Master thesis

Delineating the role of autophagy in shaping the autoreactive phenotype of monocytes from patients with Systemic Lupus Erythematosus (SLE) upon IFNa signaling.

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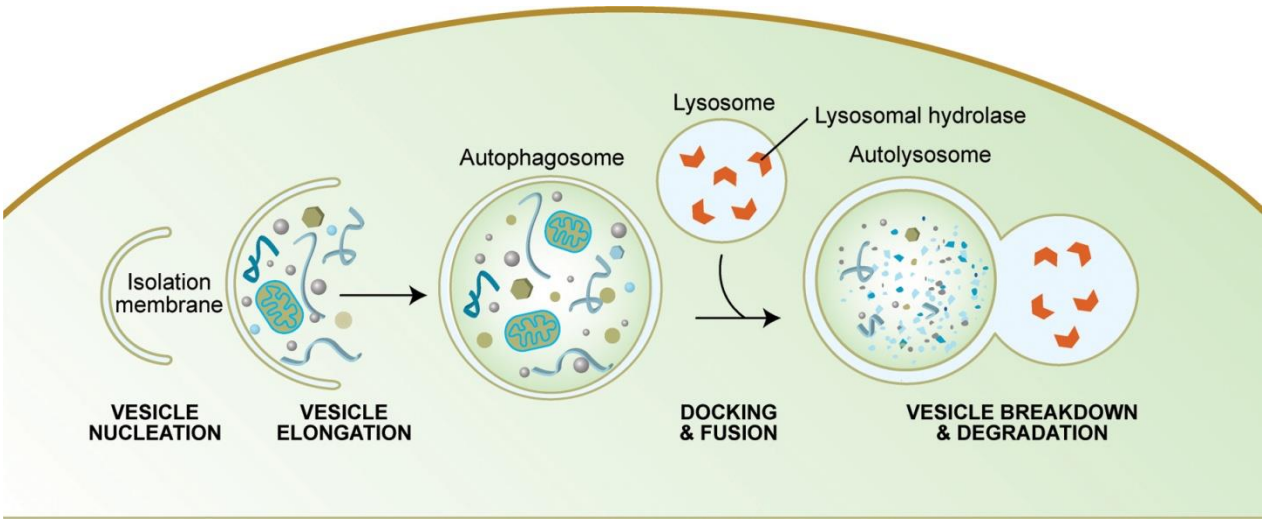

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Η παρούσα εργασία εκπονήθηκε υπό την επιστημονική επίβλεψη της Δρ. Κατερίνας Γκιρτζιμανάκη και του Δρ. Παναγιώτη Βεργίνη

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I. Αντί προλόγου

Ολοκληρώνοντας τη μεταπτυχιακή μου εργασία δε θα μπορούσα να μην ευχαριστήσω ορισμένα άτομα που με βοήθησαν και με στήριξαν καθ' όλη τη διάρκεια του μεταπτυχιακού μου και μου έδωσαν τα απαραίτητα εφόδια ώστε να μπορώ να συνεχίσω για τη διδακτορική μου διατριβή.

Αρχικά, θα ήθελα να ευχαριστήσω τον κ. Δημήτριο Μπούμπα και τον κ. Γεώργιο Μπερτσιά οι οποίοι μου δώσανε την ευκαιρία να δουλέψω στο εργαστήριο και να νιώσω εξαρχής κομμάτι της ερευνητικής ομάδας. Τον κ. Παναγιώτη Βεργίνη για όλη την επιστημονική καθοδήγηση, τις εποικοδομητικές συζητήσεις και τα "μαθήματα" ανοσολογίας. Δεν θα μπορούσα όμως να μην ευχαριστήσω ιδιαίτερα την Κατερίνα Γκιρτζιμανάκη που με τεράστια υπομονή και σχεδόν πάντα με χαμόγελο απαντούσε σε όλες μου τις απορίες, γιατί ομολογώ η ανικανότητά μου να κάνω έναν μαθηματικό υπολογισμό ξεπερνάει τα όρια της ανθρώπινης υπομονής.. Την ευχαριστώ ιδιαίτερα για όλες τις τεχνικές που με επιμέλεια μου έμαθε αλλά πιο πολύ γιατί δίπλα της έμαθα ότι τίποτα δεν πρέπει να θεωρείται δεδομένο. Ο επιστημονικός τρόπο σκέψης της ξεπερνά τα μέχρι τώρα όρια της γνώσης και όλα είναι πιθανά… "αρκεί να το αποδείξεις". Παρ' όλες τις εντάσεις νιώθω ιδιαίτερα τυχερή που ήμουν δίπλα της και ελπίζω κάποια στιγμή να ξανασυνεργαστούμε στο μέλλον. Φυσικά θα έσφαλα, αν δεν ευχαριστούσα και όλα τα υπόλοιπα παιδιά από το εργαστήριο για την στήριξή τους και τις όμορφες ώρες που περάσαμε μαζί.

Τέλος, ένα ευχαριστώ δε θα ήταν αρκετό για να εκφράσω την ευγνωμοσύνη μου στην οικογένειά μου που ευθύνεται για όλα όσα έχω κάνει στη ζωή μου αλλά και τους φίλους μου, για την ανοχή τους όλο αυτόν τον καιρό και κυρίως για την ψυχική ηρεμία που μου προσφέρουν.

"The important thing is not to stop questioning.

Curiosity has its own reason for existing."

- Albert Einstein

II. Περίληψη

Ο συστηματικός ερυθηματώδης λύκος (ΣΕΛ) είναι μια χρόνια, συστηματική αυτοάνοση διαταραχή, που κατά κύριο λόγο προσβάλει γυναίκες, στην οποία η ανοσολογική ανοχή σε αυτόλογα νουκλεϊκά οξέα και πρωτεΐνες χάνεται, με αποτέλεσμα την εμμένουσα φλεγμονή και την παραγωγή αυτοαντισωμάτων που προκαλούν ιστική βλάβη. Ο παθογενετικός ρόλος των ιντερφερονών τύπου Ι στον ΣΕΛ υποστηρίζεται από την ύπαρξη μιας "γονιδιακής υπογραφής", ενός συνόλου γονιδίων η έκφραση των οποίων ρυθμίζεται υπό την επίδραση ιντερφερονών, στα λευκοκύτταρα του περιφερικού αίματος των ασθενών με ΣΕΛ. Επίσης, τα υψηλά επίπεδα ιντερφερόνης-α στον ορό ασθενών όπου ανιχνεύονται συμβαδίζουν με την ενεργότητα αλλά και την σοβαρότητα της νόσου. Τα αυτοδραστικά μονοκύτταρα στον ΣΕΛ χαρακτηρίζονται από τον εξαρτώμενο από ιντερφερόνη-α φαινότυπό τους και η διαφοροποίησή τους αλλά και η παρουσίαση των αυτοαντιγόνων από τις πρωτεΐνες του μείζονος σύμπλοκου ιστοσυμβατότητας τύπου ΙΙ έχει δειχθεί ότι εξαρτάται από την κυτταρική αυτοφαγία. Η αυτοφαγία είναι μια καταβολική διαδικασία του κυττάρου που περιλαμβάνει την αποικοδόμηση των περιττών και μη-λειτουργικών συστατικών του και βασίζεται στη συνεργασία των αυτοφαγοσωμάτων με τα λυσοσώματα.

Σκοπός της παρούσας μελέτης είναι η διαλεύκανση του ρόλου της αυτοφαγίας στο σχηματισμό του αυτοδραστικού φαινοτύπου των μονοκυττάρων από ασθενείς με ΣΕΛ μέσω της σηματοδότησης της ιντερφερόνης-α. Γι' αυτό το σκοπό, $CD14^+$ μονοκύτταρα απομονώθηκαν από ασθενείς με ΣΕΛ αλλά και από υγιείς εθελοντές αιμοδότες και τα επίπεδα των αυτοφαγικών πρωτεϊνών LC3 και P62 αναλύθηκαν μέσω ανοσοαποτύπωσης κατά western και συνεστιακής μικροσκοπίας. Τα επίπεδα των αγγελιοφόρων RNA για τα αυτοφαγικά γονίδια *ATG5* και *P62* αναλύθηκαν μέσω πραγματικού χρόνου αλυσιδωτή αντίδραση πολυμεράσης. Αντίστοιχα πειράματα πραγματοποιήθηκαν και με *ex vivo* χειρισμό μονοκυττάρων και επώασής τους με 10% $^{v}/_{v}$ ορό από ασθενείς με ΣΕΛ και υγιείς αιμοδότες αλλά και με χορήγηση ανασυνδυασμένης ιντερφερόνης-α σε συγκέντρωση 10^4U/ml. Τα αποτελέσματα έδειξαν ότι τα μονοκύτταρα ασθενών με ΣΕΛ παρουσιάζουν αυξημένα επίπεδα αυτοφαγίας σε σχέση με τα υγιή δείγματα. Επίσης, η επώαση υγιών μονοκυττάρων με ορό από ασθενείς με ΣΕΛ ή με ανασυνδυασμένη ιντερφερόνη-α προκάλεσε την αύξηση της αυτοφαγίας. Η αντιγονοπαρουσιαστική ικανότητα των υγιών μονοκυττάρων μετά τη χορήγηση της ιντερφερόνης-α ήταν επίσης αυξημένη με βάση την έκφραση των μεμβρανικών δεικτών HLA-DR και CD-86 σε πειράματα κυτταρομετρίας ροής.

Τέλος, πραγματοποιώντας πειράματα συνεστιακής μικροσκοπίας μονοκυττάρων από ασθενείς με ΣΕΛ σε σύγκριση με υγιή δείγματα αλλά και υγιών μονοκυττάρων στα οποία χορηγήθηκε ιντερφερόνη-α για διαφορετική χρονική διάρκεια, κατέδειξαν ότι η ολοκλήρωση της αυτοφαγίας είναι διαταραγμένη στο περιβάλλον της νόσου. Η μη ολοκληρωμένη καταστροφή του περιεχομένου των αυτοφαγολυσσωσομάτων αποτελεί πηγή αυτοαντιγόνων, τα οποία δεσμεύονται από το σύμπλοκο ιστοσυμβατότητας τύπου ΙΙ και μπορούν να προκαλέσουν αυτοάνοση ανοσολογική απάντηση. Είναι μείζονος σημασίας να διαλευκανθεί ένας πιθανός μηχανισμός μέσω του οποίου η ιντερφερόνη-α μπορεί να προκαλέσει απορρύθμιση της αυτοφαγίας και να οδηγήσει συνεπώς στην παραγωγή αυτοαντισωμάτων, όχι μόνο στην περίπτωση του ΣΕΛ αλλά και σε άλλα αυτοάνοσα νοσήματα. Αποκτώντας αυτή τη γνώση, πιο στοχευμένη θεραπεία μπορεί να αναπτυχθεί σε αυτές τις χρόνιες ασθένειες.

III. Abstract

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. **The pathogenic role of type I IFNs in SLE** is supported by a signature of IFN-induced genes in the peripheral blood of patients and from high IFN-a serum levels in active SLE patients, which is associated with high disease activity and severity. Autoreactive monocytes in SLE are characterized by IFNa-dendritic cell-like phenotype and their differentiation and capacity to present self-antigen by major histocompatibility class (MHC-) II proteins has been shown to depend on cell autophagy. Autophagy is a cellular catabolic process that involves degradation of unnecessary or dysfunctional cellular components and relies on the cooperation of autophagosomes with lysosomes.

The aim of this project was to delineate the role of autophagy in shaping the autoreactive phenotype of SLE monocytes upon IFNa signaling. For this purpose, $CD14^+$ monocytes were isolated both from SLE patients and healthy donors and the levels of the autophagic proteins LC3 and P62 were assessed by immunobloting analysis and confocal microscopy. *ATG5* and *P62* mRNA levels were assessed by Real Time PCR. The same experimental approach was followed in *ex vivo* treatments of $CD14^+$ healthy monocytes with 10% $^{v}/_{v}$ SLE or healthy serum and recombinant human IFNa 10^4U/ml. The results from these experiments indicated that SLE monocytes exhibit increased levels of autophagy and that treatment of healthy monocytes with SLE serum or recombinant IFNa can induce autophagy. The antigen presenting capacity of healthy monocytes treated with rIFNa was assessed by flow cytometry for the expression of HLA-DR and CD86 surface markers. The results showed that monocytes have increased antigen presenting capacity when treated with rIFNA. Finally, alterations in the autophagolysosomal pH were observed by conducting immunofluoresence analysis of SLE monocytes compared to healthy controls but also of healthy monocytes treated with rIFNa at different time-points. The acidic autophagolysosomal pH sensitive dye, Lysotracker Red-DND99, exhibited significantly reduced staining implying that the autophagic flux may be deregulated in the lupus milieu. As a consequence, not fully processed self-antigens inside autophagolysosomes could be loaded in the Major Histocompatibility Complex II (MHCII) and subsequently trigger an autoimmune response. It is of great importance to unravel a possible mechanism through which IFNa can result in

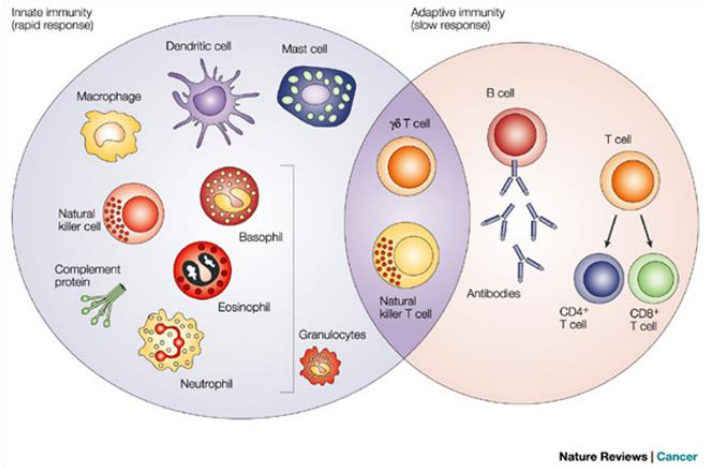
deregulation of autophagy and the production of autoantibodies not only in the context of lupus but also in other autoimmune diseases. By acquiring this knowledge, more targeted therapeutic approaches could be established to these incurable diseases up until now.

IV. 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 protective 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 pathogen antigens on MHC (Major Histocompatibility Complex) class 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]. (Scheme 1)



Scheme 1: Cell types participating in innate and adaptive immunity

B. Antigen presenting cells (APCs)

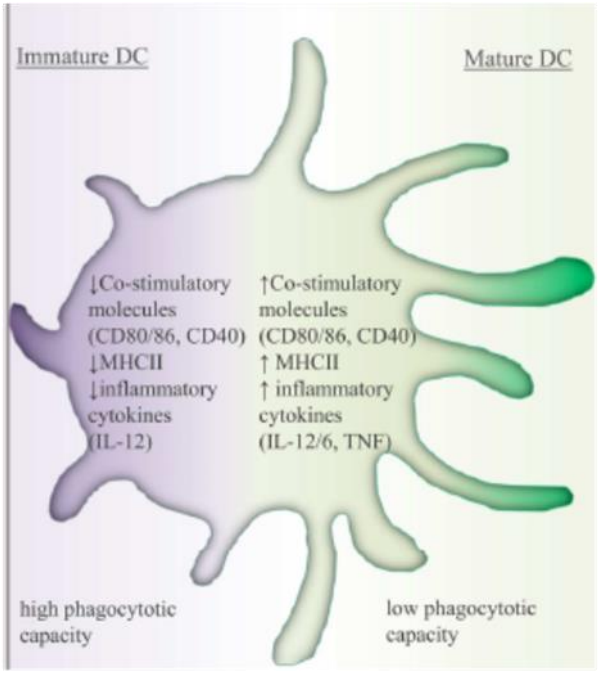
Monocytes develop in the bone marrow and enter the blood stream until they are recruited to extravascular compartments not only during inflammatory processes but also under steady-state conditions, to maintain the homeostasis of the monocytic cell compartment. Monocytes were originally described as key 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 give rise to a subset of dendritic cells (DCs) during infection or inflammation, when high levels of proinflammatory mediators such as TNF-a, IL-1β and IFN-a are produced[3]. This observation 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 to 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 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 costimulatory molecules and secrete large amounts of immunostimulatory cytokines[13].

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]. (Scheme 2)



Scheme 2: Immature vs Mature Dendritic cells

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[15].

1. Autophagic machinery - key players

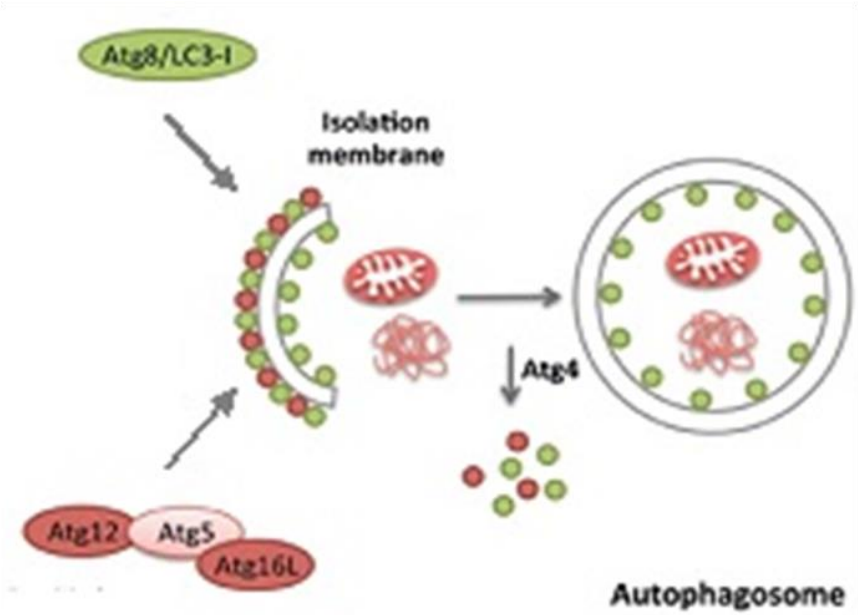
Autophagy involves 3 morphological stages: **initiation** (formation of phagophores), **elongation and closure** (increase in the size of the phagophore and its closure into a completed autophagosome), and maturation (conversion of autophagosomes into degradative organelles, termed **autophagolysosomes**, 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 **A**u**T**opha**G**y-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

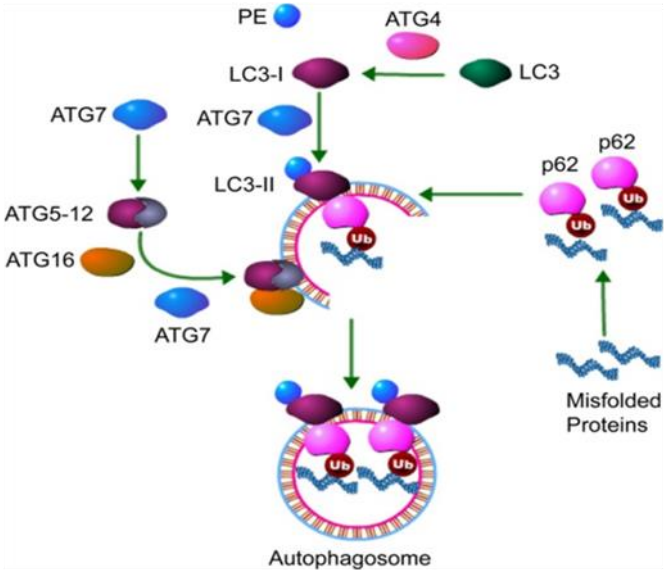
conjugate complexes with Atg16L; this complex dimerizes and associates with the exterior membrane of the phagophore. Upon completion of autophagosome formation, the Atg12–Atg5–Atg16 complex is released into the cytosol[20].

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. (Scheme 3)



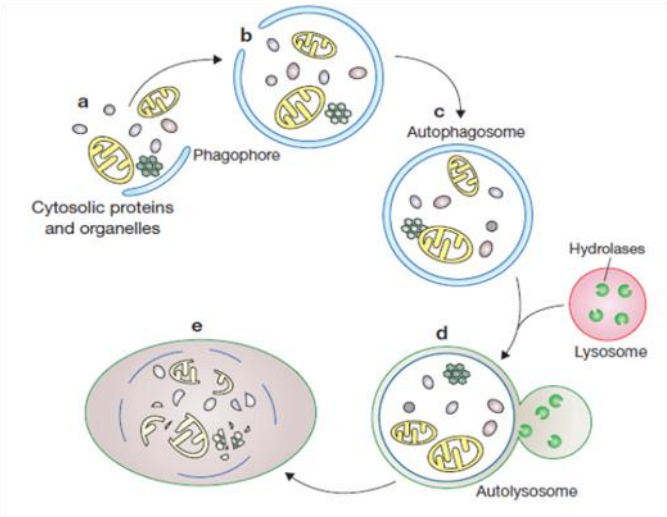
Scheme 3: Autophagosome biosynthesis

LC3-II recruits the cargo adaptor protein p62. The **p62 protein**, also called **sequestosome 1 (SQSTM1)**, is commonly found in inclusion bodies containing poly-ubiquitinated 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]. (Scheme 4)



Scheme 4: The adaptor protein p62/SQSTM1

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, are degraded and the resulting macromolecules are released into the cytosol through lysosomal membrane permeases for recycling[19]. (Scheme 5)



Nature Cell Biology, 2007

Scheme 5: Autophagic pathway

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].

2. 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.

Mitochondria are primarily responsible for producing ATP via oxidative phosphorylation in the inner mitochondrial membrane. They also play central roles in both necrosis and apoptosis and can quickly change from a source of energy for sustaining contraction to an organelle that promotes cell death. In response to changes in the environment, ATP synthesis is disrupted, and mitochondria become producers of excessive reactive oxygen species (ROS) and release proteins that participate in cell death pathways. Interestingly, cells have developed a defense mechanism against aberrant mitochondria, which can cause harm to the cell. This mechanism involves selective sequestration and subsequent degradation of the dysfunctional mitochondrion before it causes activation of cell death. This occurs through a process known as mitochondrial autophagy or **mitophagy**[42]

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[43,44].

D. Type I interferons (IFNs)

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[45]. 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[46]. Sources of type I IFNs include fibroblasts, NK cells, T cells, dendritic cells and a group of specialized leukocytes, the plasmacytoid monocytes[47]. 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[48].

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[49]. 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[46].

E. 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[50]. 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[51]. 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[50].

1. 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[52], an association with risk alleles involved in TLR and IFN pathways[53], disease acceleration by exogenous IFN-α in several lupus models[54] and disease amelioration in some lupus-prone mouse strains that have been rendered deficient for the type I IFN receptor[55]. 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[56]. 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 SLE[57]. 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[57].

2. SLE Monocytes Act as DCs

$CD14^+$ blood cells are normally immunologically quiescent monocytes that are unable to mount the so-called mixed lymphocyte reaction (MLR). However, in SLE these cells are able to induce the proliferation of alloreactive T cells, a property characteristic of DCs[57]. 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 of monocytes into DCs. Furthermore, normal serum spiked with recombinant IFN-a/b can induce the differentiation of monocytes into DCs[58].

3. Autoantibodies

Immune reaction against self-antigens is primarily prevented during maturation of T-lymphocytes in 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[59]. 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)[60]. 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)[61].

DCs acquire antigens via phagocytosis, receptor mediated endocytosis, and macropinocytosis that lead to the presentation of these antigens to T cells[11]. Autoimmune diseases are associated with multiple auto antigens against which the tolerance is broken[62]. 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, can result in DC activation[63]. 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[64]. 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[65]. 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.

4. Anti-DNA antibodies

Anti-DNA antibodies constitute a subgroup of antinuclear antibodies that bind to either **single-stranded or double stranded DNA**[66]. 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 and prognostic criterion for SLE (60–70% of patients are positive for such antibodies) and for monitoring the clinical course of the patient[67]. 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 practical and widely-used method. Anti-dsDNA antibodies, in particular of the IgG isotype, have an important pathogenetic role in SLE. A strong relationship exists, for example, between anti-dsDNA antibodies (R4A antibody) and disease activity in lupus nephritis[68]. 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 chromatin fragments in glomerular basement membrane[69].

5. 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 has demonstrated that TECs displayed high levels of constitutive autophagy, suggesting that autophagy could shape the T-cell repertoire during thymic selection[70]. 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[71]

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[72]. Activation of mTOR signaling has recently emerged as a key factor in abnormal activation of lymphocytes in SLE[73]. Moreover, genome-wide association studies have linked SNPs in Atg5 to SLE susceptibility[74]. 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[75].

Finally, hydroxychloroquine is one of the most effective treatments for mild SLE manifestations and it is also used in preventing the occurrence of SLE exacerbations. Hydroxychloroquine is an antimalarial drug that 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 acts at the autolysosomes too. Thus, a link between autophagy deregulation and SLE pathogenesis is biologically plausible, although not yet proven.

F. Aim of the project

Research on the cellular and molecular procedures leading to SLE development has led to the conclusion that signaling pathways and processes influencing the generation of self-antigens and their presentation causing 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 the mechanisms involved in the generation of this pool of self-antigens with ultimate aim of finding a way to inhibit their presentation so as to suppress SLE manifestations and pathogenesis.

The first objective of this study was to characterize the autophagic flux of SLE monocytes compared to healthy individuals. For this reason, autophagy induction was assessed in peripheral blood $CD14^+$ monocytes isolated from 8 SLE patients with active disease (SLEDAI≥ 8) and 8 age- and sex-matched healthy controls using immunofluoresence microscopy (LC3 puncta/cell), immunoblotting for LC3 and p62 (LC3II/I, LC3II/actin and p62/actin ratios) and real-time PCR for ATG5.

A second objective of this study was to assess whether treatment of healthy monocytes with SLE serum can reproduce the same "autophagic" phenotype as in SLE monocytes. A **final objective** was to identify the soluble factor in SLE serum that mediates these effects and to assess the role of autophagy in shaping the autoreactive phenotype of SLE monocytes and in presentation of autoantigens following IFNa signaling. In order to assess the above mentioned goals, $CD14^+$ monocytes from healthy donors were isolated and treated ex vivo with SLE serum (10%) or recombinant hIFNa (10^4U/ml) and were analyzed as far induction of autophagy using the same experimental setup as mentioned above. The differentiation/activation of IFNa-treated monocytes was assessed by flow cytometry using the CD14, HLA-DR, CD86 surface markers. Finally, the lysosomal acidification was examined in SLE $CD14^+$ monocytes as well as in healthy monocytes treated with recombinant hIFNa by confocal microscopy using the pH sensitive dye, Lysotracker Red-DND99.

Preliminary results indicate that SLE monocytes exhibit increased levels of autophagy and treatment of healthy monocytes with SLE serum and recombinant hIFNa can induce autophagy and also increase their antigen presenting capacity. Finally, due to reduced Lysotracker Red-DND99 intensity in SLE monocytes and in monocytes treated with recombinant hIFNa, there is some evidence that the autophagic flux may be deregulated. As a consequence, a new source of unprocessed self-antigens is created which in turn could trigger an autoimmune response in the context of lupus. It is of great importance to unravel a potent mechanism through which IFNa can result in deregulation of autophagy not only in

the context of lupus but also in other autoimmune diseases. By acquiring this knowledge, more targeted therapeutic approaches could be established to these incurable diseases up until now.

V. Materials and Methods

A. Patients

Eight (8) 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 enrollment.

B. Serum collection

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

C. $CD14^+$ Monocyte Cell Isolation

1. Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Histopaque density gradient centrifugation of whole blood. 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, 30 minutes, 24^{o}C with no brake. 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.

2. *CD14$^+$ monocyte positive cell separation*

PBMCs 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, respectively. 3 washes with 500μl or 3ml MACS buffer (for MS or LS column) 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.

3. *Cell cultures*

Purified monocytes were cultured in 24-well treated plates (10^6 cells/well) 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), LPS (5μg/ml), chloroquine (CQ: 46.1μM), wortmanin (WM: 1μM). Healthy or SLE serum treatments were used in 10% v/v in serum free RPMI-1640/L-glutamine medium.

D. 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 the FlowJo software. The antibodies that were used were anti-HLADR (APC) and anti-CD86 (FITC)

E. Immunofluoresence

After completion of the monocyte isolation, cells were plated on coverslips pretreated with poly-L-lysine at a concentration of 250.000 cells/slide. When the appropriate treatments were completed, slides were washed once with PBS and fixed with 4% PFA for 20mins at RT. The slides were washed twice with PBS and fixed again with 100% ice-cold MetOH for 10mins at RT. After washing once with blocking solution (BS: 0,1% saponin and 2% BSA) was added at slides and incubated for 20mins at RT. Primary antibody or a combo for the double staining, were added to slides and incubated for 1hr at RT. Slides were washed trice with BS and the secondary antibody or a combo of them were added to slides and incubated for 1hr at RT, always kept in the dark. Slides were then washed four times with BS and counterstained with DAPI (300nM) for 3mins at RT in the dark. The slides were then washed twice with BS, once with PBS, once with dH_2O and mounted with MOWIOL. Samples were sealed with nail-polish and kept at 4^oC for confocal microscopy analysis. Primary antibodies were used as follows: anti-LC3 (nanotools, 1:20 dilution), anti-p62 (MBL, 1:500 dilution). Secondary antibodies Alexa-fluor 488 anti-mouse and Alexa-fluor 555 anti-rabbit were used in 1:500 dilution respectively.

F. Lysosomal acidification staining

The presence of acidic lysosomes was assessed with Lysotracker Red-DND99 in 1μM final concentration. 20mins prior the end of the appropriate treatment Lysotracker was added to slides. After the completion of treatments slides were washed trice with PBS and fixed with 4% PFA for 20mins at RT. Slides were washed twice with PBS, once with dH_20 and mounted with MOWIOL. Sealing of slides were done using nail polish and samples were immediately analysed by confocal microscopy.

G. Confocal microscopy

For immunofluoresence 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.

H. Immunoblotting

RIPA buffer with protein inhibitors was used for collection of total protein lysates. SDS-PAGE electrophoresis was performed and 40µgs 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. HRP-conjugated secondary antibodies used were: anti-mouse and anti-rabbit at 1:2000 dilution.

I. RNA isolation

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

J. Reverse transcriptase and real-time PCR

cDNA was prepared from isolated RNA using PrimeScript™ 1st strand cDNA Synthesis Kit according to the manufacturer's protocol. 500µgs of RNA was used as a template for every reaction and was 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^{o}C for 45 minutes and at 95^{o}C 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^{o}C. 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 20uls. The following PCR conditions were used: 95°C for 3 minutes, 39 cycles at 95°C for 3 seconds and annealing temperature at 60°C for 30seconds. GAPDH was used as a reference gene and data were analyzed with the $2^{-\Delta\Delta CT}$ method.

Primer sets		
Gene ID	**Forward primer**	**Reverse primer**
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'
GAPDH	5'- CAT GTT CCA ATA TGA TTC CAC C-3'	5'- GAT GGG ATT TCC ATT GAT GAC-3'

K. Statistical analysis

Statistical analysis was performed using unpaired t-test with Welch's correction in Graph Pad Prism 5 software. P value < 0.05 was considered as indicative of statistical significance.

VI. Results

C. 1: Increased autophagy induction in SLE monocytes compared to healthy controls

For the assessment of autophagy, total protein lysate and RNA were collected from $CD14^+$ freshly isolated monocytes both from healthy donors and SLE patients and they were subjected to immunoblotting and real time PCR analysis, respectively. ATG5 mRNA levels were used as a measure of autophagic induction[20] and the LC3II/I ratio as a measure of autophagic index[26] (how much of the cytocolic fraction of LC3 is used for the autophagosome formation). Significantly increased LC3II/I ratios and ATG5 mRNA levels were found in monocytes from SLE patients. (Figure C.1)

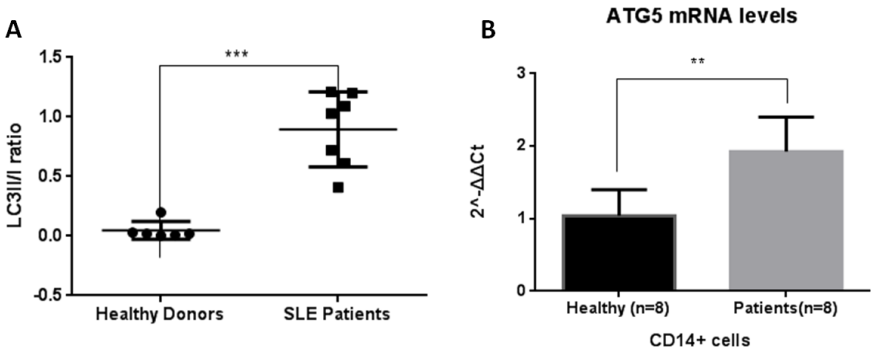


Figure C.1.: ***Autophagy is upregulated in SLE monocytes*** A) $CD14^+$ cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-LC3 and anti-actin antibodies. B) Real time pcR for ATG5 mRNA levels from $CD14^+$ cells Data are mean ± SD values, * $p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$ (Student's t test)

C.2: Disrupted autophagy completion in SLE monocytes compared to healthy controls

Since p62 is itself a substrate of the autophagic machinery and is degraded inside the autophagolysosomes upon successful completion of autophagy[19], 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, real time PCR analysis and immunofluoresence microscopy. Increased p62 protein levels were observed in SLE monocytes by western blot analysis (Figure C.2.1 A-B). These results could be interpreted either by increased production of p62 at the transcriptional level and/or by defective degradation of p62 inside the autophagolysosomes. In order to verify which of the two was

the case, p62 mRNA levels were quantified by realtime PCR. There was no difference observed between p62 mRNA levels in SLE monocytes compared to healthy controls (Figure C.2.1.C), thus p62 increased protein levels were likely due to its defective degradation. All results were confirmed using confocal microscopy too (Figure C.2.2). More specifically, the increased colocalization of p62 with LC3 further confirms that increased p62 levels are autophagy-related and do not originate from other intracellular pathways.

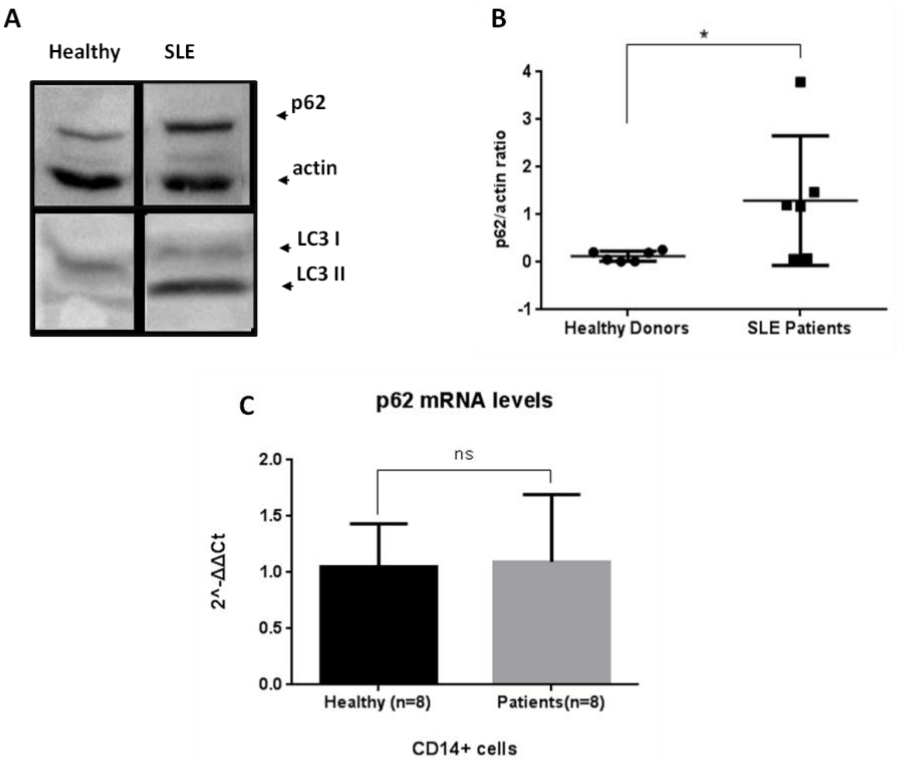


Figure C.2.1.: ***Autophagy completion is disrupted in SLE monocytes***. A) Representative image of SDS-PAGE immunoblotting of $CD14^+$cell lysates from heatlhy donor and SLE patient with the indicative antibodies. B) Quantitational analysis of p62 protein levels from 6 healthy donors compared to 6 SLE patients. C)Real time pcR for p62 mRNA levels from $CD14^+$ cells. Data are mean ± SD values, * $p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$ (Student's t test)

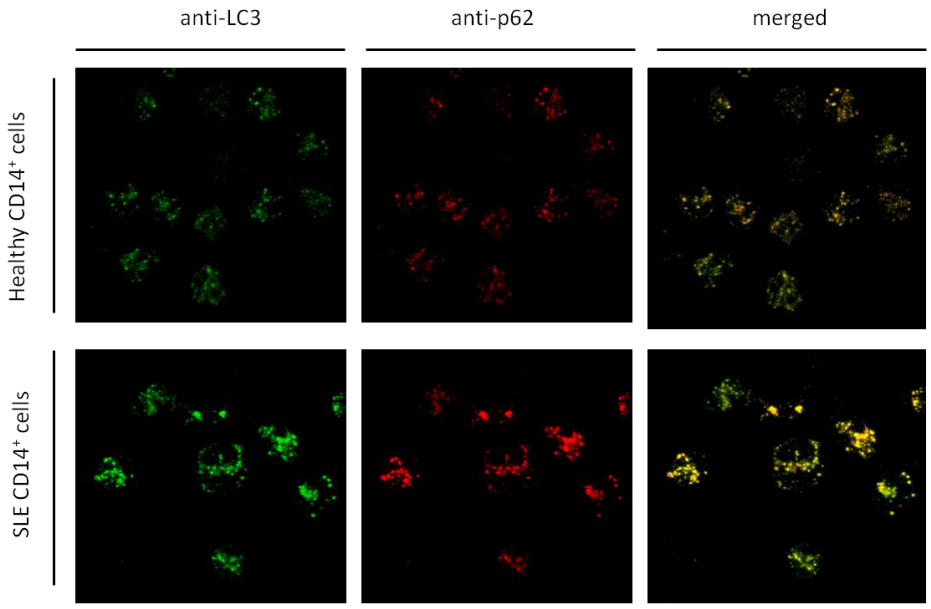


Figure C.2.2: ***Autophagy is induced but its completion is disrupted in SLE monocytes***. Immunofluoresence microscopy of freshly isolated healthy and SLE CD14^{+} cells. Cells were stained with anti-LC3 and p62 antibodies.

C.3: SLE serum induces autophagy in healthy monocytes but disrupts its completion

In order to assess whether autophagy induction was due to genetic predisposition in SLE patients or/and due to soluble factors in 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 immunoblot and real time PCR analysis. Western blot results showed increased LC3II/I ratio in the samples treated with SLE serum compared to healthy serum (Figure C.3.1 A-B), suggesting that SLE serum can induce autophagy in healthy monocytes. Additionally, p62 protein levels were similarly increased (Figure C.3.1.C), as in freshly isolated SLE CD14^{+} monocytes, but its mRNA levels indicated no difference between the samples that were treated with healthy serum compared to those treated with SLE serum (Figure C.3.1.D). Together, these observations imply that SLE serum is capable of inducing autophagy in healthy monocytes but disrupts its completion. All results were confirmed by confocal microscopy too (Figure C.3.2).

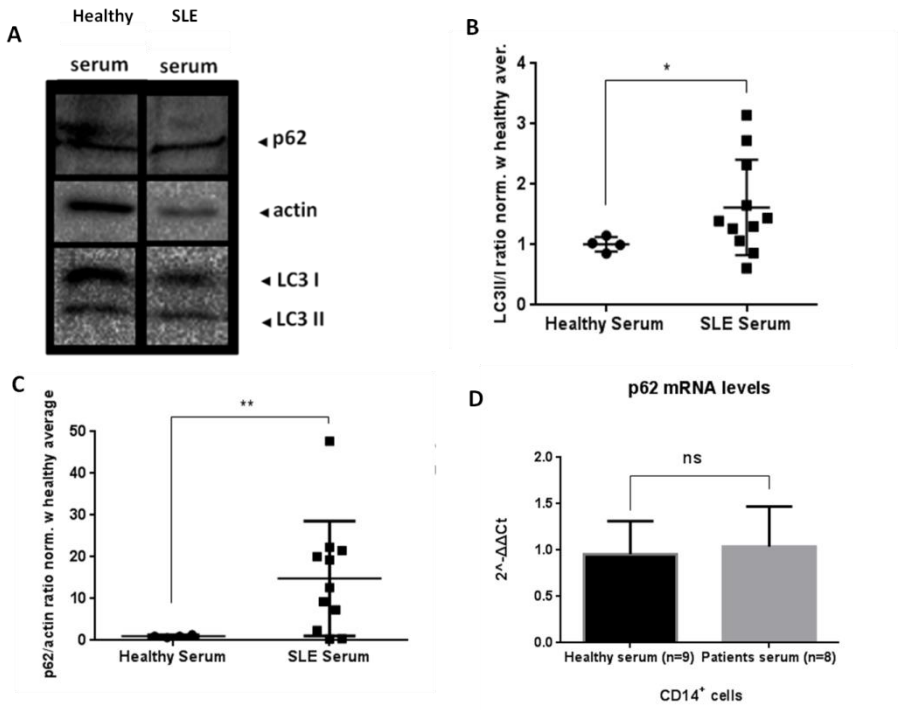


Figure C.3.1: ***SLE serum induces autophagy in healthy monocytes but disrupts its completion***. A) Representative image of SDS-PAGE immunoblotting of healthy CD14⁺cell lysates treated with 10% healthy or SLE serum for 24hrs. B,C) Quantitational analysis of LC3II/I ratio and p62 protein levels from 4 and 10 different healthy and SLE sera respectively. D) Real time pcR for p62 mRNA levels from heathy 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)

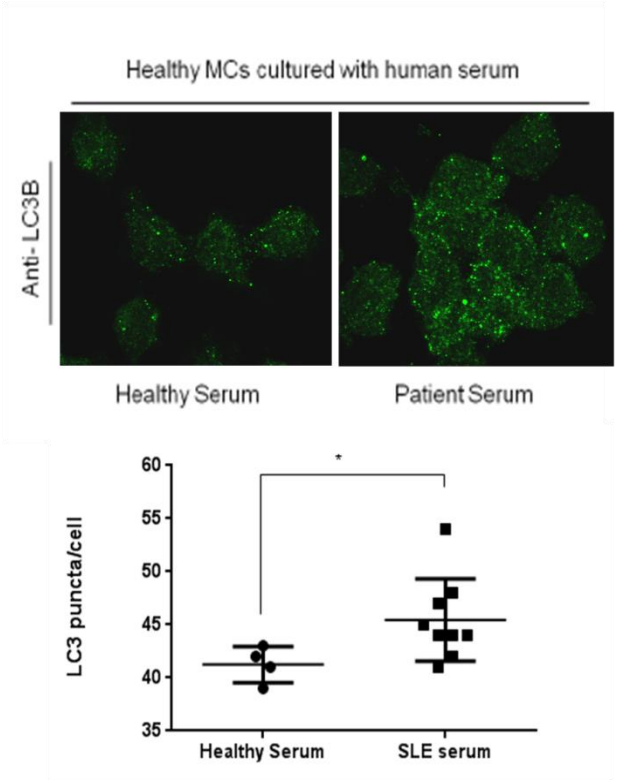


Figure C.3.2:
SLE serum induces autophagy in healthy monocytes.
A)Immunofluoresence microscopy of freshly isolated healthy CD14⁺ cells treated with 10% healthy or SLE serum for 24hrs. Cells were stained with anti-LC3 antibody.
B) Quantification of LC3 puncta/cell from 4 and 9 different samples treated with healthy or SLE serum respectively. Data are mean ± SD values, * $p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$ (Student's t test)

C.4: Recombinant human IFNa can induce autophagy in healthy monocytes but deregulates its completion

Since type I IFNs and specifically IFNa have been demonstrated to be increased in the blood of SLE patients[52] and monocytes cultured with SLE serum gain the ability to induce an MLR and are characterized as IFNa-DCs[57], we made the hypothesis that IFNa is the soluble factor in SLE sera that causes the induction of autophagy and is responsible for its deregulation. For this purpose, healthy $CD14^+$ freshly isolated monocytes were treated with human recombinant rIFNa (10^4U/ml) for different time-points (4hrs, 18hrs, 24hrs) and total protein lysate and RNA were collected. Immunoblot analysis showed that both LC3II and p62 levels were increased upon rIFNa treatment (Figure C.4.1 A, C), consistent with the results obtained when healthy monocytes were treated with SLE serum (Figure C.3.1 A, B). p62 protein levels were similarly increased but its mRNA levels indicated no difference upon treatment of healthy monocytes with rIFNa (Figure C.4.1. B, C), indicating disruption of autophagy completion. All results were confirmed by confocal microscopy (Figure C.4.2). Representative images of each experiment are shown below.

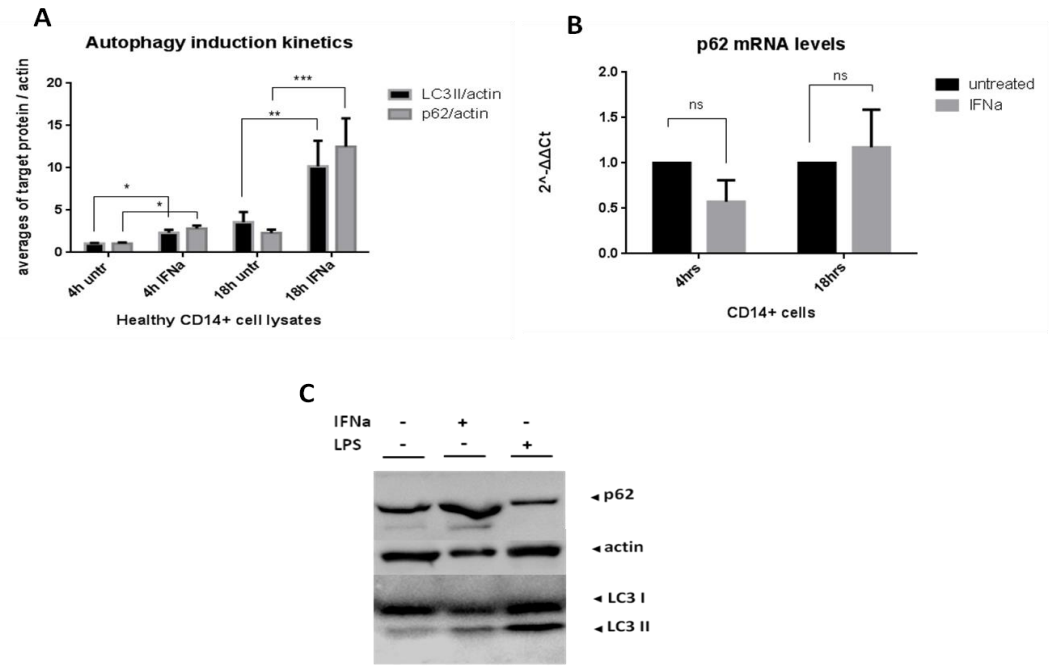


Figure C.4.1: ***Treatment of healthy monocytes with recombinant IFNa induces autophagy but deregulates its completion***. A) Quantitation of LC3II and p62 protein levels detected by immunolbotting upon 4hrs and 18hrs treatment of healthy monocytes. B) Real time pcR for p62 mRNA levels from heathy $CD14^+$ cells treated with rIFNa for 4hrs and 18hrs . C) Representative image of SDS-PAGE immunoblotting of healthy monocytes treated with rIFNa for 4hrs. LPS was used as a positive control of induced and succesfully completed autophagy. Data are mean ± SD values, * $p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$ (Student's t test)

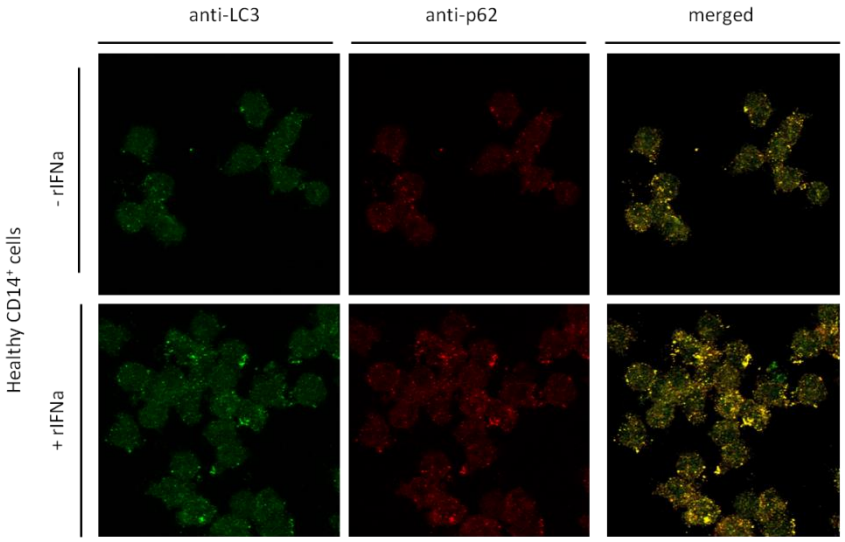


Figure C.4.2: ***Autophagy is induced but its completion is disrupted in healthy monocytes treated with rIFNa***. Immunofluoresence microscopy of healthy $CD14^+$ cells treated with rIFNa for 24hrs. Cells were stained with anti-LC3 and p62 antibodies.

C.5.: IFNa affects autophagy completion through its effect on autophagolysosomal pH.

During the final step of autophagy, autophagosomes fuse with lysosomes to form the mature **autophagolysosome**, exposing the inner compartment to lysosomal hydrolases which digest proteins, lipids, and nucleic acids in an acidic microenvironment[25]. In order to examine why p62 is not degraded inside the autophagolysosome, we examined the autophagolysosomal pH using a pH sensitive marker Lysotracker Red-DND99. Healthy monocytes were treated with rIFNa for different time-points and stained with Lysotracker Red-DND99. Lysotracker's intensity was examined by confocal microscopy. Monocytes treated with rIFNa exhibited reduced intensity of Lysotracker (Figure C.5.1), indicative alkalinized pH compared to untreated monocytes or LPS treated monocytes where Lysotracker stainining was intense, implying normal acidic pH inside autophagolysosomes (Figure C.5.2).

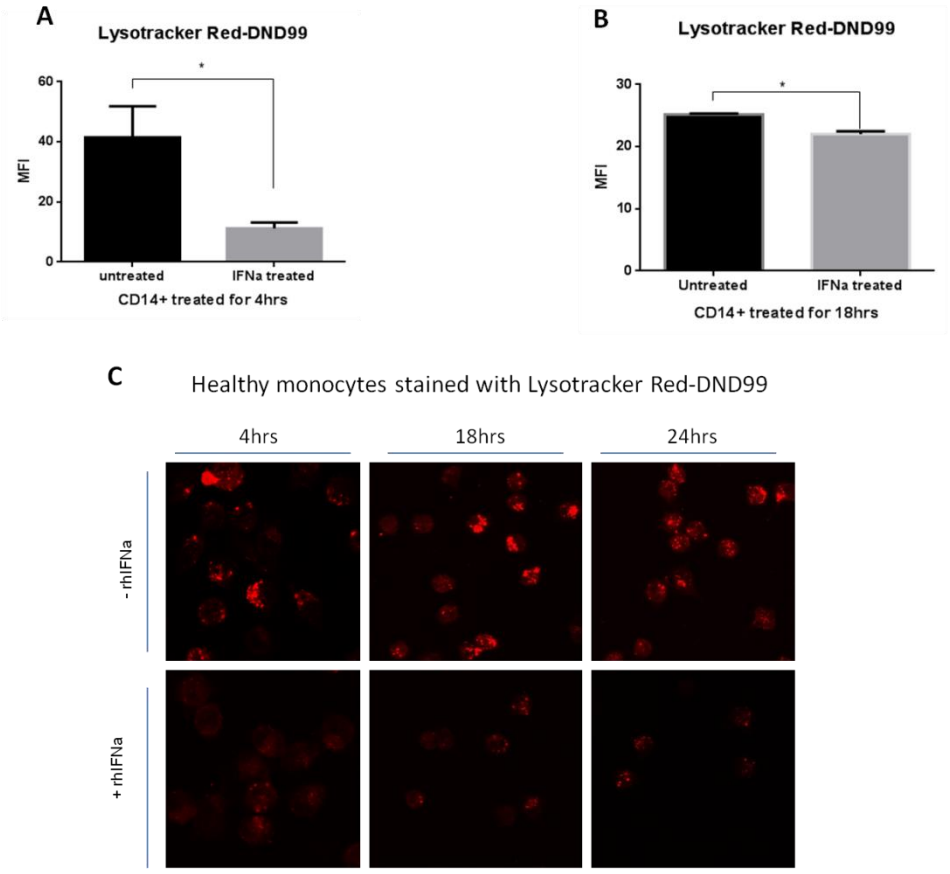


Figure C.5.1.: ***Recombinant IFNa affects autophagolysosomal pH in healthy monocytes.*** A,B) Quantitative analysis of Lysotracker Red-DND99 mean fluoresence intensity (MFI) of healhy monocytes upon treatment with rIFNa in indicative timepoints. C) Representative confocal images of healthy monocytes stained with Lysotracker Red-DND99

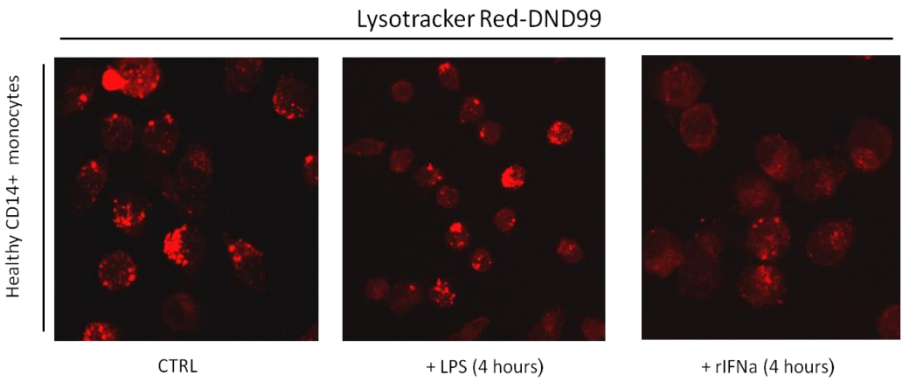


Figure C.5.2.: ***Recombinant IFNa affects autophagolysosomal pH in healthy monocytes.*** Representative confocal images of healthy monocytes stained with Lysotracker Red-DND99 upon treatment with LPS of rIFNa for 4hrs.

C.6: Autophagolysosomal pH is affected in freshly isolated SLE monocytes

Since treatment of healthy monocytes with rIFNa can reproduce the autophagic phenotype of SLE monocytes we wanted to examine whether SLE monocytes have alterations in autophagolysosomal pH compared to healthy controls. For this reason, freshly isolated $CD14^{+}$ cells from an SLE patient were stained with Lysotracker Red-DND99 and fluorescence intensity was assessed by confocal microscopy (Figure C.6). Interestingly, SLE monocytes had remarkably reduced staining of Lysotracker Red-DND99 compared to healthy control.

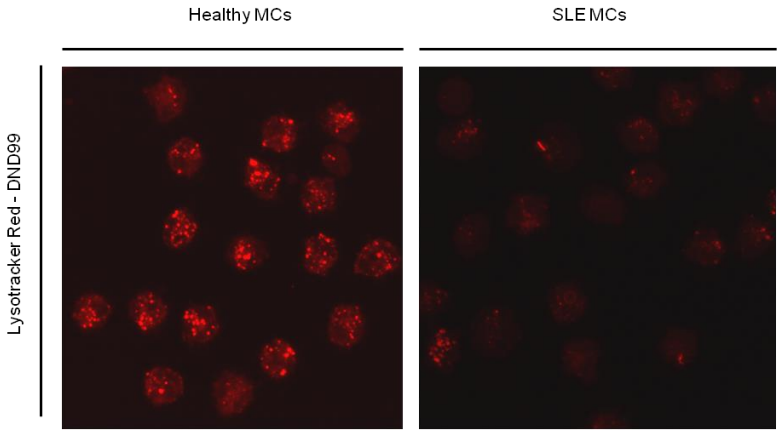


Figure C.6: ***SLE monocytes have more alkalinized autophagolysosomal pH compared to healthy control***. Freshly isolated CD14+ cells from a healthy donor and a SLE patient were stained with Lysotracker Red-DND99 and examined by confocal microscopy.

C.7: CD14^{+} monocytes treated with recombinant human IFNa obtain DC characteristics in an autophagy depended manner.

Previous studies had demonstrated that autophagy induction is essential for macrophage differentiation of human monocytes[41] and that treatment of healthy monocytes with rIFNa results in a DC-like phenotype of monocytes[58]. Thus, we wanted to test whether CD14^{+} monocytes treated with rIFNa obtain this DC-like phenotype in an autophagy dependent manner. Healthy CD14^{+} monocytes were treated with rIFNa (10^{4}U/ml), stained with anti-HLA-DR and anti-CD86 markers and assessed by flow cytometry. Wortmanin(WM), a PI3K inhibitor[76] and chloroquine (CQ) which affects lysosomal pH[77] and autophagolysosomal fusion were used as autophagy inhibitors. Results obtained from FACS analysis, indicated an increase in HLA-DR and CD-86 membrane markers in monocytes upon rIFNa treatment (Figure C.7), implying that these DC-like characteristics are obtained in an autophagy dependent manner. Treatment of monocytes with autophagy inhibitors and rIFNa "reversed" the DC-like phenotype since a decrease in both markers is observed.

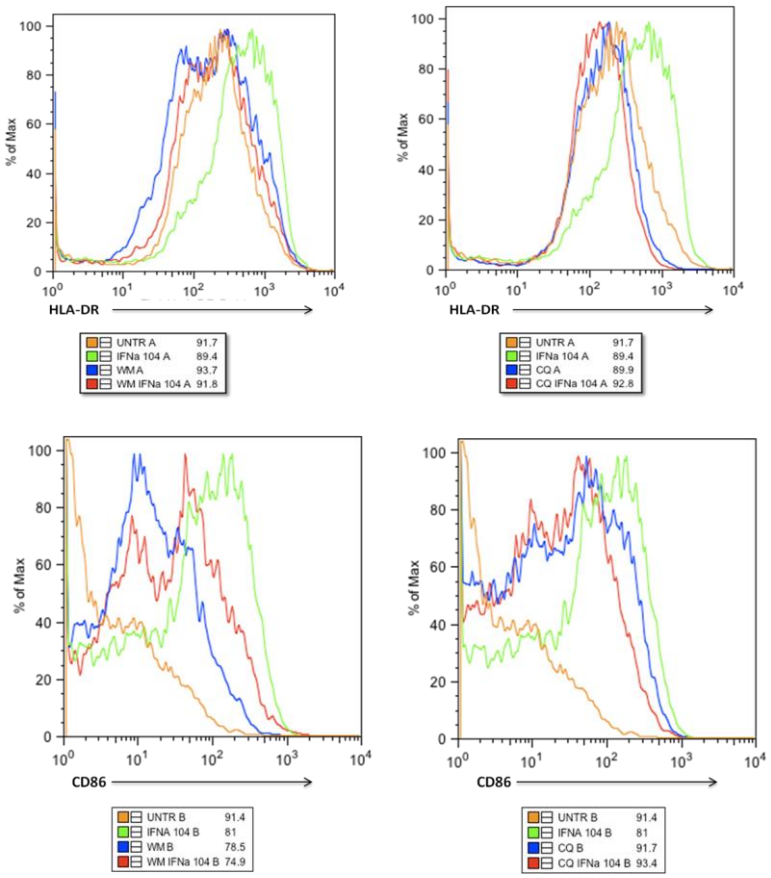


Figure C.7: ***Healthy CD14⁺ monocytes obtain DC characeristics upon treatment with rIFNa in an autophagy dependend manner***. Healthy CD14⁺ cells were treated with rIFNa and stained with HLA-DR and CD86 membrane markers and assessed by flow cytometry. Wortmanin (WM) and Chloroquine (CQ) were used as autophagy inhibitors.

VII. Conclusions - Discussion

Autophagy is activated under stress conditions such as starvation, ischemia/reperfusion and pathogen infection and is deregulated in various pathological conditions, including cancer, neurodegenerative and autoimmune diseases[78,79]. Autophagy is implicated in the differentiation, survival, and activation of both myeloid and lymphoid cells and its role in antigen presenting cells has been extensively studied[33]. Specifically, in SLE GWAS studies have linked genetic polymorphisms in autophagy related genes (ATG genes) and specifically in *ATG5* to the pathogenesis of the disease[74]. Additionally, T cells from SLE patients or from two distinct SLE-prone mouse strains, NZB/W F1 and MRL lpr/lpr mice, were reported to contain increased amounts of autophagosomes as compared to T cells from control patients or mice[80]. Also, autophagy is a mechanism for survival of SLE autoreactive B cells and is required for plasmablast differentiation[81]. Finally, autophagy induction in bone-marrow dendritic cells (DCs) has been shown to enhance presentation of mycobacterial antigen and mice immunized with rapamycin-treated DCs showed stronger T cell response upon challenge with *Mtb*[82]. Thus, dendritic cell-mediated antigen presentation in the context of MHC-II is perhaps an area where the role of autophagy could directly influence autoimmune diseases like SLE.

As shown in Figure C.1 and C.2 autophagy is upregulated in SLE monocytes compared to healthy controls but its completion is disrupted as indicated by the increased levels of p62. This phenomenon is also reproduced when healthy monocytes are treated with SLE serum as shown in Figure C.3.1 and C.3.2.

SLE serum has been reported to have significantly higher levels of proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-6[83]. High IFNγ and IFNa serum levels have also been reported in SLE[84] and patients with active SLE often present flu-like symptoms such as fever and fatigue, both of which may reflect high serum IFNa levels and are relevant to disease activity and severity[85]. Thus, we hypothesized that IFNa could be the soluble factor mediating this autophagy induction and deregulation, when SLE serum is added in healthy monocytes. Ex vivo experiments shown in Figure C.4.1 and Figure C.4.2 confirmed that indeed, recombinant IFNa can cause autophagy induction in healthy monocytes at different time-points. Moreover, rIFNa can cause alterations in the pH of lysosomes and autophagolysosomes (Figure C.5.1 and C.5.2). Since autophagy completion

is also deregulated as depicted from increased protein levels of p62 after treatment of healthy monocytes with rIFNa (Figure C.4.1 A, B and Figure C.4.2), we can assume that alterations in autophagolysosomal pH are responsible for the inefficient degradation of p62. In order to verify that IFNa is indeed the soluble factor in SLE serum that causes 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, will be performed expecting that these autophagy effects would be diminished.

An important observation is that freshly isolated SLE monocytes have remarkably reduced staining of Lysotracker Red-DND99 compared to healthy control (Figure C.6). This result implies that autophagy deregulation and increased protein levels of p62 as depicted in Figure C.2.1.B and C.2.2 may be due to defective degradation of autophagolysosomal constituents.

It is already published that SLE monocytes have IFNa-DC like phenotype[57] and interestingly our results depict that these characteristics (high expression of HLA-DR and CD-86 surface markers) are reproduced upon rIFNa treatment in an autophagy dependent manner as shown in Figure C.7, we can conclude that induction of autophagy is responsible for the DC phenotype of SLE $CD14^+$ monocytes. Extensive analysis of SLE monocytes, ex vivo treated with autophagy inhibitors, should be made by flow cytometry in order to verify these observations.

Previous studies have shown that MHC class II antigen loading compartments continuously receive input from autophagosomes[86] and that LC3-II molecules are 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[71]. Since SLE monocytes are capable of inducing the proliferation of alloreactive T cells[57], meaning that they can efficiently present antigens in the context of MHCII and since autophagy is required for cellular "housekeeping" by recycling misfolded proteins and whole organelles, like mitochondria, the non complete degradation of autophagolysosomal constituents may generate a large pool of self-antigens that can meet the MHCII compartment leading to their self-presentation in SLE environment.

Furthermore, the presentation of self-antigens at the periphery, similarly to the thymus, is carried out by multiple antigen presenting cells (APCs) such as dendritic cells (DCs)[61]. 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[67] and their existence is correlated with the presence of high IFNa levels, as patients either with other autoimmune diseases or cancer who received type I IFN therapy, exhibited anti-DNA antibody production[87,88]. Taken into consideration all the above, it could be suggested that high IFNa levels in SLE serum could be responsible for the deregulation of the autophagic degradation of self DNA leading thus to the brake of tolerance and the production of anti-dsDNA antibodies.

Future experiments could focus on the molecular identification of how IFNa changes the lysosomal pH and what consequences this might have to the MHCII repertoire. Moreover, identifying the signaling cascade that IFNa results in the upregulation of the autophagic machinery in monocytes can serve as fruitful targets for drug development not only in the context of lupus but also in other incurable autoimmune diseases.

Finally, preliminary evidence also indicates a role of IFNa in maturation and isotype switching of SLE B cells highlighting new roads for experimental studies. As a result, it would be of outmost interest to delineate the role of IFNa in the regulation of autophagy in SLE B cells, since these cells are responsible for the production of the anti-dsDNA antibodies.

VIII. References

1. Janis Kuby. *Immunology*. (W.H. Freeman and Company, 2000).

2. Gordon, S. & Taylor, P. R. Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* **5,** 953–64 (2005).

3. Dauer, M. *et al.* Mature Dendritic Cells Derived from Human Monocytes Within 48 Hours: A Novel Strategy for Dendritic Cell Differentiation from Blood Precursors. *J. Immunol.* **170,** 4069–4076 (2003).

4. León, B., López-Bravo, M. & Ardavín, C. Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania. *Immunity* **26,** 519–31 (2007).

5. Le Borgne, M. *et al.* Dendritic cells rapidly recruited into epithelial tissues via CCR6/CCL20 are responsible for CD8+ T cell crosspriming in vivo. *Immunity* **24,** 191–201 (2006).

6. Serbina, N. V, Salazar-Mather, T. P., Biron, C. A., Kuziel, W. A. & Pamer, E. G. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* **19,** 59–70 (2003).

7. Tezuka, H. *et al.* Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. *Nature* **448,** 929–33 (2007).

8. Ginhoux, F. *et al.* Langerhans cells arise from monocytes in vivo. *Nat. Immunol.* **7,** 265–73 (2006).

9. Landsman, L., Varol, C. & Jung, S. Distinct differentiation potential of blood monocyte subsets in the lung. *J. Immunol.* **178,** 2000–7 (2007).

10. Banchereau, J. *et al.* Immunobiology of dendritic cells. *Annu. Rev. Immunol.* **18,** 767–811 (2000).

11. Steinman, R. M. & Banchereau, J. Taking dendritic cells into medicine. *Nature* **449,** 419–26 (2007).

12. Sallusto, F. & Lanzavecchia, A. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. *Immunol. Rev.* **177,** 134–40 (2000).

13. Cella, M., Engering, A., Pinet, V., Pieters, J. & Lanzavecchia, A. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* **388,** 782–7 (1997).

14. Sozzani, S. *et al.* Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties. *J. Immunol.* **161,** 1083–6 (1998).

15. Mizushima, N., Yoshimori, T. & Ohsumi, Y. The role of Atg proteins in autophagosome formation. *Annu. Rev. Cell Dev. Biol.* **27,** 107–32 (2011).

16. Codogno, P., Mehrpour, M. & Proikas-Cezanne, T. Canonical and non-canonical autophagy: variations on a common theme of self-eating? *Nat. Rev. Mol. Cell Biol.* **13,** 7–12 (2012).

17. Zoncu, R., Efeyan, A. & Sabatini, D. M. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat. Rev. Mol. Cell Biol.* **12,** 21–35 (2011).

18. Hamasaki, M. *et al.* Autophagosomes form at ER-mitochondria contact sites. *Nature* **495,** 389–93 (2013).

19. Xie, Z. & Klionsky, D. J. Autophagosome formation: core machinery and adaptations. *Nat. Cell Biol.* **9,** 1102–9 (2007).

20. Walczak, M. & Martens, S. Dissecting the role of the Atg12-Atg5-Atg16 complex during autophagosome formation. *Autophagy* **9,** 424–5 (2013).

21. Kabeya, Y. *et al.* LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* **19,** 5720–8 (2000).

22. Longatti, A. & Tooze, S. A. Vesicular trafficking and autophagosome formation. *Cell Death Differ.* **16,** 956–65 (2009).

23. Kuusisto, E., Salminen, A. & Alafuzoff, I. Ubiquitin-binding protein p62 is present in neuronal and glial inclusions in human tauopathies and synucleinopathies. *Neuroreport* **12,** 2085–90 (2001).

24. Pankiv, S. *et al.* p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J. Biol. Chem.* **282,** 24131–45 (2007).

25. Kroemer, G. & Jäättelä, M. Lysosomes and autophagy in cell death control. *Nat. Rev. Cancer* **5,** 886–97 (2005).

26. Kadowaki, M. & Karim, M. R. Cytosolic LC3 ratio as a quantitative index of macroautophagy. *Methods Enzymol.* **452,** 199–213 (2009).

27. Kroemer, G., Mariño, G. & Levine, B. Autophagy and the integrated stress response. *Mol. Cell* **40,** 280–93 (2010).

28. Levine, B. & Kroemer, G. Autophagy in the pathogenesis of disease. *Cell* **132,** 27–42 (2008).

29. Levine, B. & Klionsky, D. J. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev. Cell* **6,** 463–77 (2004).

30. Meléndez, A. *et al.* Autophagy genes are essential for dauer development and life-span extension in C. elegans. *Science* **301,** 1387–91 (2003).

31. Vellai, T. *et al.* Genetics: influence of TOR kinase on lifespan in C. elegans. *Nature* **426,** 620 (2003).

32. Regulation, T., Kuma, A., Hatano, M. & Matsui, M. The role of autophagy during the early neonatal starvation period. **432,** 1–5 (2004).

33. Ma, Y., Galluzzi, L., Zitvogel, L. & Kroemer, G. Autophagy and cellular immune responses. *Immunity* **39,** 211–27 (2013).

34. Schmeisser, H. *et al.* Type I interferons induce autophagy in certain human cancer cell lines. *Autophagy* **9,** 683–96 (2013).

35. Tang, D., Kang, R., Coyne, C. B., Zeh, H. J. & Lotze, M. T. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. *Immunol. Rev.* **249,** 158–75 (2012).

36. Su, C. *et al.* Helminth infection impairs autophagy-mediated killing of bacterial enteropathogens by macrophages. *J. Immunol.* **189,** 1459–66 (2012).

37. Apel, A., Herr, I., Schwarz, H., Rodemann, H. P. & Mayer, A. Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. *Cancer Res.* **68,** 1485–94 (2008).

38. Xu, T., Su, H., Ganapathy, S. & Yuan, Z.-M. Modulation of autophagic activity by extracellular pH. *Autophagy* **7,** 1316–22 (2011).

39. Boya, P., Reggiori, F. & Codogno, P. Emerging regulation and functions of autophagy. *Nat. Cell Biol.* **15,** 713–20 (2013).

40. Nimmerjahn, F. *et al.* Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. *Eur. J. Immunol.* **33,** 1250–9 (2003).

41. Zhang, Y., Morgan, M. J., Chen, K., Choksi, S. & Liu, Z. Induction of autophagy is essential for monocyte-macrophage differentiation. *Blood* **119,** 2895–905 (2012).

42. Green, D. R., Galluzzi, L. & Kroemer, G. Mitochondria and the autophagy-inflammation-cell death axis in organismal aging. *Science* **333,** 1109–12 (2011).

43. Zhou, R., Yazdi, A. S., Menu, P. & Tschopp, J. A role for mitochondria in NLRP3 inflammasome activation. *Nature* **469,** 221–5 (2011).

44. Nakahira, K. *et al.* Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat. Immunol.* **12,** 222–30 (2011).

45. Haven, N. Innate immunity : impact on the adaptive immune response Ruslan Medzhitov and Charles A Janeway Jr. *Curr. Opin. Immunol.* (1997).

46. Bogdan, C. The function of type I interferons in antimicrobial immunity. *Curr. Opin. Immunol.* **12,** 419–24 (2000).

47. Cella, M. *et al.* Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat. Med.* **5,** 919–23 (1999).

48. Siegal, F. P. *et al.* The nature of the principal type 1 interferon-producing cells in human blood. *Science* **284,** 1835–7 (1999).

49. N, J. *et al.* Functional Role of Type I and Type 11 in Antiviral Interferons Defense. **264,** 1918–1921 (1994).

50. Liu, Z. & Davidson, A. Taming lupus-a new understanding of pathogenesis is leading to clinical advances. *Nat. Med.* **18,** 871–82 (2012).

51. Pascual, V., Farkas, L. & Banchereau, J. Systemic lupus erythematosus: all roads lead to type I interferons. *Curr. Opin. Immunol.* **18,** 676–82 (2006).

52. Bennett, L. *et al.* Interferon and Granulopoiesis Signatures in Systemic Lupus Erythematosus Blood. *J. Exp. Med.* **197,** 711–723 (2003).

53. Bauer, J. W. *et al.* Elevated serum levels of interferon-regulated chemokines are biomarkers for active human systemic lupus erythematosus. *PLoS Med.* **3,** e491 (2006).

54. Ramanujam, M. *et al.* Interferon-alpha treatment of female (NZW x BXSB)F(1) mice mimics some but not all features associated with the Yaa mutation. *Arthritis Rheum.* **60,** 1096–101 (2009).

55. Agrawal, H. *et al.* Deficiency of type I IFN receptor in lupus-prone New Zealand mixed 2328 mice decreases dendritic cell numbers and activation and protects from disease. *J. Immunol.* **183,** 6021–9 (2009).

56. Rönnblom, L. E., Alm, G. V & Oberg, K. Autoimmune phenomena in patients with malignant carcinoid tumors during interferon-alpha treatment. *Acta Oncol.* **30,** 537–40 (1991).

57. Blanco, P., Palucka, a K., Gill, M., Pascual, V. & Banchereau, J. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. *Science* **294,** 1540–3 (2001).

58. Blanco, P. Induction of Dendritic Cell Differentiation by IFN- ⌴ in Systemic Lupus Erythematosus. **1540,** (2013).

59. Kyewski, B. & Klein, L. A central role for central tolerance. *Annu. Rev. Immunol.* **24,** 571–606 (2006).

60. Mueller, D. L. Mechanisms maintaining peripheral tolerance. *Nat. Immunol.* **11,** 21–7 (2010).

61. Turley, S. J., Fletcher, A. L. & Elpek, K. G. The stromal and haematopoietic antigen-presenting cells that reside in secondary lymphoid organs. *Nat. Rev. Immunol.* **10,** 813–25 (2010).

62. Doyle, H. A. & Mamula, M. J. Post-translational protein modifications in antigen recognition and autoimmunity. *Trends Immunol.* **22,** 443–449 (2001).

63. Behrens, E. M. *et al.* Complement receptor 3 ligation of dendritic cells suppresses their stimulatory capacity. *J. Immunol.* **178,** 6268–79 (2007).

64. Doyle, H. A. & Mamula, M. J. Autoantigenesis: the evolution of protein modifications in autoimmune disease. *Curr. Opin. Immunol.* **24,** 112–8 (2012).

65. Zamvil, S. S. *et al.* T-cell epitope of the autoantigen myelin basic protein that induces encephalomyelitis. *Nature* **324,** 258–60

66. Hahn, B. H. Antibodies to DNA. *N. Engl. J. Med.* **338,** 1359–68 (1998).

67. Bootsma, H. *et al.* Prevention of relapses in systemic lupus erythematosus. *Lancet* **345,** 1595–9 (1995).

68. Yung, S. & Chan, T. M. Autoantibodies and resident renal cells in the pathogenesis of lupus nephritis: getting to know the unknown. *Clin. Dev. Immunol.* **2012,** 139365 (2012).

69. Rekvig, O. P. *et al.* Autoantibodies in lupus: culprits or passive bystanders? *Autoimmun. Rev.* **11,** 596–603 (2012).

70. Mizushima, N., Yoshimori, T. & Levine, B. Methods in mammalian autophagy research. *Cell* **140,** 313–26 (2010).

71. Kasai, M. *et al.* Autophagic compartments gain access to the MHC class II compartments in thymic epithelium. *J. Immunol.* **183,** 7278–85 (2009).

72. Pierdominici, M., Vacirca, D., Delunardo, F. & Ortona, E. mTOR signaling and metabolic regulation of T cells: new potential therapeutic targets in autoimmune diseases. *Curr. Pharm. Des.* **17,** 3888–97 (2011).

73. Fernandez, D. & Perl, A. mTOR signaling: a central pathway to pathogenesis in systemic lupus erythematosus? *Discov. Med.* **9,** 173–8 (2010).

74. Harley, J. B. *et al.* Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. *Nat. Genet.* **40,** 204–10 (2008).

75. Pierdominici, M. *et al.* Role of autophagy in immunity and autoimmunity, with a special focus on systemic lupus erythematosus. *FASEB J.* **26,** 1400–12 (2012).

76. Blommaart, E. F., Krause, U., Schellens, J. P., Vreeling-Sindelárová, H. & Meijer, A. J. The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. *Eur. J. Biochem.* **243,** 240–6 (1997).

77. Colombo, M. I. & Bertini, F. Properties of binding sites for chloroquine in liver lysosomal membranes. *J. Cell. Physiol.* **137,** 598–602 (1988).

78. Scherz-Shouval, R. & Elazar, Z. Regulation of autophagy by ROS: physiology and pathology. *Trends Biochem. Sci.* **36,** 30–8 (2011).

79. Mizushima, N., Levine, B., Cuervo, A. M. & Klionsky, D. J. Autophagy fights disease through cellular self-digestion. *Nature* **451,** 1069–75 (2008).

80. Gros, F. *et al.* Macroautophagy is deregulated in murine and human lupus T lymphocytes. *Autophagy* **8,** 1054–1053 (2012).

81. Clarke, A. J. *et al.* Autophagy is activated in systemic lupus erythematosus and required for plasmablast development. *Ann. Rheum. Dis.* 1–9 (2014). doi:10.1136/annrheumdis-2013-204343

82. Jagannath, C. *et al.* Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. *Nat. Med.* **15,** 267–76 (2009).

83. al-Janadi, M., al-Balla, S., al-Dalaan, A. & Raziuddin, S. Cytokine profile in systemic lupus erythematosus, rheumatoid arthritis, and other rheumatic diseases. *J. Clin. Immunol.* **13,** 58–67 (1993).

84. Bengtsson, A. A. *et al.* Activation of type I interferon system in systemic lupus erythematosus correlates with disease activity but not with antiretroviral antibodies. *Lupus* **9,** 664–71 (2000).

85. Wenzel, J., Zahn, S., Bieber, T. & Tüting, T. Type I interferon-associated cytotoxic inflammation in cutaneous lupus erythematosus. *Arch. Dermatol. Res.* **301,** 83–6 (2009).

86. Schmid, D., Pypaert, M. & Münz, C. Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. *Immunity* **26,** 79–92 (2007).

87. Kälkner, K. M. *et al.* Antibodies against double-stranded DNA and development of polymyositis during treatment with interferon. *QJM* **91,** 393–9 (1998).

88. Bonaci-Nikolic, B. *et al.* Anti-double stranded DNA and lupus syndrome induced by interferon-beta therapy in a patient with multiple sclerosis. *Lupus* **18,** 78–80 (2009).