAE as non-acetylated peptides failed to stimulate T cells or induce the disease [**143**]. Similar post translational modifications were involved in the autoimmune process in

lupus. Importantly, these modified proteins could be produced and/or taken up by DCs for presentation to T and B cells.

H4) Anti-DNA antibodies in SLE

Anti-DNA antibodies constitute a subgroup of antinuclear antibodies that bind to either single-stranded or double stranded DNA [**144**]. Both subtypes of DNA-binding antibodies may be found in SLE. Due to their high specificity, anti-dsDNA antibodies are universally used as a diagnostic criterion for SLE (70–98% of patients are positive for such antibodies) and for monitoring the clinical course of the patient [**145**]. Immunofluoresence (IF) on *Crithidia luciliae*, radio-immuno assay (RIA), and ELISA are the most commonly used assays to detect anti-dsDNA antibodies. IF-based *Crithidia* assay is probably the most specific technique, but ELISA is the most ractical and clinically relevant method. Anti-dsDNA antibodies, in particular of the IgG isotype, have an important pathogenetic role in SLE. A clear-cut relationship exists, for example, between anti-dsDNA antibodies (R4A antibody) and disease activity in lupus nephritis [**146**]. Anti-DNA-DNA immune complexes can deposit in the mesangial matrix and their subsequent complement activation leads to inflammation and mesangial nephritis. Moreover, anti-dsDNA antibodies also contribute to the end-stage lupus nephritis by directly binding exposed chromatine fragments in glomerular basement membrane [**147**].

H5) Autophagy in Autoimmunity and SLE

Autophagy has been implicated in many physiological and pathological processes. During T-cell development in the thymus, scanning of peptide/MHC molecule complexes on the surface of thymic epithelial cells (TECs) ensures that only useful (self-MHC restricted) and harmless (self-tolerant) thymocytes survive. Interestingly, a recent study had demonstrated that TECs displayed high levels of constitutive autophagy, suggesting that autophagy could shape the T-cell repertoire during thymic selection [**148**]. Additionally, LC3-II molecules have been shown to be colocalized with lysosomal compartments, in which MHC class II complexes are formed, thus implying that autophagy could intersect the MHC class II presentation pathway and consequently play an important role in presenting self-antigens to immature T cells in the thymus [**149**].

The autophagy process has not been directly explored in SLE, although a number of studies from different laboratories have suggested that autophagy-related abnormalities may be involved in the pathogenesis of the disease. mTOR signaling has been implicated as a major integrator of signals related to cellular nutrient and energy status, playing a crucial role in the regulation of cellular metabolism [**150**]. Activation of mTOR signaling has recently emerged as a key factor in abnormal activation of lymphocytes in SLE [**151**]. Moreover, genome-wide association studies have linked SNPs in Atg5 to SLE susceptibility [**152**]. Although the effects of these SNPs on Atg5 expression and function are not known, loss of Atg5-dependent effects, including regulation of proinflammatory cytokine secretion, clearance of dying cells, and cell antigen presentation, might contribute to the autoimmunity and inflammation associated with SLE [**153**].

Finally, hydroxychloroquine (CQ) is one of the most effective treatments of mild SLE manifestations and it is also used in preventing the occurrence of new mild SLE manifestations. Hydroxychloroquine is considered as an antimalarial drug which inhibits lysosome function, thereby inhibiting TLR activation leading to a down-regulation of IFN-α and decreasing the antigen processing necessary for autoantigen presentation. Since, lysosomes are the final step of the autophagic machinery it is therefore possible that hydroxychloroquine as a treatment acts to the autolysosomes too. Thus, a link between autophagy deregulation and SLE pathogenesis is biologically plausible, although not yet proven [**154**].

H6) Mitochondria in SLE T cells

A link between systemic lupus erythematosus (SLE) and disturbances in metabolic and organelle homeostasis, importantly within the mitochondrial compartment, have been recently identified in lupus T cells [**155**]. SLE T cells exhibit mitochondrial dysfunction characterized by increased mitochondrial mass, high $\Delta\psi_m$ as well as reduced production of ATP [**156**]. It is proposed that accumulation of defective mitochondria may occur as a result of defective autophagic turnover of these organelles (mitophagy) [**157**] and increased nitric oxide (NO)-dependent biogenesis (the latter one masks the effect of IL-15 on mitochondrial biogenesis and ATP production) [**158**] [**Fig.H6**]. These features allow for sustained T cell activation [**159, 160**]. Therefore, mitochondrial dysfunction can serve as a biomarker for SLE and act as a potential target for therapy.

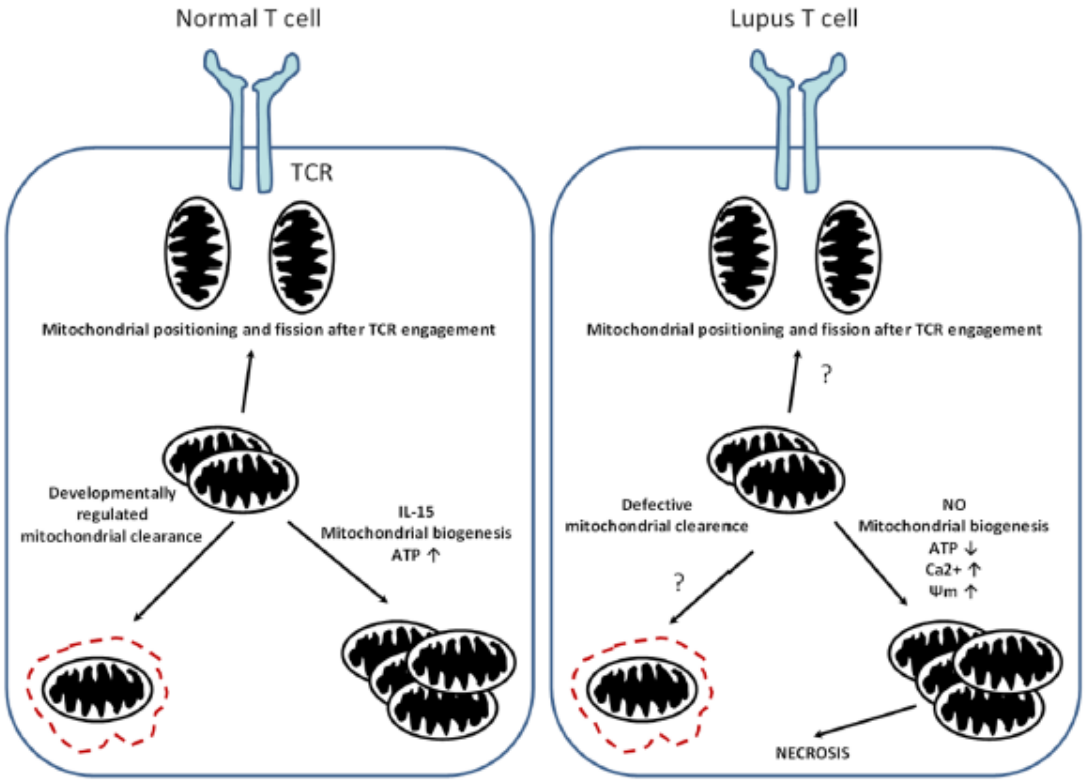


Figure H6: Mitochondrial homeostasis in normal and lupus T cells. *Clin Immunol*., Perl et.al., 2012

IV) Aim of the project

Research on the cellular and molecular processes leading to SLE development has led to the conclusion that the signaling pathways which influence the generation of self-antigens and their presentation (leading to the “effector” phenotype of DCs), represent a crucial part of SLE pathophysiology. Hence, it would be of utmost importance to elucidate the molecular pathways and mechanisms involved in the generation of this pool of self-antigens with the ultimate aim of finding a way to inhibit their presentation, so as to suppress SLE manifestations and pathogenesis.

According to previous results of the lab, increased induction of the autophagic flux combined with disrupted autophagy completion has been recorded in monocytes derived from SLE patients with respect to monocytes from healthy individuals. Furthermore, treatment of healthy monocytes with SLE serum reproduced the same ‘autophagic phenotype’ that was observed in monocytes from SLE patients.

Since SLE patients are characterized by increased IFNα signaling and IFNα is a soluble factor found in high titers in SLE serum, **the first objective** of the study was to investigate if this certain cytokine could be responsible for the occurrence of the aforementioned events and if rapamycin treatment could reverse the effect of incomplete autophagy. **The second objective** of the study was to assess the ‘mitophagic’ phenotype of SLE monocytes upon IFNα signaling. Finally, **the third objective** of the study was to identify the potential link between autophagy, mitophagy and the autoreactive phenotype of SLE monocytes for the subsequent presentation of autoantigens following IFNα signaling.

V) Materials and Methods

A) Patients

15 active SLE patients diagnosed according to the American College of Rheumatology 1982 criteria and followed up at the Rheumatology Department of the University Hospital of Heraklion (Crete) were studied. Active SLE was defined as an SLE Disease Activity Index score (SLEDAI) higher than 8. Patients had not received steroids for at least 24 hours before blood sampling. Healthy age- and sex-matched volunteers from the Department of Transfusion Medicine of the University Hospital of Heraklion (Crete) served as controls. All subjects gave written informed consent prior to study.

B) Serum collection

Healthy and/or SLE peripheral blood was added in a collection tube without anticoagulants. The tube was centrifuged at 2500 rpm for 15mins (Room Temperature) and the serum was collected under sterile conditions.

C) $CD14^+$ monocyte cell isolation

C1) Peripheral Blood Mononuclear Cell (PBMC) isolation

Peripheral blood mononuclear cells were isolated from human peripheral blood by Ficoll-Histopaque density gradient centrifugation. Peripheral blood from healthy volunteers or SLE patients, supplemented with anticoagulants (heparin or EDTA), was diluted with 1 volume PBS. The diluted blood was overlayed onto Ficoll-Histopaque (dilution 1:2) and was centrifuged at 1800 rpm (600g), 30 minutes, 21^{o}C with no brakes. The mononuclear cell layer (white interphase) was aspirated with a Pasteur pipette and was washed twice with PBS (1500 rpm for 5 min centrifugation). After resuspending the cell pellet in PBS, a volume of cells was diluted with Trypan Blue and placed onto Neubauer haemocytometer so as to be counted by light microscopy.

C2) $CD14^+$ monocyte positive cell separation

Isolated peripheral blood mononuclear cells, diluted in MACS buffer (ACD, 7.5% BSA, Sodium Bicarbonate, PBS) were incubated with appropriate volume of $CD14^+$ microbeads for 15 minutes at 4^{o}C. (80ul MACS buffer and 20ul $CD14^+$ microbeads per 10^7 total PBMCs). After undergoing one wash with MACS buffer, the cells were resuspended with 500µl or 3ml MACS buffer and the

suspension was applied onto a MACS MS or LS column, properly placed in the magnetic field of a MiniMACS™ Separator, repsectively. 3 washes with 500μl or 3ml MACS buffer (for MS or LS column respectively) were performed prior to the removal of the column from the separator. The column was then placed into a suitable collection tube and 0.5ml or 3ml MACS buffer (for MS or LS column) was added onto the column. The magnetically labeled $CD14^+$ monocyte cells were flushed out by firmly pushing the plunger into the column. Monocytes were mixed with Trypan Blue and the suspension was placed onto Neubauer haemocytometer in order to be counted by light microscopy. Purity ($CD14^+$ cells/total separated live cells) was evaluated by flow cytometry.

C3) Cell culture

Purified monocytes at a concentration of 10^6 cells/well were cultured in 12-well or 24-well treated plates in RPMI-1640/L-glutamine, supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin and 100μg/mL streptomycin. The concentrations used for the treatments were: rhIFNa (10^4U/ml), chloroquine (CQ: 46.1μM), rapamycin (Rapa: 1 μM), MitoTEMPO (2nM). Healthy or SLE serum treatments were used in 10% v/v in serum free RPMI-1640/L-glutamine medium.

D) RNA isolation

Total RNA from monocytes was collected using the TRIZOL extraction protocol. Total RNA was treated with DNAse in order to eliminate any genomic DNA contaminations. Turbo DNAse kit (Ambion) was used according to manufacturer's protocol.

E) Real time-PCR (RT-PCR)

cDNA was prepared from isolated RNA using PrimeScript™ 1st strand cDNA Synthesis Kit according to manufacturer's protocol. 500ngs of RNA were used as a template for every reaction and were mixed initially with appropriate volume of Oligo dT primers and mixture of dNTPs. After incubation for 5 minutes at 65^o C at a Veriti 96 well thermal cycler, the samples were cooled immediately on ice. Next, appropriate volumes of 5x PrimeScript buffer, RNase inhibitor, PrimeScript Reverse Transcriptase and RNase-free water were added and the samples were incubated at 50^oC for 45 minutes and at 95^oC for 5 minutes at a Veriti 96 well thermal cycler, followed by cooling on ice. RNAse H (2U/reaction) was added in order to clean the resulting cDNA from any RNA and incomplete cDNA products. cDNA was stored at -20^oC. PCR amplification of the resulting cDNA

samples was performed using appropriate volumes of KAPA SYBR® FAST Universal 2x qPCR Master Mix and specific for each gene primers at a CFX Connect™, Real-Time System. Total volume of each pcR reaction was 20µl. The following PCR conditions were used for LC3, ATG5, p62 and GAPDH: 95°C for 3 minutes, 39 cycles at 95°C for 3 seconds and annealing temperature at 60°C for 30seconds. The following PCR conditions were used for PINK1 and GAPDH: 95°C for 3 minutes, 39 cycles at 95°C for 3 seconds and annealing temperature at 57°C for 30seconds. The following PCR conditions were used for Parkin and GAPDH: 95°C for 3 minutes, 39 cycles at 95°C for 3 seconds and annealing temperature at 53°C for 30seconds. GAPDH was used as a reference gene for normalization and data were analyzed with the $2^{-\Delta\Delta CT}$ method.

Gene ID	Forward primer	Reverse primer
LC3	5'- CTG TTG GTG AAC GGA CAC AG-3'	5'- CTG GGA GGC ATA GAC CAT GT-3'
ATG5	5'- TGA CGT TGG TAA CTG ACA AAG TG-3'	5'- AAT GCC ATT TCA GTG GTG TG-3'
p62	5'- AGC AGA TGA GGA AGA TCG CC-3'	5'- CTG TAG ACG GGT CCA CTT CTT-3'
PINK1	5'- GGA GTA TGG AGC AGT CAC TTA CAG-3'	5'- GGC AGC ACA TCA GGG TAG TC-3'
PARKIN	5'- CGA CCC TCA ACT TGG CTA CT -3'	5' - TCT TTA ATC AAG GAG TTG GGA CA- 3'
GAPDH	5'- CAT GTT CCA ATA TGA TTC CAC C-3'	5'- GAT GGG ATT TCC ATT GAT GAC-3'

F) Immunoblotting

RIPA lysis buffer including both protease and phosphatase inhibitors was used for collection of total protein lysates. SDS-PAGE electrophoresis was performed and 40-60µgs of protein lysate were loaded in each well. PVDF-membrane was used for protein transfer and membranes were incubated for 1hr with primary antibodies at RT. 3 washes with PBST were performed before the addition of the HRP-conjugated secondary antibody. Membranes were incubated with ECL and band intension was analyzed with Fiji. Mouse anti-LC3 and rabbit anti-p62 antibodies were used at 1:1000 dilution and mouse anti-actin at 1:5000 dilution. The HRP-conjugated secondary antibodies used were: anti-mouse and anti-rabbit at 1:2000 dilutions.

G) Flow cytometry

Treated monocytes were scraped from the 24-well plate and washed once with PBS by undergoing centrifugation at 1500 rpm for 10 minutes at 4^{o}C. Next, they were stained with appropriate volume of monoclonal anti-human antibodies according to the manufacturer's protocol. After incubating the cells for 20 minutes at RT in the dark, the cells were washed with 0.5 ml PBS/FBS 5% and centrifuged at 1500 rpm for 10 minutes at 4^{o}C. Then, the cells were subjected to flow cytometry. Analysis was performed with FlowJo software. The antibodies that were used were anti-HLADR and anti-CD86. Moreover, the MitoSOX Red mitochondrial superoxide indicator (M36008) was used according to manufacturer's instructions.

H) ATP Assay

Treated monocytes (10^6 cells/ condition) were harvested and washed according to the kit protocol (ab83355) and samples were then used in 1:4 dilution. The colorimetric assay was used according to the instructions provided. The calculation of ATP levels of the samples occurred according to the formula given: $[ATP]\ (nmol\ per\ \mu L\ or\ mM) = (Ts/Sv) * D$

Where: Ts = ATP amount from standard curve (nmol or mM).

Sv = sample volume added in sample wells (μL).

D = sample dilution factor.

I) DNA Isolation

DNA was isolated and prepared with the use of QIAamp DNA Micro kit (56304) following exactly the manufacturer's protocol.

J) Confocal microscopy

For immunofluorescence analysis Leica SP8 inverted confocal microscope was used. The analysis of pictures taken was 1024 x 1024, speed scan was set at 700Hz and bidirectional mode was on. 63x objective was used with zoom ranking between 2.0-3.0. Hybrid detectors were used and z-step size was set at 0.5μm for z-scan series.

K) TaqMan Assay

For the quantification of mitochondrial DNA copy number a multiplex RT-PCR assay was used according to the publication: 'Simultaneous quantification of mitochondrial DNA copy number and deletion ration' [**161**]. DNA samples (50ng/reaction) were used together with appropriate volumes of TaqMan^{R} Fast Universal PCR Master Mix (2x), No AmpErase^{R} UNG (Applied Biosystems) and specific volumes for each gene primer and probe (according to the manufacturer) at a CFX Connect™, Real-Time System. Total volume of each multiplex RT-PCR reaction was 25μl.

Gene ID	Forward primer	Reverse primer
β2M	5'- TTA ACG TCC TTG GCT GGG TC-3'	5'- ACT GGA AGA CAA AGG GCT CG-3'
mt (major arc)	5'- CTG TTC CCC AAC CTT TTC CT-3'	5'- CCA TGA TTG TGA GGG GTA GG-3'

Nuclear huβ2M probe (HEX)	5'- CAG ATG CAG TCC AAA CTC TCA CT-3'
Mitochondrial probe (FAM)	5'- GAC CCC CTA ACA ACC CCC-3'

L) ELISA

The Interleukin-6 (IL-6) protein levels were assessed with the use of the kit: Human IL-6 ELISA Ready-Set-Go (e-Bioscience 88-7066). Samples were used in 1:2 dilutions. The Tumor Necrosis Factor-α (TNF-α) protein levels were assessed with the use of the kit: Human TNF-α ELISA Ready-Set-Go (e-Bioscience 88-7346). Samples were used in 1:2 dilutions.

M) Statistical Analysis

Statistical analysis was performed using unpaired t-test in Graph Pad Prism 6 software. P value < 0.05 was considered as indicative of statistical significance.

VI) Results

A) Autophagy in the context of SLE

A1: Increased induction of the autophagic flux in SLE monocytes compared to healthy controls (previous results of the lab)

For the experimental monitoring of autophagy, RNA and total protein lysate were collected from $CD14^+$ freshly isolated monocytes both from healthy donors (as a control) and SLE patients and were subjected to real time PCR analysis and immunoblotting. ATG5 mRNA levels were quantified regarding the autophagic induction and LC3II/I ratio regarding the autophagic index. Significantly increased ATG5 mRNA levels [**Fig.A1.1**] and LC3II/I ratio [**Fig.A1.2**] were observed in monocytes from SLE patients, indicating induction of the autophagic flux.

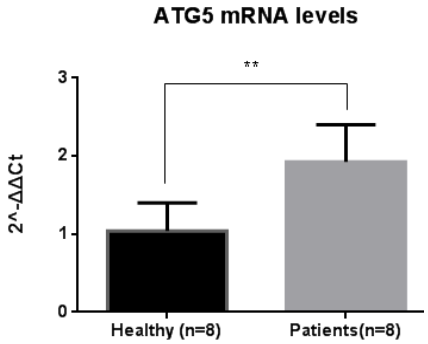


Figure A1.1: Increased ATG5 mRNA levels in SLE $CD14^+$ monocytes. Real-time PCR for ATG5 mRNA levels of $CD14^+$ Monocytes. Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test).

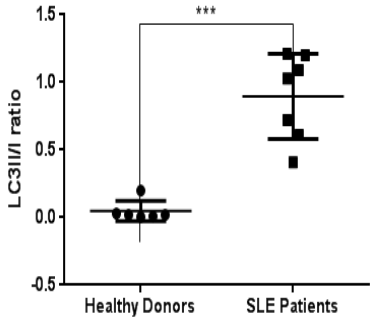


Figure A1.2: Increased LC3II/I ratio in SLE $CD14^+$ Monocytes. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-LC3 and anti-actin antibodies. Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test).

A2: Disrupted autophagy completion in SLE monocytes compared to healthy controls (previous results of the lab)

Since p62 is itself a substrate of the autophagic machinery and is degraded inside the autophagolysosomes upon successful completion of autophagy [**Fig.A2.1**], p62/actin ratio was quantified by immunoblotting, in order to assess autophagy completion. Total protein lysate and RNA were collected form $CD14^+$ freshly isolated monocytes both from healthy donors and SLE patients and were subjected to immunoblotting and Real-time PCR analysis. Increased p62 protein levels were observed in SLE monocytes by western blot analysis [**Fig.A2.2 A, B**]. These results could be interpreted either by increased production of p62 in transcriptional level or by defective degradation of p62 inside the autophagolysosomes. In order to assess this, p62 mRNA levels were quantified by Real-time PCR. There was no difference observed between p62 mRNA levels in SLE monocytes compared to healthy controls [**Fig.A2.2 C**], thus it is supported that increased p62 protein levels were due to its defective degradation.

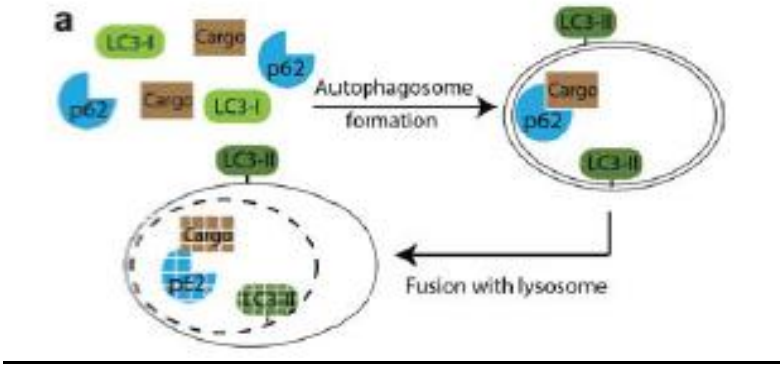


Figure A2.4: Degradation of p62 inside the autophagolysosomes. Image by *Nature Immunology*. Ahmed et al., 2014

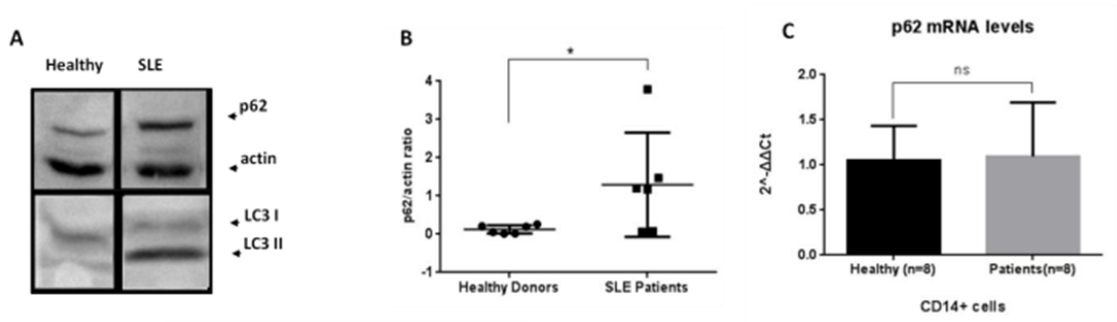


Figure A2.2: Autophagy completion is disrupted in SLE monocytes. A) Representative image of SDS-PAGE immunoblotting of CD14$^+$ cell lysates from healthy donor and SLE patient with the indicative antibodies. B) Quantitational analysis of p62 protein levels from 6 healthy donors compared to 6 SLE patients. C) Real-time PCR for p62 mRNA levels from freshly isolated healthy/SLE CD14$^+$ Monocytes. Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test).

A3: SLE serum induces autophagy in healthy monocytes but disrupts its completion (previous results of the lab)

In order to assess whether autophagy induction was due to some genetic predisposition of SLE patients and/or due to soluble factors inside their serum, healthy CD14$^+$ freshly isolated monocytes were treated with 10% $^v/_v$ healthy or SLE serum for 24hrs and total protein lysate and RNA were collected and subjected to immunoblotting and Real-time PCR analysis, respectively. Western blot results showed increased LC3II/I ratio in the cells treated with SLE serum compared to healthy serum [**Fig. A3 A-B**], indicating that SLE serum can induce autophagy in healthy monocytes. Additionally, p62 protein levels were similarly increased [**Fig.A3 C**], as in SLE CD14$^+$ freshly isolated monocytes, but its mRNA levels indicated no difference between the samples that were treated with healthy serum with respect to those treated with SLE serum [**Fig.A3 D**]. Together these observations imply that SLE serum is capable of inducing autophagy in healthy monocytes but disrupts its completion.

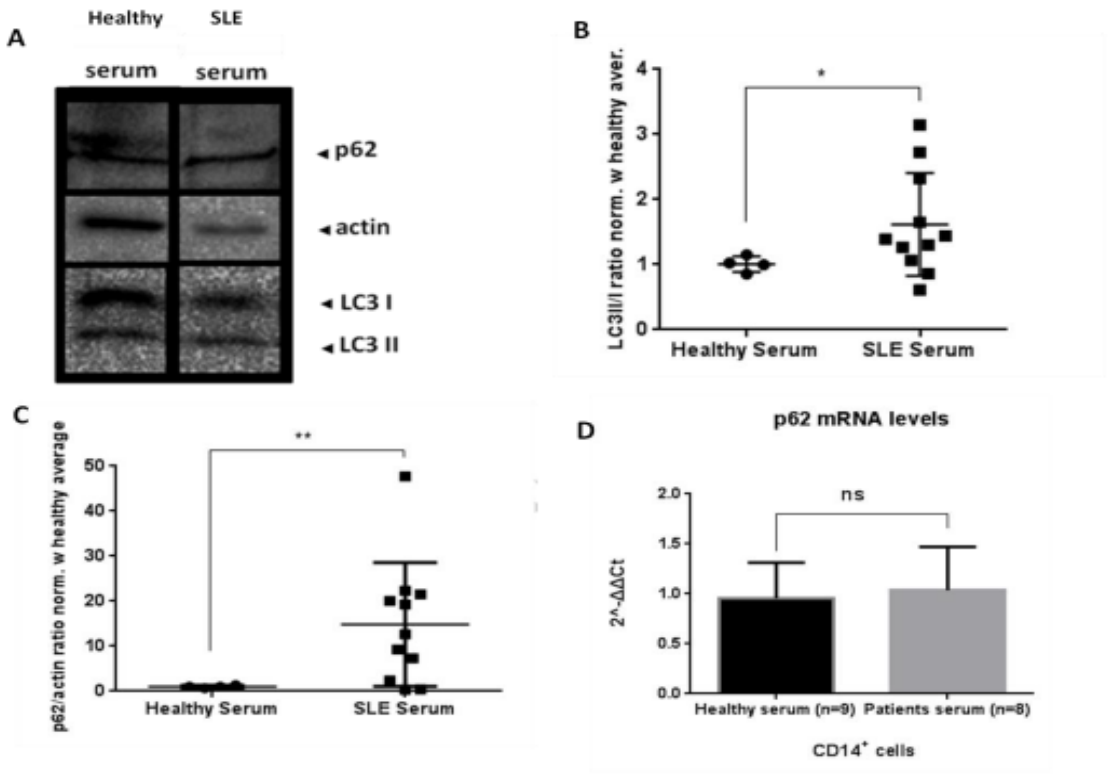


Figure A3: SLE serum induces autophagy in healthy monocytes but disrupts its completion. A) Representative image of SDS-PAGE immunoblotting of healthy CD14$^+$ cell lysates treated with 10% healthy or SLE serum for 24hrs. B, C) Quantitational analysis of LC3II/I ratio and p62 protein levels from 4 and 10 different healthy and SLE sera respectively. D) Real-time PCR for p62 mRNA levels from healthy CD14$^+$ cells treated with 10% healthy or SLE serum for 24hrs. Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test).

A4: Recombinant human IFNα induces the autophagic flux in healthy monocytes but deregulates its completion

Since type I IFNs and more specifically IFNα have been demonstrated to be increased in the blood of SLE patients [**130**] and monocytes cultured with SLE serum gain the ability to induce an MLR and are characterized as IFNa-DCs [**135**], we speculated that IFNα might be the soluble factor inside SLE serum that causes the induction of autophagy and is responsible for its deregulation as far as its completion is concerned. For this purpose, healthy CD14$^+$ freshly isolated monocytes were treated with human recombinant rIFNα in different timepoints (4hrs, 18hrs) and RNA as well as total protein lysate were collected. The mRNA levels of ATG5 and LC3b were significantly increased 18hrs after treatment with rIFNα with respect to the control (untreated) [**Fig.A4 A, B**].

Western blot analysis showed that both LC3II and p62 levels were increased upon rIFNα treatment (**Fig.A4 D**). This was consistent with the results obtained when healthy monocytes were treated

with SLE serum (**Fig.A3 A, B**). p62 protein levels were similarly increased but its mRNA levels displayed no difference upon treatment of healthy monocytes with rIFNα [**Fig. A4 C**], indicating disruption of autophagy completion.

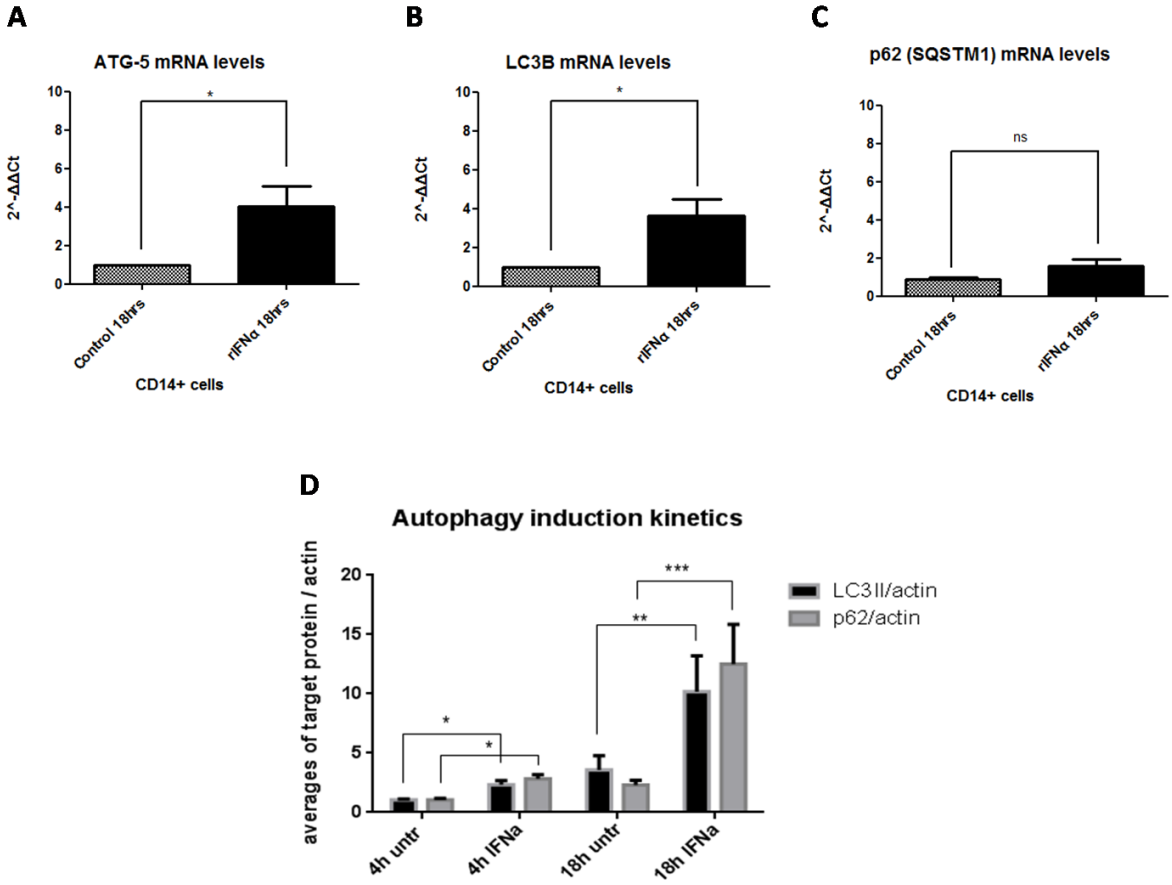


Figure A4: Treatment of healthy monocytes with recombinant IFNα induces autophagy but deregulates its completion. A-C) Real-time PCR for ATG5, LC3b and p62 mRNA levels from healthy CD14$^+$ cells treated with rIFNα for 18hrs. D) Quantitation of LC3II and p62 protein levels detected by immunoblotting upon 4hrs and 18hrs treatment of healthy monocytes with rIFNα. Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test).

A5) Rapamycin treatment reverses the effect of rIFNα on deregulation of autophagy completion

Since rapamycin is an established inducer of the autophagic flux, it was hypothesized that it might be able to reverse the effect of incomplete autophagy upon IFNα signaling. Therefore, healthy $CD14^+$ freshly isolated monocytes were treated with human recombinant rIFNα, rapamycin (Rapa), chloroquine (CQ) or not treated at all (negative control) for 18hrs and total protein lysate was collected. Chloroquine was used as a positive control, since it is known that it blocks autophagy in the level of autophagolysosomes and leads to accumulation of p62 protein. The 18hr timepoint was chosen for this purpose, because according to the previous results depicted above [**Fig.A4 D**] the greatest effect of rIFNα regarding incomplete autophagy was observed in 18hrs. Interestingly, western blot analysis revealed that p62 protein levels were decreased after simultaneous treatment of healthy monocytes with rIFNα and rapamycin compared to treatment with rIFNα alone, indicating reverse in deregulation of autophagy completion [**Fig.A5 A, B**].

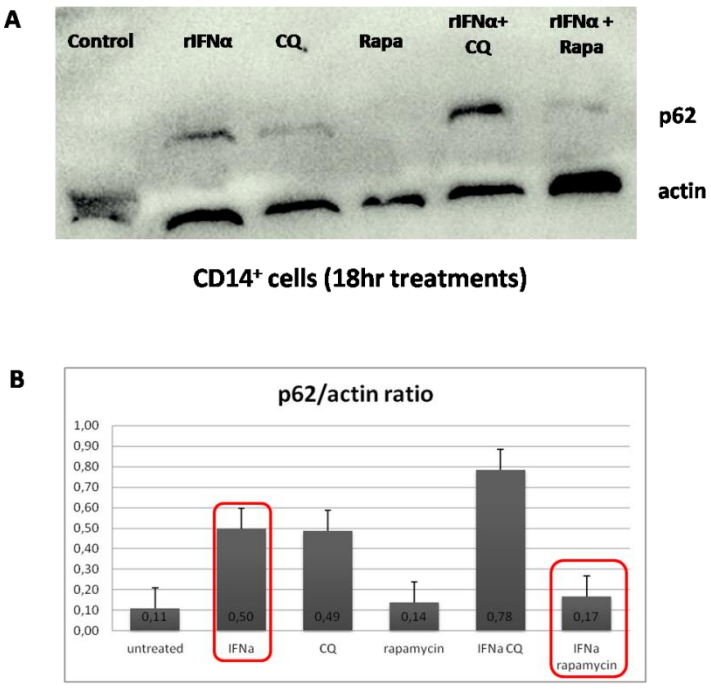


Figure A5: Treatment of healthy monocytes with rapamycin reverses the effect of rIFNα on incomplete autophagy. A) Representative image of SDS-PAGE immunoblotting of healthy $CD14^+$ cell lysates treated with rIFNα, rapamycin, CQ and combos for 18hrs. B) p62/actin ratio obtained by immunoblotting after 18hrs treatment of healthy monocytes with rIFNα, rapamycin, CQ and combos.

B) Mitophagy in the context of SLE

B1) Increased induction of the mitophagy pathway in SLE monocytes compared to healthy controls

So far, it has been shown that both SLE monocytes and healthy monocytes treated with SLE serum and/or rIFNα displayed induction of the autophagic flux. At this point, it was speculated that the mitophagy pathway for the selective clearance of the superfluous or defective mitochondria could be induced as well upon IFNα signaling. Hence, for the experimental monitoring of mitophagy, RNA was collected from $CD14^+$ freshly isolated monocytes both from healthy donors (as a control) (n=8) and SLE patients (n=8) and was subjected to Real-Time PCR analysis. The mRNA levels of the two major players of the mitophagy pathway, PINK1 and Parkin, were examined. Although Parkin mRNA levels did not display any difference between healthy and SLE monocytes, a significant increase in PINK1 mRNA levels was observed in SLE monocytes compared to healthy controls [**Fig.B1**]. Since PINK1 is considered as the basic sensor of mitochondrial dysfunction inside the cell [**113**], it is proposed that mitochondria of SLE monocytes might display damage and thus, the mitophagy pathway is induced.

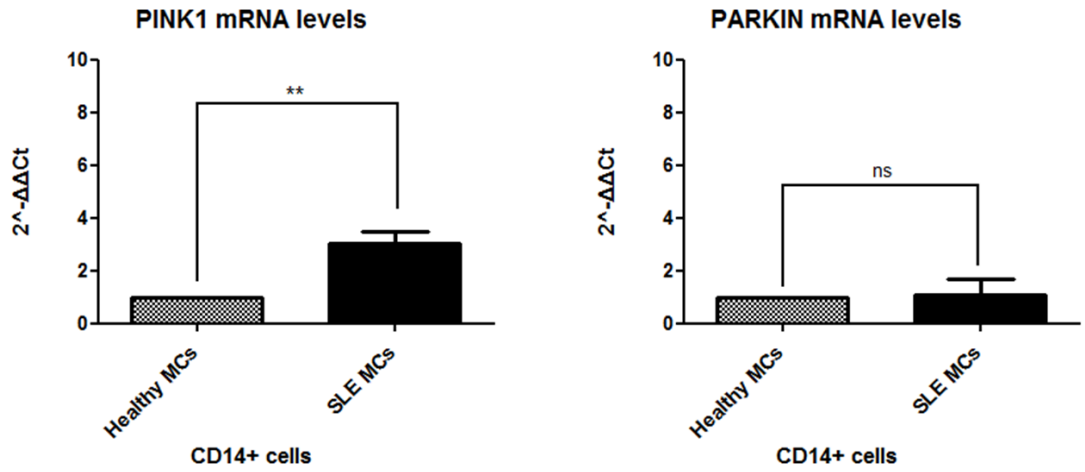


Figure B1: SLE monocytes exhibit increased induction of the mitophagy pathway compared to healthy controls. Real-time PCR for PINK1 and Parkin mRNA levels from freshly isolated $CD14^+$ SLE (n=8) and healthy (n=8) monocytes. Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test).

B2) SLE serum induces the Parkin-dependent mitophagy pathway in healthy monocytes

In order to assess whether mitophagy induction was due to some genetic predisposition of SLE patients and/or due to soluble factors inside their serum, healthy $CD14^+$ freshly isolated monocytes were treated with 10% $^v/_v$ healthy (n=4) or SLE serum (n=4) for 18hrs and 24hrs and RNA was collected and subjected to Real-Time PCR analysis. The mRNA levels of both PINK1 and Parkin were increased 24hrs and 18hrs, respectively, after treatment of healthy monocytes with SLE serum compared to those of monocytes treated with healthy serum [**Fig.B2**]. This result implied that SLE serum is able to induce alone the mitophagy pathway in healthy monocytes. Also, the significant increase in Parkin mRNA levels indicated induction of the Parkin-dependent pathway of mitophagy.

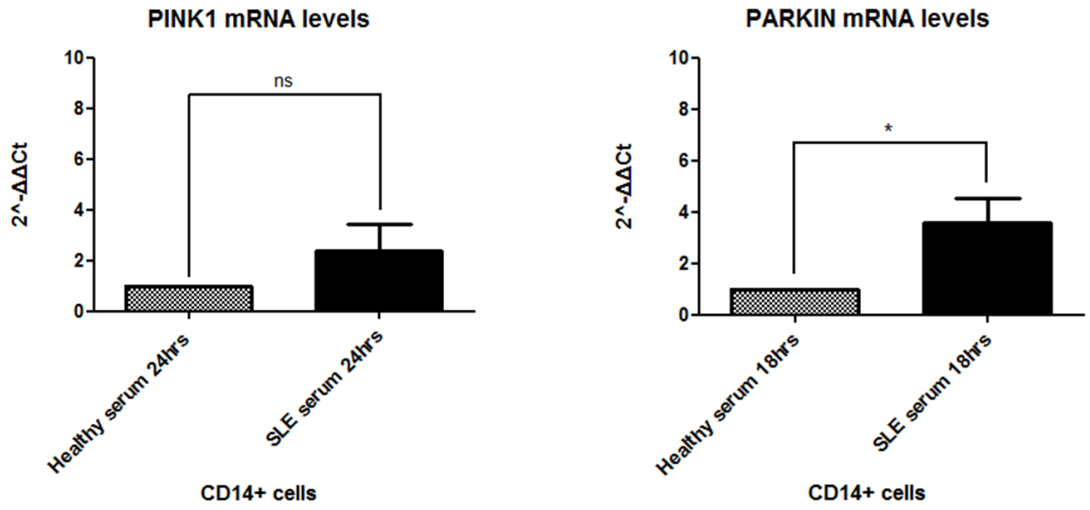


Figure B5: Healthy monocytes treated with SLE serum display increased induction of the Parkin-dependent mitophagy pathway with respect to those treated with healthy serum. Real-time PCR for PINK1 and Parkin mRNA levels from freshly isolated $CD14^+$ healthy monocytes treated with 10% v/v healthy (n=4) and SLE (n=4) serum for 18hrs and 24hrs. Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test).

B3) Recombinant human rIFNα leads to mitochondrial hyperpolarization and induction of the Parkin-dependent mitophagy pathway

In order to assess if mitochondria display damage upon IFNα signaling and thus, the mitophagy pathway is subsequently induced, freshly isolated $CD14^+$ healthy monocytes were treated with rIFNα for 8hrs and 18hrs and then stained with the JC-1 dye, a mitochondrial membrane potential probe. Also, RNA was collected and subjected to Real-Time PCR analysis. Interestingly, a significant increase in the number of JC-1 aggregates (proportional to $\Delta\psi_m$) was observed in healthy monocytes treated with rIFNα compared to the untreated ones [**Fig.B3 A**]. This result implied that mitochondria are hyperpolarized in monocytes upon IFNα signaling. Furthermore, the mRNA levels of Parkin were increased in monocytes treated with rIFNα for 8hrs (but not 18hrs) with respect to the untreated ones [**Fig.B3 B**], indicating an early induction of the Parkin-dependent mitophagy pathway upon IFNα signaling.

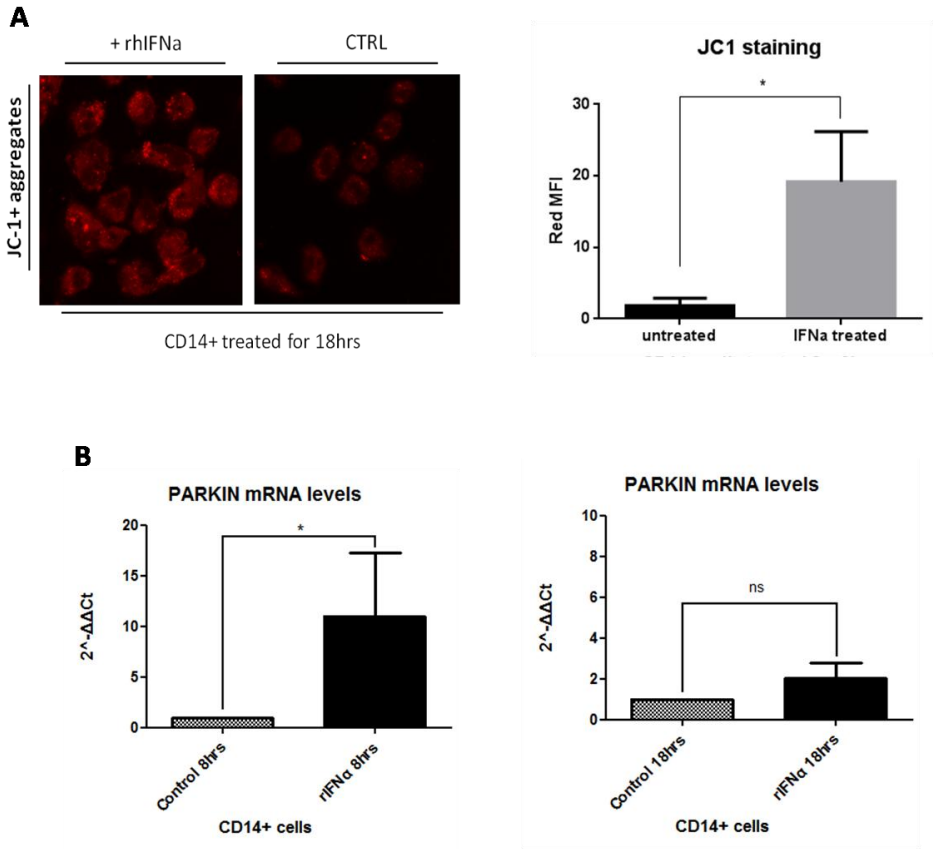


Figure B6: Healthy monocytes treated with rIFNα display hyperpolarization and increased induction of the Parkin-dependent mitophagy pathway compared to the untreated ones. A) Immunofluorescence microscopy of $CD14^+$ healthy monocytes treated with rIFNα for 18hrs. Cells were stained with the JC-1 dye. Representative image (left panel). Quantification (right panel). B) Real-time PCR for Parkin mRNA levels from freshly isolated $CD14^+$ healthy monocytes treated with rIFNα for 8hrs and 18hrs. Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test).

B4) Recombinant human rIFNα leads to increased mitochondrial ROS production and altered mitochondrial activity. Rapamycin treatment reverses this effect

The association between mitochondrial hyperpolarization and increased ROS production together with decreased ATP levels in SLE patients has been already described [**162**]. Since rIFNα leads to mitochondrial hyperpolarization according to the results shown above [**Fig.B3 A**], it was hypothesized that rIFNα could possibly affect ROS generation and ATP production by the Electron Transfer Chain (ETC) too. Also, it was speculated that rapamycin treatment could ameliorate this effect caused by rIFNα. For this purpose, freshly isolated $CD14^{+}$ healthy monocytes were treated with rIFNα, rapamycin and their combination for 2hrs and 18hrs. Afterwards, the cells were either stained with MitoSOX dye for the examination of ROS production (2hrs treatments) or used for the assessment of ATP levels (18hrs treatments). As it was expected, healthy monocytes treated with rIFNα exhibited increased ROS generation [**Fig.B4 A**] and decreased ATP production [**Fig.B4 B**] compared to the controls. Interestingly, rapamycin treatment reversed the effect caused after rIFNα treatment [**Fig.B4 A, B**]. These results indicate that besides mitochondrial hyperpolarization, upon IFNα signaling mitochondria are characterized by reverse electron flow in the ETC (↑ROS, ↓ATP), which is responsible for the subsequent mitochondrially-derived oxidative stress and altered mitochondrial function.

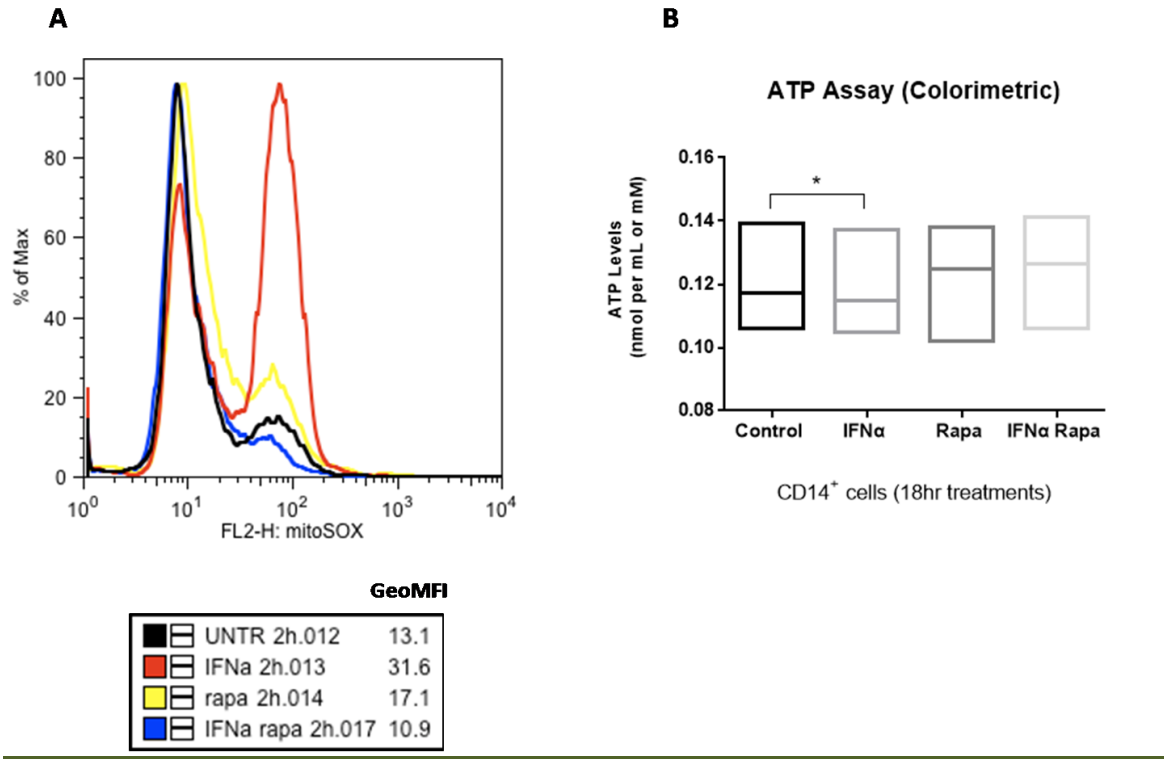


Figure B7: Healthy monocytes treated with rIFNα display increased mitochondrial ROS generation and decreased ATP production. A) Healthy CD14$^+$ monocytes were treated with rIFNα, rapamycin and their combination for 2hrs, stained with MitoSOX and assessed by flow cytometry. B) Healthy CD14$^+$ monocytes were treated with rIFNα, rapamycin and their combination for 18hrs and were used for examination of the ATP levels. Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test).

B5) Accumulation of dysfunctional mitochondria in SLE monocytes

Since SLE monocytes display deregulated autophagy completion, it was hypothesized that a similar effect regarding the proper completion of mitophagy might also take place in the SLE environment. Therefore, CD14$^+$ freshly isolated monocytes both from healthy donors (as a control) (n=5) and SLE patients (n=5) were subjected to staining with Mitotracker CMXRos (mitochondrion-selective probe) for the assessment of the mitochondrial mass in these two groups. The result of the Immunofluorescence microscopy revealed that monocytes derived from SLE patients are characterized by higher accumulation of defective mitochondria compared to those from healthy controls [**Fig.B5 A**]. In order to confirm the previous result, total DNA from CD14$^+$ freshly isolated monocytes both from healthy donors (as a control) (n=3) and SLE patients (n=3) was collected and used for the quantification of mitochondrial (FAM probe) to nuclear (HEX probe) DNA ratio through multiplex Real-Time PCR (TaqMan assay). It is known that in this sensitive type of reaction the lower the cycle (Cq) ratio is, the highest the amount of mitochondrial DNA is as well [**161**]. Interestingly,

SLE monocytes are characterized by lower FAM Cq/ HEX Cq ratio with respect to healthy controls [**Fig.B5 B**]. So, the amount of mitochondrial DNA was found higher in SLE monocytes and this was a second indication for the accumulation of defective mitochondria together with their potentially damaged DNA in the SLE environment. Therefore, it is implied that mitophagy completion is also deregulated in SLE monocytes.

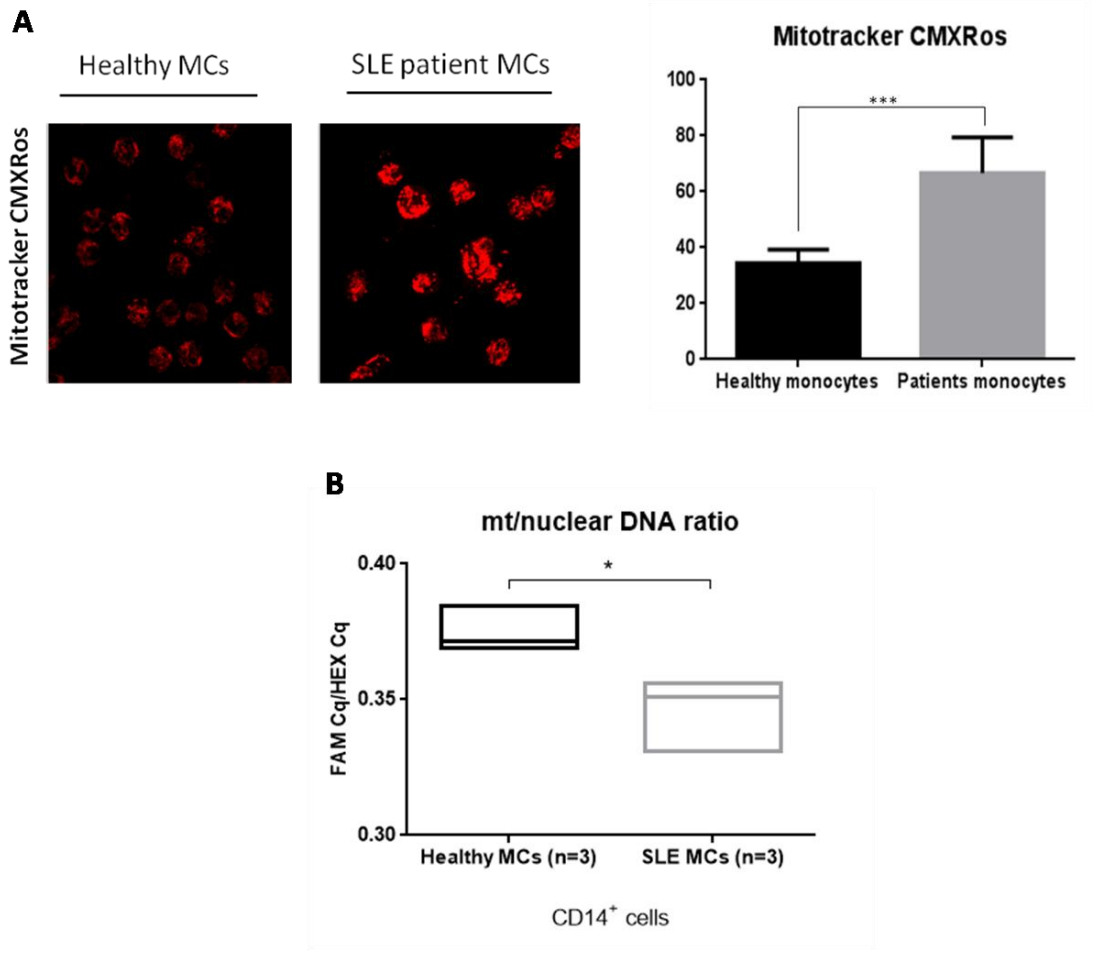


Figure B8: SLE monocytes are characterized by accumulation of defective mitochondria. A) Immunofluorescence microscopy of freshly isolated CD14$^+$ SLE (n=5) and healthy (n=5) monocytes. Cells were stained with the Mitotracker CMXRos probe. Representative image (left panel). Quantification (right panel). B) Multiplex Real-Time PCR (TaqMan assay) with DNA samples from freshly isolated CD14$^+$ SLE (n=5) and healthy (n=5) monocytes. Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test).

B6) Healthy monocytes treated with either SLE serum or rIFNα exhibit accumulation of dysfunctional mitochondria

At this point, it was speculated that accumulation of defective mitochondria inside SLE monocytes was an effect attributed to a soluble factor present inside the SLE serum and not due to some genetic predisposition of SLE patients. In order to validate this, healthy $CD14^+$ freshly isolated monocytes were treated with 10% $^v/_v$ healthy (n=3) or SLE serum (n=3) for 18hrs and total DNA was collected and used for the quantification of mitochondrial (FAM probe) to nuclear (HEX probe) DNA ratio through multiplex Real-Time PCR (TaqMan assay). As it was hypothesized, healthy monocytes treated with SLE serum displayed lower FAM Cq/ HEX Cq ratio with respect to healthy controls [**Fig.B6 A**], indicating that higher amount of mitochondrial DNA and thus, accumulation of dysfunctional mitochondria, characterize monocytes following treatment with patient serum. Next, the potential effect of IFNα (as a soluble factor contained in SLE serum) on accumulation of damaged mitochondria was decided to be assessed. In order to address this, freshly isolated $CD14^+$ healthy monocytes were treated with rIFNα for 18hrs and again total DNA was collected from these cells and was used for the quantification of mitochondrial (FAM probe) to nuclear (HEX probe) DNA ratio through multiplex Real-Time PCR (TaqMan assay). The result of this experiment revealed a small decrease in FAM Cq/ HEX Cq ratio upon IFNα signaling with respect to the control (untreated cells) [**Fig.B6 B**]. Accordingly, higher amount of mitochondrial DNA present in monocytes treated with rIFNα was observed. Additionally, in order to verify that IFNα does have an effect on mitochondrial accumulation, freshly isolated $CD14^+$ healthy monocytes were treated with rIFNα for 18hrs and were subjected to staining with Mitotracker CMXRos. The result obtained by immunofluorescence microscopy depicted increased accumulation of mitochondria in monocytes upon IFNα signaling with respect to the controls [**Fig.B6 C**]. All together, these data suggest the idea of defective mitophagy completion upon IFNα signaling in the same way that autophagy completion is deregulated.

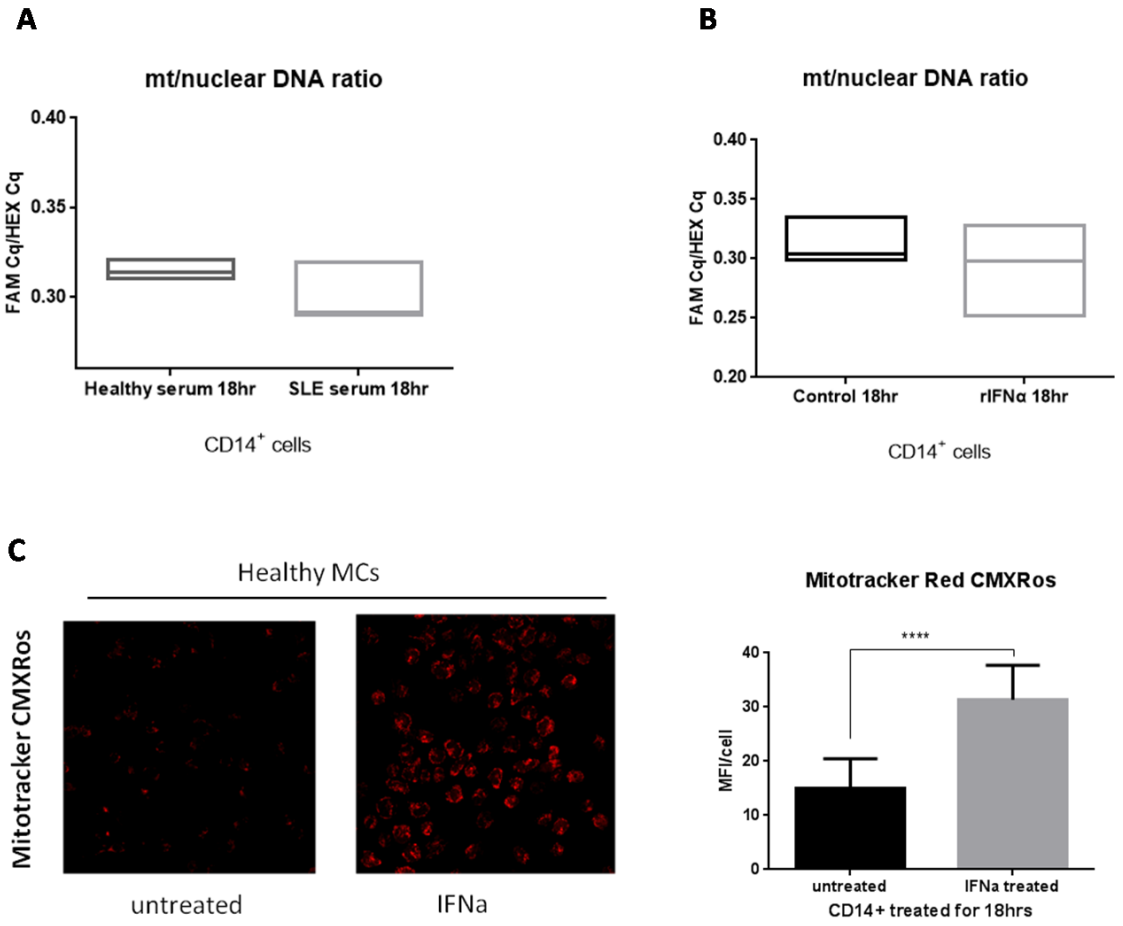


Figure B9: Increased accumulation of damaged mitochondria in healthy monocytes treated with either SLE serum or rIFNα compared to the controls. A) Multiplex Real-Time PCR (TaqMan assay) with DNA samples from freshly isolated CD14$^+$ monocytes treated with SLE serum (n=3) or healthy serum (n=3) for 18hrs. B) Multiplex Real-Time PCR (TaqMan assay) with DNA samples from freshly isolated CD14$^+$ monocytes treated with rIFNα or not (control) for 18hrs. C) Immunofluorescence microscopy of freshly isolated CD14$^+$ monocytes treated with rIFNα or not (control) for 18hrs. Cells were stained with the Mitotracker CMXRos probe. Representative image (left panel). Quantification (right panel). Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test).

C) Autoreactive phenotype of monocytes upon IFNα signaling

C1) CD14$^+$ monocytes treated with rIFNα obtain DC characteristics in an autophagy-dependent manner

Previous studies have demonstrated that autophagy induction is essential for macrophage differentiation of human monocytes [**41**] and that treatment of healthy monocytes with rIFNα results in a DC-like phenotype of monocytes [**136**]. Thus, we wanted to test whether CD14$^+$ monocytes treated with rIFNα obtain this DC-like phenotype in an autophagy-dependent manner. For this reason healthy CD14$^+$ monocytes were treated with rIFNα for 18hrs, stained with anti-HLA-DR and anti-CD86 markers and assessed by flow cytometry. Chloroquine (CQ), which affects lysosomal pH and autophagolysosomal fusion, was used as an autophagy inhibitor. Results obtained from FACS analysis, indicated an increase in HLA-DR [**Fig.C1 A**] and CD-86 [**Fig.C1 B**] membrane markers in monocytes upon IFNα signaling, implying that these DC-like characteristics are obtained in an autophagy-dependent manner. Concomitant treatment of monocytes with chloroquine and rIFNα "reversed" the DC-like phenotype, since a decrease in both markers was observed [**Fig.C1 A, B**].

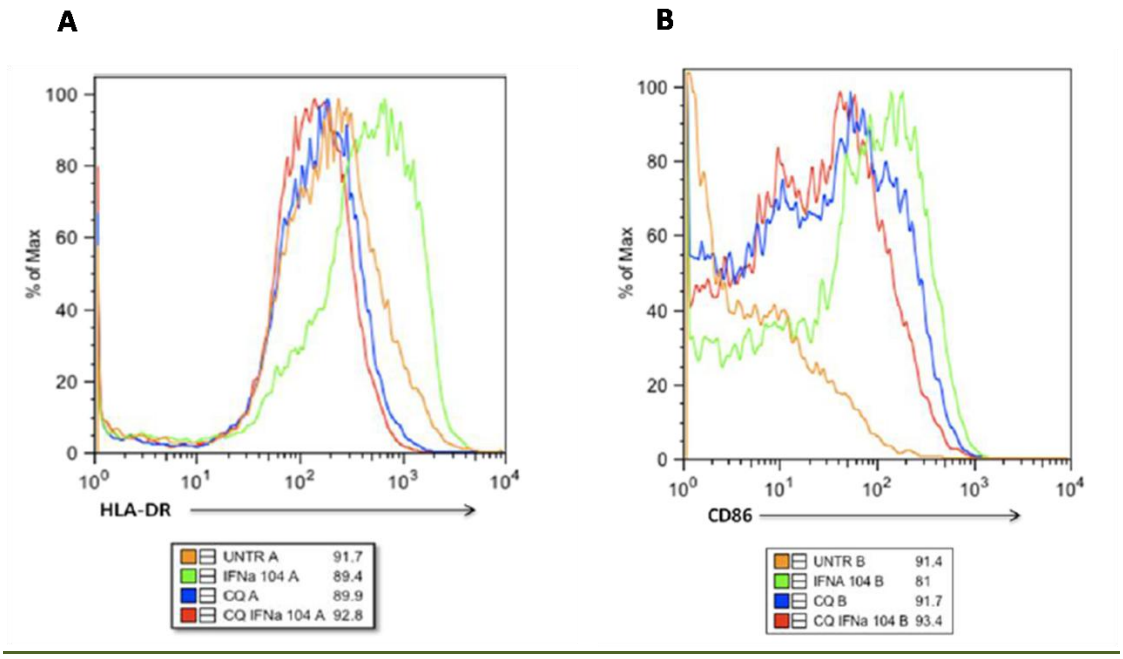


Figure C1: Healthy monocytes treated with rIFNα acquire DC characteristics in an autophagy-dependent manner. Representative image of healthy CD14$^+$ monocytes that were treated with rIFNα for 18hrs, stained with anti-HLA-DR (A) and anti-CD86 (B) antibodies and then subjected to flow cytometry analysis.

C2) Rapamycin and MitoTEMPO improve the autoreactive phenotype of monocytes upon IFNα signaling

As it was shown previously, monocytes exhibit elevated expression of the surface markers HLA-DR and CD86 upon IFNα signaling and thus, they are characterized by increased antigen-presenting capacity in an autophagy-dependent manner. Therefore, it was speculated that rapamycin might be able to reverse the effect of IFNα on the autoreactive phenotype of monocytes. Also, the potential association between mitochondrial ROS production and the autoimmune phenotype of monocytes upon IFNα signaling was decided to be examined as well. For this purpose, healthy $CD14^{+}$ monocytes were treated with rIFNα alone or combined with rapamycin and MitoTEMPO (mitochondrial ROS scavenger) for 18hrs, stained with anti-HLA-DR and anti-CD86 markers and assessed by flow cytometry. Interestingly, both rapamycin and MitoTEMPO decreased the expression of HLA-DR [**Fig.C2 A**] and CD-86 [**Fig.C2 B**] in the presence of IFNα signaling. However, rapamycin treatment reversed the autoreactive phenotype of monocytes more efficiently compared to MitoTEMPO [**Fig.C2 A, B**].

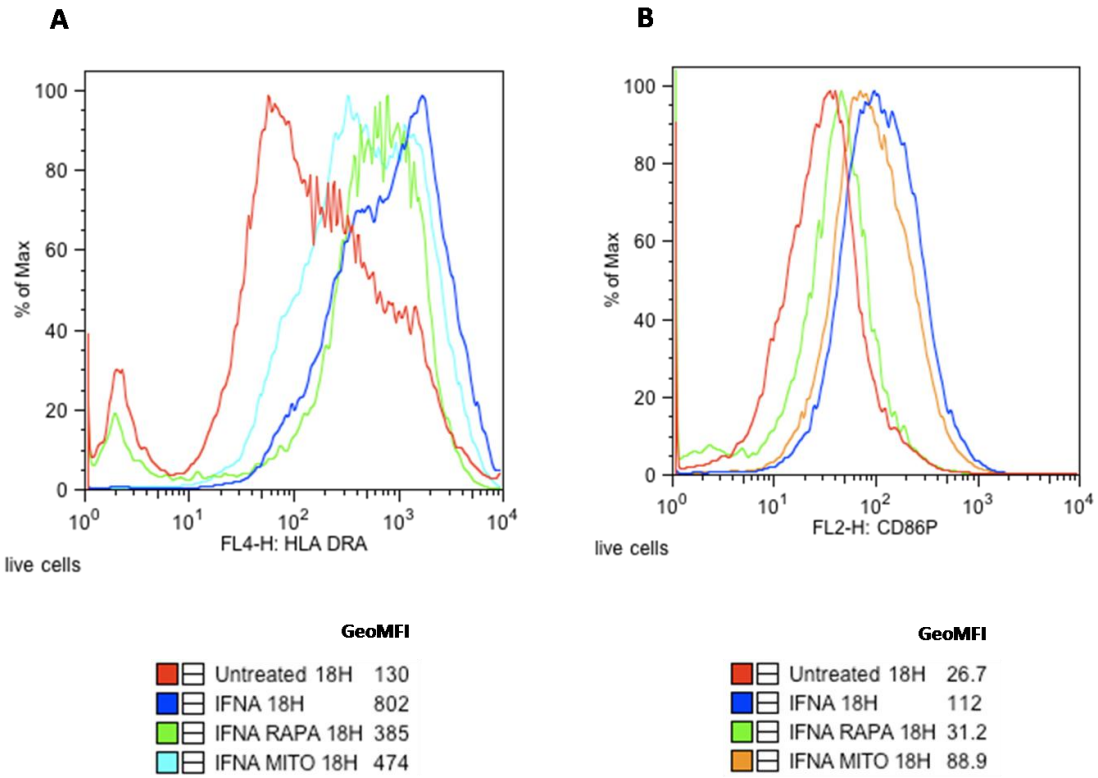


Figure C2: Improvement of the autoreactive phenotype of monocytes following IFNα signaling by rapamycin and MitoTEMPO. Representative image of healthy CD14$^+$ monocytes that were treated with rIFNα alone or in combination with rapamycin and MitoTEMPO for 18hrs, stained with anti-HLA-DR (A) and anti-CD86 (B) antibodies and subjected to flow cytometry analysis. Geometrical mean fluorescence intensity (GeoMFI) is provided.

C3) Increased proinflammatory cytokine secretion by monocytes upon IFNα signaling compared to control

Besides the antigen presenting capacity, the autoreactive phenotype of monocytes by means of proinflammatory cytokine production and secretion upon IFNα signaling was tested. Healthy CD14$^+$ monocytes were treated with rIFNα or not (control) for 18hrs, the culture medium was collected and subjected to ELISA. Remarkably, a significant increase in the levels of the secreted proinflammatory cytokines Interleukin 6 (IL-6) [**Fig.C3 A**] and Tumor Necrosis Factor alpha (TNF-α) [**Fig.C3 B**] was observed after treatment of monocytes with rIFNα compared to the control. This result further confirmed the autoreactive status of monocytes upon IFNα signaling.

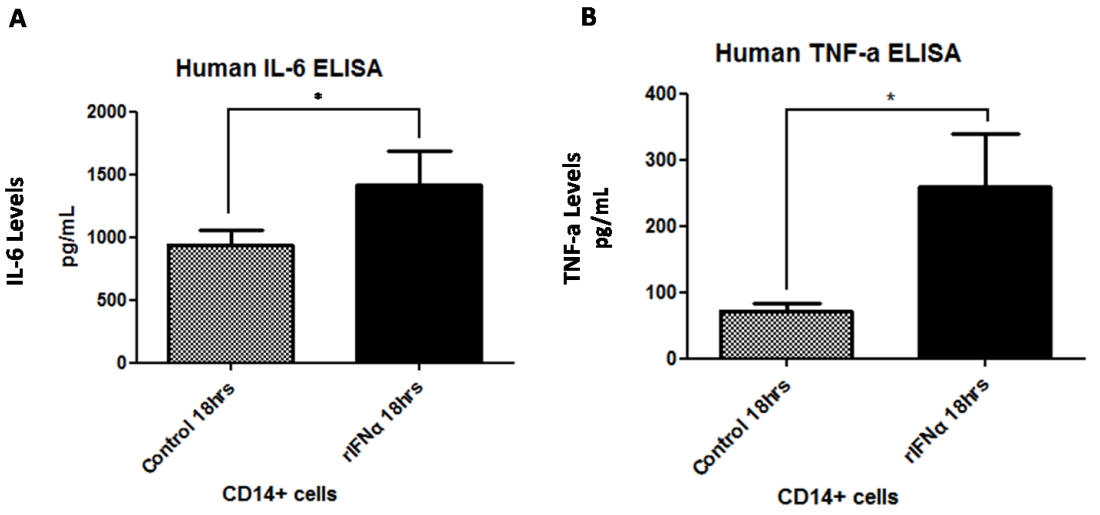


Figure C3: Healthy monocytes treated with rIFNα secrete higher amounts of proinflammatory cytokines with respect to control. Sandwich ELISA in culture medium derived from healthy monocytes treated with rIFNα or not (control) for 18hrs. The levels (pg/mL) of IL-6 (A) and TNF-α (B) are depicted. Data are mean ± SD values, *p<0.05, **p<0.01, and ***p<0.001 (Student's t test).

VII) Discussion

Autophagy is an evolutionary conserved and highly regulated catabolic process activated under stress conditions such as starvation, ischemia/reperfusion and pathogen infection. Also, autophagy is implicated in the differentiation, survival and activation of both myeloid and lymphoid cells and its role in antigen presenting cells (APC) has been studied extensively [**33**]. However, deregulation of the autophagic flux has been described in certain pathological conditions including cancer, neurodegenerative and autoimmune diseases [**163, 164**]. More specifically, GWA studies concerning systemic lupus erythematosus (SLE) have linked genetic polymorphisms in ATG genes (such as ATG5) to the pathogenesis of the disease [**151**].

Mitophagy is a selective type of autophagy, whereby damaged or superfluous mitochondria are eliminated to maintain proper mitochondrial numbers as well as quality control. While mitophagy shares key regulatory factors with the general macroautophagy pathway, it also involves distinct steps that are specific for mitochondrial removal. Recent findings indicate that parkin and the phosphatase and tensin homolog-induced putative kinase protein 1 (PINK1), which have already been implicated in the pathogenesis of neurodegenerative disorders such as Parkinson's disease, also regulate mitophagy and function to maintain mitochondrial homeostasis. In the context of SLE, though, the implication of the pathway of mitophagy is not well understood so far [**165**].

Moreover, many studies in the last few years attempt to elucidate the pathogenic role of type I interferons (IFNs) in SLE. Importantly, a great inference of the role of type I IFNs in SLE has been raised due to the identification of a signature of IFN-induced genes in the peripheral blood of SLE patients [**130**]. Additionally, IFNa not only induces the development of mature DCs from monocytes in vitro, but also high serum levels of IFN-a have been shown to be associated with the acquisition of DC characteristics by monocytes isolated from peripheral blood of SLE patients [**135**].

It should be mentioned that until now, IFNα signaling together with the autophagy and mitophagy pathways have been studied independently in the context of SLE. However, they might consist different parts of the same puzzle that are somehow interconnected and therefore, the potential link among them was decided to be studied and analyzed in the present study.

According to previous results of the lab, autophagy is upregulated in SLE monocytes compared to healthy controls but its completion is disrupted as indicated by the increased levels of the autophagy marker p62. Interestingly, this phenomenon is reproduced in healthy monocytes treated with SLE serum. This was the first indication implying the existence of a certain soluble factor inside the SLE serum that is responsible for the aforementioned effect on the autophagic flux. Also, by this way the possibility of a genetic predisposition of SLE patients was excluded.

Since high titers of IFNα have been identified in the serum of SLE patients and are strongly associated with disease activity and severity, it was hypothesized that IFNα might be this specific soluble factor that mediates autophagy induction and deregulation when SLE serum is added in healthy monocytes. Ex vivo experiments confirmed that recombinant human IFNα (rIFNα) induces autophagy in healthy monocytes at different timepoints but disrupts its completion, with the greatest effect observed at 18hrs. Of course, in order to make sure that IFNα is the soluble factor in SLE serum responsible for these effects, ex vivo treatments of healthy monocytes with SLE serum together with a soluble receptor of IFNa, like B18R (used as an inhibitor of IFNa signaling), should be performed expecting that these autophagy effects would be diminished.

Regarding disrupted autophagy completion, it was speculated that rapamycin might skew the autophagic flux towards its completion and reverse the effect observed upon IFNα signaling, since it is as a well established autophagy inducer. Indeed, treatment of healthy monocytes with the combination of rapamycin and rIFNα led to decreased levels of p62 and thus, to an improved autophagic phenotype concerning the completion compared to treatment of monocytes with rIFNα alone.

Since the autophagy pathway is influenced by SLE environment and more specifically upon IFNα signaling, and mitophagy is considered as a selective type of autophagy, we wondered whether IFNα can affect the mitochondrial status and subsequently the mitophagic flux in monocytes too. First of all, the mRNA levels of the two major players of mitophagy, PINK1 and Parkin, were examined in freshly isolated SLE monocytes and were compared to healthy controls. As it was shown, SLE monocytes exhibit higher mRNA levels of the sensor of mitochondrial damage PINK1, although a difference in Parkin mRNA levels was not observed. This result was the first indication that mitochondria in SLE monocytes might display a dysfunction and the mitophagy pathway could be induced.

Additionally, the PINK1 mRNA levels were increased in healthy monocytes treated with SLE serum compared to the controls, implying that the environment of SLE might be responsible for a potential defect of mitochondria in monocytes. Also, the significant increase in the mRNA levels of Parkin this time indicated that the parkin-dependent mitophagy pathway is induced in healthy monocytes treated with SLE serum.

Therefore, the question that was raised was the following one: is serum IFNα this particular factor of SLE environment that is implicated in the dysfunction of mitochondria and the subsequent induction of the mitophagic flux? In order to address this, healthy monocytes were treated with rIFNα and the mitochondrial status and the induction of mitophagy were examined. Interestingly, it was observed that mitochondria in monocytes are hyperpolarized upon IFNα signaling, with increased ROS generation and decreased ATP production by the ETC with respect to the controls. This was an indication that the ETC is deregulated (reverse electron flow). Hence, the increased Parkin mRNA levels in monocytes following treatment with rIFNα implied that the Parkin-dependent mitophagy pathway is induced due to the damage caused in mitochondria upon IFNα signaling (mitochondrially-derived oxidative stress).

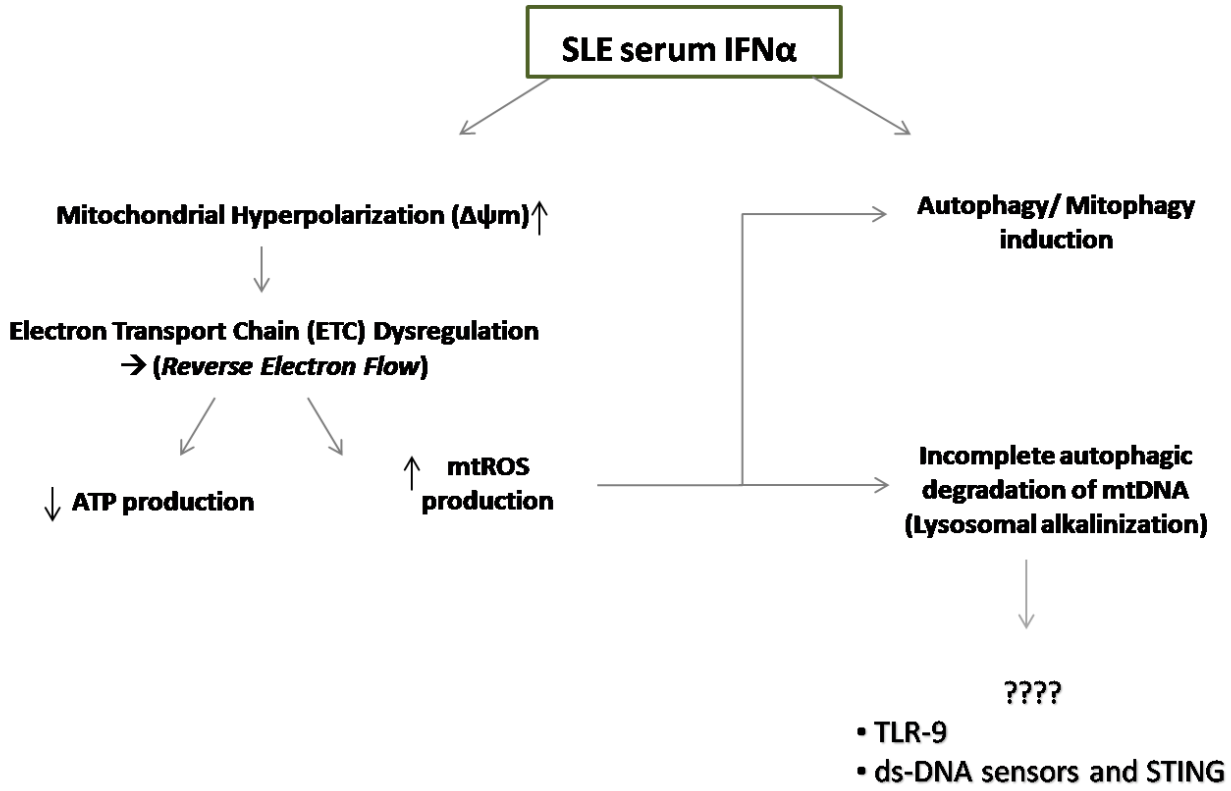
Another interesting finding was related to the accumulation of mitochondria in SLE monocytes due to the observation of higher mitochondrial mass as well as mitochondrial DNA copy numbers compared to healthy controls. This phenomenon was reproduced in healthy monocytes treated with SLE serum or rIFNα and indicated potential disruption of mitophagy completion in the same way that autophagy completion was deregulated.

Finally, it was identified that monocytes display higher expression of the membrane markers HLA-DR and CD86 upon IFNα signaling compared to the control, meaning that they obtain effector-DC characteristics regarding antigen presenting capacity. As it was shown, this occurs in an autophagy-dependent manner, but rapamycin and the mitochondrial ROS scavenger MitoTEMPO are able to reverse the effect by decreasing the expression of these two markers. The autoreactive phenotype of monocytes upon IFNα signaling was further supported by the observation that healthy monocytes treated with rIFNα secrete significantly larger amounts of the proinflammatory cytokines Interleukin-6 (IL-6) and Tumor Necrosis Factor alpha (TNF-a) with respect to the controls.

VIII) Proposed Model/ Future Directions

Taken together, all these data suggest that in the context of SLE and upon IFNα signaling, the autophagy and mitophagy pathways are induced in human monocytes. At the same time, serum IFNα leads to mitochondrial hyperpolarization and deregulation of the ETC function (reverse electron flow), resulting in decreased ATP levels and increased ROS generation. It is already known that ROS are inducers of the autophagic and mitophagic flux and thus, their increased production upon IFNα signaling results in a feedback loop as it concerns induction of the autophagy/mitophagy pathways. At the same time, ROS affect the oxidation status of the cargo that is destined for elimination. Also, IFNα disrupts the completion of autophagy/mitophagy pathways, possibly due to the effect of elevated ROS levels on lysosomal pH (alkalinization). By this way, all the cytosolic constituents (including damaged mitochondria and their components such as oxidized mitochondrial DNA) that are targeted for degradation are actually not properly removed. On the contrary, they accumulate inside autolysosomes. This chain of reactions creates a fundamental pool of self-antigens, the escape of which might provoke a subsequent autoimmune response.

To date, we are aware of the fact that the immune system can be triggered by sterile inflammation. It should be mentioned that the appearance of DNA in the cytoplasm after tissue damage can lead to its specific detection by receptors and the initiation of downstream signaling pathways. Notably, the immune recognition of intracellular DNA is sequence independent and does not require that the DNA lacks cytosine methylation for maximal activity. DNA-containing immune complexes that are closely associated with inflammation in SLE can activate both Toll-like Receptor (TLR) 9-dependent and TLR 9-independent pathways [**166**]. More specifically, there is evidence supporting the idea that mitochondrial DNA can escape from autophagy and cause TLR 9-mediated inflammatory response and heart failure [**167**]. In an alternative scenario, mitochondrial DNA could escape, become recognized by other cytoplasmic DNA receptors such c-GAS and then activate the stimulator of Interferon genes (STING) for further induction of type I IFNs production [**166**].



Proposed Model of the study

Conclusively, it is considered of great importance to unravel in the future a potent mechanism through which IFNa can result in deregulation of autophagy and mitophagy not only in the context of SLE, but also in other autoimmune diseases, so that more targeted therapeutics to be established. Future experiments in order to identify how IFNa changes the lysosomal pH and what consequences this might have in the MHCII repertoire, or by which exact signaling cascade IFNa can result in the upregulation of the autophagic and mitophagic machinery in monocytes could serve as key targets for drug development. Furthermore, it would be of outmost interest to delineate the role of IFNa in the regulation of autophagy and mitophagy in SLE B cells, since these cells are responsible for the production of the anti-dsDNA antibodies. Preliminary evidence also indicates a role of IFNa in maturation and isotype switching of SLE B cells, highlighting new roads for experimental studies.

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