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Delineating the impact of IFNα-mediated autophagy in the regulation of the expression and function of BAFF of monocytes in active SLE

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Εργασία Μεταπτυχιακού Τίτλου Ειδίκευσης

Διερεύνηση της δράσης της IFNα μεσολαβούμενης αυτοφαγίας στην ρύθμιση της έκφρασης και της λειτουργίας του BAFF σε μονοκύτταρα από ενεργό Συστηματικό Ερυθηματώδη Λύκο (ΣΕΛ)

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ABSTRACT

Systemic lupus erythematosus (SLE) is a complex, chronic autoimmune inflammatory disease characterized by multiple pathophenotypes. It exhibits remarkable variation in its clinical manifestations and is characterized by the systemic activation of B and T lymphocytes, myeloid cells as well as the presence of pathogenic autoantibodies against nucleic acids and associated proteins. In this way, it is understood that there is a global loss of self-tolerance, with B cells contributing actively to the pathogenesis of the disease. Lack of B cell tolerance plays an important role in the disease, leading to accumulation of autoantibodies, causing severe complications and inflammatory lesions, affecting basic organs including the skin, joints, the central nervous system and the kidneys. Among other things, variations in the production and secretion of various cytokines contribute to proportional deregulation of the immune system.

Type I interferons, and in particular Interferon-α (IFNα), play a pivotal role in the pathogenesis of the disease, through intense up-regulation of multiple type I IFN-inducible genes, also known as IFNα "signature", manifested by lupus patients. In addition, high levels of IFNα have been correlated with the severity and activity of the disease, as well as the production of circulating autoantibodies in SLE patients. Autoreactive lupus monocytes acquire a dendritic cell(DC)-like phenotype, showing a strong antigen-presenting capacity through the action of IFNα. In addition, SLE and IFNα-primed DC-like monocytes are considered a major source of BAFF (B-cell activating factor), which plays a prominent role in SLE by promoting the differentiation and survival of autoreactive B-cells. Antigen presentation in autoreactive monocyte is depended on cell autophagy, a normal process for maintaining cellular homeostasis, which presents defects in patients with lupus. To this end, the possible role of autophagy in regulation of BAFF production by monocytes in the context of SLE remains elusive. Of note, BAFF targeting by monoclonal anti-BAFF antibody (Belimumab) represents the first approved biologic therapy in SLE.

The aim of this study was to investigate the role of autophagy in SLE monocytes, presenting an IFNα-DC like phenotype, in the regulation and secretion of BAFF, thus contributing to the differentiation of auto-reactive B cells through class-switch-recombination. Indeed, we observed an induction of BAFF membrane, protein and mRNA expression, upon IFNα signaling, which was involved through autophagic modulation, in CD14+ monocytes. Unraveling a possible molecular mechanism of regulating BAFF differentiation upon IFNα signaling may provide with a wider armamentarium for putative SLE-targeting therapies.

ΠΕΡΙΛΗΨΗ

Ο Συστηματικός Ερυθηματώδης Λύκος (ΣΕΛ), αποτελεί μια πολύπλοκη, χρόνια αυτοάνοση ασθένεια με ευρύ φάσμα παθολογικών φαινοτύπων. Παρουσιάζει ετερογένεια ως προς τις κινικές εκδηλώσεις του και χαρακτηρίζεται από συστηματική ενεργοποίηση των Β και Τ λεμφοκυττάρων και μυελικών κυττάρων, καθώς επίσης την παρουσία παθογόνων αυτο-αντισωμάτων έναντι των νουκλεϊκών οξέων και των πρωτεϊνών που προσδένουν αυτές. Κατά αυτό τρόπο, γίνεται αντιληπτό ότι υπάρχει διαταραχή της ανοσολογικής ανοχής, με αυτή των Β κυττάρων να συμβάλλει ενεργά στην παθογένεια της νόσου. Η έλλειψη ανοχής των Β κυττάρων παίζει σημαντικό ρόλο στην ασθένεια, οδηγώντας στην συσσώρευση αυτοαντισωμάτων, τα οποία προκαλούν φλεγμονώδη αντίδραση και ιστικές βλάβες επηρεάζοντας βασικά όργανα του σώματος συμπεριλαμβανομένων του δέρματος, των αρθρώσεων, του νευρικού συστήματος και των νεφρών.Μεταξύ άλλων, διαφοροποιήσεις στην παραγωγή και έκκριση διάφορων κυτταροκινών συμβάλλουν στην ανοσολογική απορρύθμιση.

Ειδικότερα , η Ιντερφερόνη-α (IFNα) φαίνεται διαδραματίζει καθοριστικό ρόλο στην αίτιο-παθογένεια της νόσου, μέσω της έκφρασης Ιντερφερονο-επαγόμενων γονιδίων , γνωστής και ως γονιδιακής "υπογραφής" της Ιντερφερόνης -α, που παρουσιάζουν οι ασθενείς με Λύκο. Επιπλέον, τα υψηλά επίπεδα της ιντερφερόνης-α έχει αναφερθεί ότι σχετίζονται με την σοβαρότητα και την ενεργότητα της ασθένειας καθώς και την παραγωγή των αυτοαντισωμάτων, που παρατηρούνται στον ορό των ασθενών. Στο ΣΕΛ, τα αυτοδραστικά μονοκύτταρα –μέσω της δράσης της IFNα- αποκτούν ένα φαινότυπο που μοιάζει με αυτόν των δενδριτικών κυττάρων, παρουσιάζοντας έντονη αντιγόνο-παρουσιαστική ικανότητα. Ωστόσο, έχει επίσης δειχθεί πως στην αντιγόνο-παρουσίαση των αυτοδραστικών κυττάρων , καθοριστικό ρόλο παίζει η κυτταρική αυτοφαγία, μια φυσιολογική διεργασία για την διατήρηση της κυτταρικής ομοιοστασίας, η οποία στους ασθενείς με ΣΕΛ παρουσιάζει διαταραχές. Παρά την χρόνια χορήγηση αντιφλεγμονωδών φαρμάκων για την βελτίωση της νόσου, τα τελευταία χρόνια έχει αναπτυχθεί ενδιαφέρον για πιο στοχευμένες θεραπείες, κυρίως στα Β κύτταρα. Παρόλο που η αρχική προσπάθεια εξάλειψης των Β κυττάρων δεν φάνηκε να είναι αποτελεσματική τα τελευταία χρόνια οι επιστήμονες έχουν στραφεί στην στόχευση παραγόντων κυτταρικής επιβίωσης των Β (B cell activating Factor-BAFF),αποτελώντας σημαντικό επίτευγμα στην θεραπεία του Λύκου.

Στόχος της παρούσας εργασίας ήταν να εξακριβωθεί ο ρόλος της αυτοφαγίας των μονοκυττάρων στο Λύκο , που παρουσιάζουν έναν φαινότυπο δενδριτικών κυττάρων, στην ρύθμιση και έκκριση του παράγοντα BAFF, συμβάλλοντας στη διατήρηση και διαφοροποίηση των Β κυττάρων. Παρατηρήθηκε επαγώμενη έκφραση του παράγοντα BAFF, ύστερα από σηματοδότηση με Ιντερφαρόνη α, η οποια φαίνεται να σχετίζεται με την αυτοφαγική διαμόρφωση των CD14+ μονοκυττάρων. Η αποσαφήνιση ενός μηχανισμού ρύθμισης της διαφορετικής λειτουργίας του παράγοντα BAFF, καθώς επίσης και η αποσαφήνιση του ρόλου της αυτοφαγίας των μονοκυττάρων ως πιθανού μηχανισμού "παραγωγής" αλλά και συντήρησης των αυτοδραστικών Β κυττάρων, προσφέρει ένα μεγαλύτερο εύρος πιθανών θεραπευτικών στόχων στο ΣΕΛ.

INTRODUCTION

Innate & adaptive immunity

The most important function of the immune system is to provide protection to the body against invading microbial pathogens along with elimination of abnormal cells and cellular debris. 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).

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 (Janis Kuby. Immunology. (W.H. Freeman and Company, 2013).

At the core of the immune system is the ability to tell the difference between self and non-self. A flaw can make the body unable to discriminate between self and non-self. When this happens, a harmful auto- immune response takes place (Figure 1).

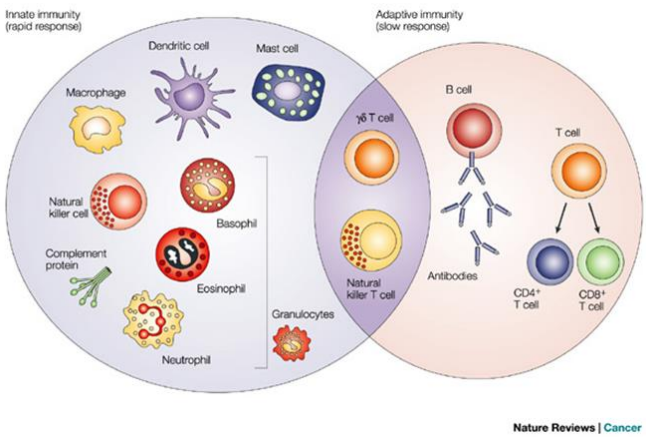


Figure 1. Innate and Adaptive immunity components (Dranoff e al., 2004)

Antigen Presenting Cells (APCs)

Antigen-presenting cells (APCs) are a heterogeneous group of immune cells that mediate the cellular immune response by processing and presenting antigens for recognition by certain lymphocytes such as T cells. Classical APCs include dendritic cells, macrophages, Langerhans cells and B cells. Dendritic cells (DCs) are professional 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 (Banchereau et al. 2000). Additionally, via communicating with various immune cells [e.g., natural killer cells (NKs)] DCs bridge the innate and adaptive arm of the immune response (Steinman 2007). Ligation of receptors for inflammatory chemokines recruits immature DCs and their blood precursors to sites of inflammation or infection (Cella et al. 1997). Upon encounter with microbial, pro inflammatory 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 immune stimulatory cytokines (Cella et al., 1997). Moreover, they 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 (Sozzani et al., 1998).

Monocytes are members of the mononuclear phagocyte system, a family of myeloid cells that comprises monocytes and two other subtypes namely: Dendritic cells (DCs) and macrophages. Monocytes are known to originate in the bone marrow from a common myeloid progenitor and they are then released into the peripheral blood where they circulate for several days before entering tissues and replenishing the tissue macrophage population (Gordon & Taylor 2005).

Monocytes may give rise to a subset of DCs during infection or inflammation, when high levels of pro-inflammatory mediators such as TNF-a, IL-1β and IFN-a are produced (Dauer et al. 2003). 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 (León & Ardavín 2008), cross prime antigen-specific $CD8^+$ T cells, exert a microbicidal action by producing tumor-necrosis factor-a (TNF-a) and iNOS (Serbina et al. 2003) and regulate IgA production by B cells. Recent studies have also revealed that monocytes are the precursors for some important DC subsets found in the steady state, such as Langerhans cells (Ginhoux et al. 2006) and DC subsets present in the intestinal and respiratory mucosas.

Type I interferons

Type I and II interferons (IFNα-β and IFN-γ respectively) are key regulators of innate and adaptive immunity. Type I interferons consisting of IFN-β and at least 13 homologous of IFNα proteins, have potent antiviral activity and represent pleiotropic modulators of immune responses. Type I interferons can be produced by almost every cell type, including leukocytes, fibroblasts and endothelial cells.

The signaling pathways that lead to the induction of type I IFNs differ depending on the stimulus and the responding cell types, but they ultimately lead to the activation of some common signaling molecules including TNF receptor – associated factor 3 (TRAF3) and the transcription factors IFN regulatory factor 3 (IRF3) and IRF7. IFNα/β receptor (IFNAR) is the common heterodimeric receptor that Type I interferons signal through. Almost all cells in the bod can produce IFNα/β and this is usually occurring in response to the stimulation of receptors known as pattern recognition receptors (PRRs) by microbial products. Moreover, canonical type I IFN signaling activates the Jak kinase (JAK) signal transducer and activator of transcription (STAT) pathway leading to transcription of IFN stimulated genes (ISGs). On the binding of type, I IFNs to their receptor (IFNAR), multiple downstream signaling pathways can be induced, leading to a diverse range of biological effectors. The canonical signal transducer and activator of transcription I (STAT1)-STAT2-IFN-Regulatory-facor 9 (IRF9) signaling complex binds to IFN-stimulated response elements (ISREs)in gene promoters leading to induction of a large number of IFN stimulated genes (ISGs). Type I IFNs can also signal through STAT1 and homodimers which are more commonly associated with the IFNγ-mediated signaling pathway. Other heterodimers and homodimers may also be activated downstream including STAT3, STAT4, STAT5 and some signaling pathways that do not rely on Janus kinase (JAK) and/or STAT activity may also be activated including mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K) pathway there by leading to diverse effects on the cellα.IFNα/β affect myeloid cells, B cells, T cells, and NK cells there by enhancing the immune response more effectively resolving viral infection and improving the generation of memory responses that will allow responses to future viral challenges (Chen et al. 2017).

Autophagy

Autophagy, the process by which cells degrade intracellular components can be categorized into three main types, macro autophagy, micro autophagy and chaperone mediated autophagy (CMA). Macro autophagy (hereafter referred as 'autophagy') encapsulate and break down defective or unnecessary organelles (Rockel & Kapoor 2016). Autophagy is a conserved process by which substrates in the cytosol are transported to the lysosome through a double membrane bound vesicle intermediate termer the autophagosome. Substrates targeted by autophagy include bulk cytoplasm protein aggregates, macromolecular complexes and organelles. By the pathway of autophagy, a portion of cytoplasm (usually 0.5-1 µm diameter) is engulfed by an isolation membrane or 'phagophore' resulting in the

formation of autophagosome. The outer membrane of autophagosome ultimately fuses with the lysosome to become an autolysosome leading to degradation of autophagosomal contents by lysosomal enzymes (Yu et al. 2018; Matsuzawa-Ishimoto et al. 2018). Autophagy

not only presents cellular homeostasis in conditions of endogenous distress but also plays a primordial role in controlling intracellular pathogens in evolutionarily distant species, ranging from unicellular organisms to humans (Ma et al. 2013). Among many functions of autophagy are cellular homeostasis, anti-aging and development. Accumulating evidences suggest that autophagy influences cellular immune responses well beyond its role as cell intrinsic mechanism of defense against invading pathogens. In particular, autophagy has recently shown to influence the antigenic profile of antigen-donor cells (ADCs) and their ability to release immunogenic signals. Thus, especially what it precedes cell death autophagy alerts innate immune effectors to elicit important role in maintaining cell and tissues homeostasis through regulating the self-renewal, maturation and survival of B cells, T cells and hematopoietic stem cells (Rockel & Kapoor 2016). Autophagy is also essential for the maturation and differentiation of monocytes into macrophage (Zhang et al. 2012).

Furthermore, the process of autophagy is implicated in the intracellular processing of inflammatory cytokines. One primordial response to viral infection is the secretion of type I Interferons (IFNs). However, recent studies have shown autophagy, as well as being regulated by cytokines, can itself directly influence the transcription, processing and secretion of a number of cytokines. In particularly, disruption of normal autophagic pathways has been inked to increased secretion of the pro-inflammatory cytokines IL-1α, IL-1β, IL-23 and IL-18 through presentation of mitochondrial function (Harris & James 2011).

Autophagy in Antigen Presenting Cells (APCs)

The regulation of autophagy is vital for appropriate cell responses to stresses, including infection and inflammation. Autophagy has an important role in T-cell repertoire selection by thymic epithelial cells- as well as in T-cell activation which is regulated by the presentation of antigens (derived from autophagosomes) by MHC class II expressing antigen-presenting cells (APCs). Immune cells, such as dendritic cells, can also engulf viruses in autophagosomes. The breakdown products are then delivered to endosomes where they interact with TLRs (for example singe-stranded RNA molecules interact with TLR7) and can be located onto MHC class II proteins for antigen presentation (Rockel & Kapoor 2016). Moreover, the importance of autophagy for antigen presentation extends to other APCs, like B cells and macrophages (Ma et al. 2013). For instance, these cell types when treated with pharmacological inhibitors of autophagy are impaired to their ability to present antigens on MHC class II molecules. Thus, autophagy plays a major role in how antigens from infected or transformed cells are taken up APCs digested to form peptides that can be loaded onto MHC class I or II molecules and finally presented on T cells.

To summarize, autophagy has critical role in the development and function of immune cells, innate immune signaling and cell autonomous defense.

Furthermore, autophagy can affect the secretion of cytokines by itself. For example, loss of autophagy in macrophages or dendritic cells, either through knock down of Atg7, Atg16L1, Becin-1, or by treatment with the autophagy inhibitor 3-MA, stimulates the processing and secretion of IL-1β in response to TLR agonist. This effect may be dependent on TIR domain-containing adaptor-inducing IFN-β (TRIF) and mitochondrial ROS and/or mitochondrial DNA and at least partially dependent on NLRP3 and also may be independent of TRIF, but dependent on p38 MAPK signaling. Conversely, induction of autophagy with rapamycin

inhibits the secretion of IL-1β in murine dendritic cells in response to LPS with ATP or alum. Given that IL-1α and IL-1β have both been shown to induce autophagy; this may act as a negative feedback loop to control IL-1-induced inflammation. Similarly, the secretion of IL-18, IL-6 and TNF-α was also regulated by autophagy. Inhibition of autophagy enhanced the production of IL-18, but reduced the production of IL-6, IL-8 and TNF-α. The modulation of autophagy in the secretion of IFN in virally-infected cells is controversial. Atg5 or autophagy deficient plasmacytoid dendritic cells was failed to produce IFN-α in response to infection with vesicular stomatitis virus (VSV). In contrast, other studies have demonstrated that embryonic fibroblasts from Atg5-/- mice are more resistant to VSV infection and produce higher levels of IFN-a and IFN-b mRNA in response to VSV or stimulation with dsRNA [poly(I:C)], compared with WT controls. In hepatitis C virus infected hepatocytes, Atg7 knockdown induced IFN signal pathway, thus induced cell death.

Autophagy pathway key protein players

Autophagy involves 3 molecular events: initiation (formation of phagosomes), elongation and closure (increase in 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 process of autophagy is positively and negatively regulated by many factors during the initiation and maturation of autophagosomes. Under basal conditions, autophagy is restricted by mechanistic target of rapamycin (mTOR). mTOR is a serine/ threonine protein kinase found in two distinct complexes: mTORC1 and mTORC2. Activation of mTOR leads to the inhibition of autophagy though direct modification of the autophagic machinery. Reduction in the availability of amino acids and glucose, oxidative stress and other environmental perturbations inhibit mTOR and activate consisting ULK1 complex. Rapamycin-sensitive m TORC1 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. In mammals, the major membrane source in the formation of the autophagophore, is thought to be the endoplasmic reticulum (ER), although several other membrane sources such as mitochondria and the plasma or nuclear membrane, may also contribute (Figure 2).

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. The biogenesis of the autophagosome is orchestrated by sequential multiple activities of three key protein complexes: ULK1 complex (comprising ULK1, FIP200, ATG13, ATG101), the phosphoinositide 3 kinase catalytic subunit type II (PI3KC3)

complex (comprising Beclin-1, vacuolar protein sorting 34 [VPS34], VPS15 and ATG14L) and ATG16L complex (comprising ATG16L, ATG5, ATG12) (Figure 2). As we mentioned above autophagy can be regulated in a positive or negative way. In particular, autophagy is positively regulated by a complex of four proteins: ULK1, autophagy related protein (ATG) 13, ATG101 and RB1-inducible coiled coil protein 1 (FIP200). ATG13 stabilizes interaction of ULK1 with FIP200 resulting in initiation of autophagosome formation. ULK1 complex component to be activated by AMP kinase (AMPK) and by ULK1 which phosphorylates itself ATG13 and FIP200

promoting early autophagophore formation. Autophagophore elongation and maturation require other ATG components to be recruited to the early autophagophore. The ubiquitin-like modifier- activating enzyme ATG7 the combined ATG10, ubiquities autophagy protein 5 (ATG5) so it can form a functional complex with ubiquitin- like protein ATG12.ATG5 and ATG12 act as a E3-ubiquitin-ike-ligase-lipidating the cytoplasmic form of microtubule-associated proteins 1A/1B light chain 3A (LC3), known as LC3-I and converting it to the membrane bound phosphatidylethanolamine-conjugated form, known as LC3-II. The conversion of LC3-I and LC3-II is an essential process in the maturation of autophagosomes enabling their essential fusion with lysosomes and autophagosome cargo degradation. The lipidation of LC3 requires prior cleavage by ATG4, a cysteine protease. Importantly, ATG4 can also act on LC3-II to release LCE from PE and therefore form the autophagosomal membrane. The removal of LC3-II from the autophagosome is required for its subsequent fusion with endosome/lysosome. Since the production of LC3-II is required for the elongation of autophagophore and consequently to the autophagic machinery LC3-II is currently the most widely used autophagosome marker, as it is well known that the amount of LC3-II reflects the number of autophagosomes and autophagy related structures (Bhutia et al. 2019). As the phagophore grown the coordinated action of autophagy proteins, cargos are incorporated into the autophagosome through autophagy receptor. One example is the sequestosome 1 (SQSTM1), also known as adaptor protein p62. Simultaneously interact with LC3 and ubiquitin chains, thereby cross-linking ubiquitin-modified substrates to the nascent autophagosome. LC3-II recruits p62 which is commonly found in inclusion bodies containing polyubiquitinated protein aggregates. Thus, p62/ SQSTM1 have been shown to bind directly to LC3 in order to facilitate degradation of ubiquitinated protein aggregates by autophagy. In addition, degradation of P62 is another widely used marker to monitor autophagic activity because of the direct binding of p62 to LC3 and the selective degradation by autophagy (Bhutia et al. 2019). Maturation of autophagosome, consider as the last step of autophagy. Once autophagosome has formed subsequently fuses with lysosomes forming the mature autophagolysosome exposing inner compartment to lysosomal hydrolases which digest lipids and nucleic acids in an acidic microenvironment. 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 (Figure 2) (Hansen et al. 2018).

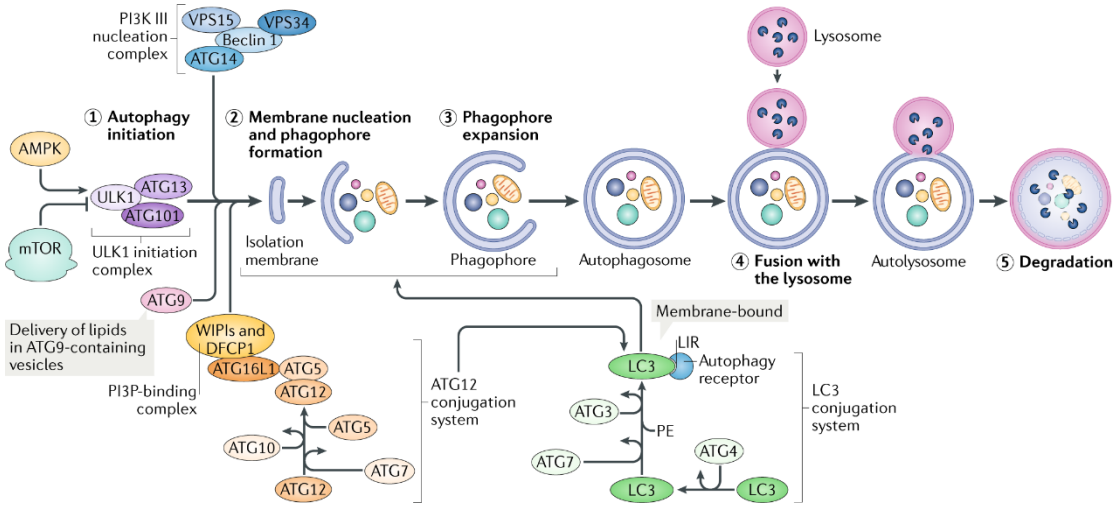


Figure 2. The Macroautophagy process (Hansen et al. 2018).

B cell activating factor (BAFF)

B cell activating factor belonging to the tumor necrosis factor (TNF) family (BAFF) was discovered a decade ago by several different scientific groups. BAFF also known as Blys (B lymphocyte stimulator, or TALL-1, THANK, TNSF13B, z TNF4, CD257) has become significant in B cell biology as a cytokine responsible for B cell survival and maturation during the early transitional stages. BAFF has also been associated with the control of tolerance and malignancy (MacKay & Schneider 2009). BAFF's influence is widespread and its effects are as numerous as varied. In fact, BAFF offers a range of variants membrane- bound or soluble, glycosylated or non-forms, monomer or trimers, homotrimers or heterotrimers, heterotrimers with another TNF family member APRIL (a proliferation inducing ligand) or heterotrimer with BAFF variants or even virus-like aggregates of 69 monomers (Vincent et al. 2013).

BAFF gene and transcripts

In humans, the Baff gene is mapped on the chromosome 13 in the q33.3 regions and contains 6 exons and 5 introns corresponding 39 kilo base (kb). The main *BAFF* transcript encoded by the gene contains 1204 base pairs (bp) with an open reading frame (ORF) of 858 bp (Genbank accession number is NM-006573) (Figure 3). In mice, Baff gene is mapped on chromosome 8A1.1 and contains 7 exons and 6 introns corresponding to 31kb. The main Baff transcript encoded by this gene contains 1710bp with an ORF of 930bp (Genbank accession number is NM-0336221). The promoter can be activated by many transcription factors. NFAT (nuclear factor of activated T cells) members and NF-κ B members can bind on BAFF promoter. In addition two other transcription factors for BAFF belong to the TNF-receptor family: CD40 and BR3 (BAFF receptor 3) (Fu et al. 2009). These two factors interact with c-Rel to activate BAFF transcription (Zhou et al. 2007; Fu et al. 2009). In human intestinal epithelial cells (EE) and IFN-γ can induce the production of both soluble and membrane-bound BAFF by JAK/STAT activation pathway and binding of phosphorylated STAT-1 to the IFN-γ activated site (GAS) element at specific position on BAFF promoter (Woo et al. 2013). Furthermore, recently has been described that BAFF expression is directly regulated by type I IFNs via IRF1 and IRF2 whereas IRF4 and IRF8 are negative regulators of BAFF expression (Sjöstrand et al. 2016).

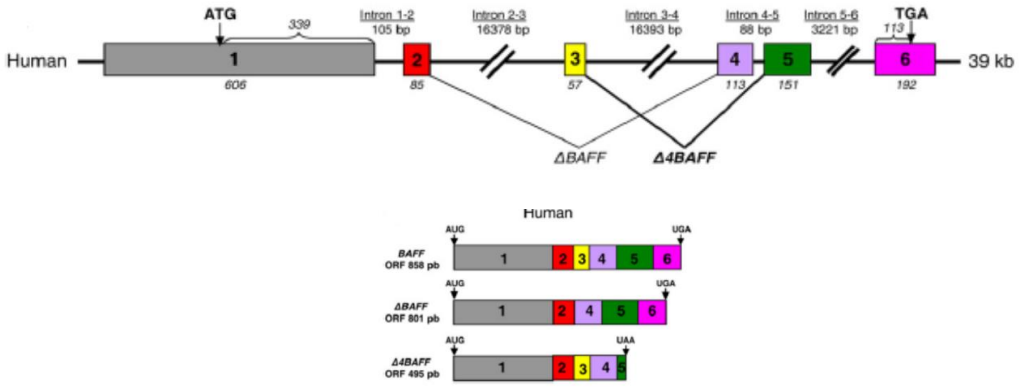


Figure 3. BAFF Human gene and transcripts (Journal of Autoimmunity 2012)

BAFF proteins and structures depending on PH contains

Human and murine BAFF are type- II transmembrane proteins of 285 amino acids (31.2 k Da) for human and 309 amino acids for murine. BAFF and APRIL share about 50% similarity within the TNF homology domain (THD) which is a C-terminal sequence characteristic for the TNFSF. BAFF is expressed on the membrane as a full-length molecule and can be cleaved by a furin like enzyme that belongs to the protein convertase family as a 152 amino acid soluble ligand (Schneider et al. 1999). This cleavage of surface BAFF results in the release of this soluble biologically active 17k Da molecule.

Furthermore, a shorter isoform termed delta BAFF exists and arises from alternative splicing (Vincent et al. 2013). Delta BAFF is also a transmembrane protein but it lacks specifically the furin protease recognition motif. In this way Delta BAFF cannot be released as a soluble molecule. Thus, Delta BAFF proteins do not bind to cells expressing BAFF receptors but rather form biologically inactive intracellular heterodimers with the full-length BAFF thereby limiting BAFF availability. Delta BAFF suppresses BAFF function and its co-expression appears to regulate ability of BAFF to appear on the cell surface and to be subsequently shed into the extracellular space (Gavin et al. 2005). All form of BAFF adopts a trimeric structure which is mediated by this THD. In addition, it is well known that BAFF protein can form trimers or 60mers. Like all TNF-ligands, the biological form of BAFF is a trimer. Interestingly there is a unique FLAP region (DE loo) of soluble BAFF that allows trimer to trimer interaction leading to a virus-like assembly of the soluble trimers (Karpusas et al. 2002). BAFF is the only member of TNF superfamily that can be organized in this higher order capsid-like assembly of 20 trimers (60 monomers) (Vincent et al. 2013). This structure contains these 20 trimers associated with each other by hydrogen and hydrophobic bonds involving 4 residues from the FLAP region seems decisive for the formation of oligomers in basic conditions. The different structural forms of BAFF descried (monomers, trimers and oligomers) are formed and deformed according to the PH levels. Indeed, at PH=6.0 BAFF exists only in a trimeric form, whereas at PH=6.5 the ratio oligomers/trimers are 1:2 and 1:1 at PH=7.0. Finally, at PH=7.4 only oligomeric form is present.

Soluble BAFF acts as a cytokine through the regulation of the survival of B cells. The oligomeric form can also induce the proliferation of B cells in vitro with the same efficiency as the trimeric form. These structural forms were detected in the supernatants of several cell lines demonstrating their existence. However, their physiological role has not yet been clearly demonstrated (Cachero et al. 2006).

Cell type	BAFF Isoforms	Release	pH	Specificity on B cell receptors	Function
Monocytes, Dendritic cells, Macrophages, neutrophils	Δ BAFF	Cannot be release	-	-	co-expression with BAFF, - regulate ability of BAFF to appear on the cell surface and to be subsequently shed into the extracellular space
	3-mer BAFF	Release after furin convertase	pH=6	BAFF-R,	Biological form of BAFF Leads to immature B cell survival , plasma cell survival
	Oligomeric form of BAFF (20mers or 60mer)	Release after furin convertase and oligomerization from 3-mer BAFF	pH=7.4	BCMA, BAFF-R, TACI	, T-cell independent antibody responses, B cell regulation, class-switch recombination

Table 1: BAFF isoforms, function, cell type of expression, release ability

BAFF receptors, BAFF/APRIL system and their role in B cell development

Two ligands, BAFF and APRIL and three receptors TNF receptor superfamily member 13C (also known as BAFF receptor [BAFF-R] or Blys receptor 3 [BR3]), TNF receptor superfamily member 17 (also known as 13-cell maturation member antigen [BCMA]) and TNF-receptor superfamily member 13B (also known as transmembrane activation and cyclophilin ligand interactor [TACI]) form the backbone of the BAFF/APRIL system. BAFF and APRIL can both interact with BCMA and TACI whereas BAFF is the sole ligand for BAFF-R (Vincent et al. 2013). Among non B cells, BAFF-R also expressed by activated and regulatory T cells and TACI is expressed by monocytes and DCs (MacKay & Schneider 2009). In addition to an important role as B cell survival factor BAFF promotes B cell proliferation, plasma cell differentiation and also IgG class switching (Litinskiy et al. 2015). Moreover, BAFF promotes the survival transitional immature B-cell subpopulation by its interaction with BAFF-R (Vincent et al. 2013).

In humans, these three receptors are mainly expressed by B cells, but expression of these receptors differs among B-cell subset. All B cells express BAFF-R except bone marrow plasma cells. Moreover, BAFF-R is essential for both survival and maturation of immature B cells. TACI is expressed by CD27+ memory B cells tonsillar and bone marrow plasma cells a subpopulation of activated CD27- non- germinal center and by a small subset of naïve B cells in the blood and tonsils. TACI is critical for T-cell independent responses of B cells to type I and type II antigen negative regulation of B cell compartment and class-switch recombination of B cells. BCMA is expressed by plasmablast and plasma cells from tonsils, spleen and bone marrow, promoting cell survival. However, TACI unlike BAFF-R is solely activated by membrane or oligomeric BAFF (60mer) to support survival of activated B cells and plasmablasts (MacKay & Schneider 2009; Vincent et al. 2013) (Figure 3).

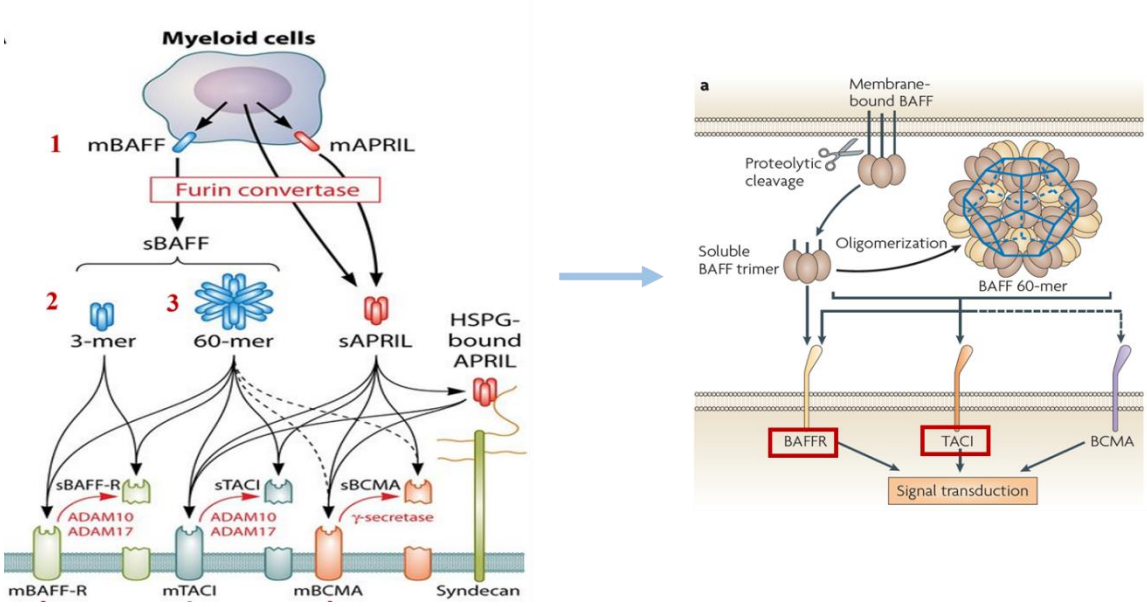


Figure 4. Schematic representation of various forms of B cell activating factor (BAFF) and APRIL and their binding to receptors, BAFF-R, TACI,BCMA (MacKay & Schneider 2009).

BAFF producing cells and is regulation upon cytokines

BAFF is produced mainly by mononuclear cells from blood, spleen and lymph nodes, although low expression is present in several tissues (Schneider et al. 1999). The main cells considered as BAFF producing cells are myeloid lineage cells, malignant B cells, activated T cells, neutrophils and bone marrow stromal cells. Myeloid cells such as monocytes, macrophages and monocyte derived DCs release BAFF after IFN-γ, IFNα and CD40 stimulation (Litinskiy et al. 2015). In these cells, membrane bound BAFF can be cleaved by a furin convertase to produce a soluble form (Nardelli et al. 2001) . However, neutrophils do not express BAFF on their surface and show a special mechanism for BAFF secretion upon stimulation with G-CSF and IFN-γ (Scapini et al. 2003). Although, BAFF is produced as a membrane bound for as well as soluble protein, it can be cleaved at the membrane in other cell types. In particular, in human neutrophils BAFF is cleaved and stored intracellularly (López et al. 2014). Bacterial component such as lipopolysaccharide (LPS) and peptidoglycan, also up-regulate BAFF secretion by macrophages, dendritic cells and monocytes. On the contrary IL-4 inhibits the expression of BAFF in monocytes (Nardelli et al. 2001; Scapini et al. 2003). CD40 ligand and IgM stimulation both induce BAFF expression in normal human B cells by activating NF- κB and NFAT binding to the BAFF promoter (Fu et al. 2009). Intestinal EC-IFN-induces the production of both soluble and membrane bound BAFF through the JAK/STAT signaling pathway and through the binding of phosphorylated STAT1 to BAFF promoter (Woo et al. 2013).

Systemic lupus erythematosus

Our immune system developed to protect us against invading pathogens and to aid tissue healing after injury. In systemic autoimmune diseases, mechanisms that regulate the balance between recognition of pathogens and avoidance of self- attack are impaired. Furthermore, control of inflammation is lost, resulting in sustained immune activation without any overt infection, with different amplitudes during flares and quiescent disease. Two hypotheses for systemic autoimmune inflammation have been suggested. First, barrier control between innate and adaptive immunity could be disturbed, fueling continuous inflammation by a positive feedforward loop. Second, impaired adaptive immunity with reactivated (auto) reactive memory by lymphocytes could result in persistent inflammation and include defects of tolerance checkpoint

Systemic lupus erythematosus (SLE; also known simply as 'lupus') is a chronic, progressive, autoimmune disorder that affects multiple organ system with a broad range of clinical (butterfly rash, nephritis, glomerulonephritis etc.) and laboratory manifestations. The etiology of SLE is multifunctional and includes contributions from the environment (hormones, ultraviolet light), stochastic factors and genetic susceptibility. In addition, it is widely studied the woman comprises 90% of most SLE cohorts compared to men (Birney et al. 2007).

SLE is characterized by the presence of multiple cellular and molecular abnormalities in the immune system including leukocyte activation and cytokine dysregulation (López et al. 2016). Aberrant innate immune responses pay as significant role in the pathogenesis of SLE, contributing both to tissue injury via release of inflammatory cytokines as well as to aberrant

activation of autoreactive T and B cells. Aberrant activation of autoreactive B cells leads to pathogenic autoantibody production and formation of immune complexes (ICs) resultant end-organ injury such as skin, lungs, nervous system, brain, kidney and blood vessels (Choi et al. 2013; Bertsias et al. 2012).

Auto antigenic nucleic acids and their binding proteins are required for self-antigen specific activation of autoreactive lymphocytes (Choi et al. 2013) and these produced autoantibodies are highly present in the serum of SLE patients(Tsokos 2011; Tsokos et al. 2016) (Figure 5). In addition, cytokines can contribute to susceptibility to SLE but are more strongly implicated in loss of tolerance and end-organ effects. Levels of many cytokines are elevated in SLE (such as TNF, IL-4, IL-6 and IL-10) with main effects on promotion of autoantibody production and inflammation. Type I and Type II IFNs have emerged as key cytokines in the pathogenesis of SLE. Furthermore, SLE activity and severity is also associated with increased activation of IFNα pathway. This IFN α production may also facilitate the SLE pathogenesis enhancing B cell activating factor (BAFF) and as result to survival of autoimmune B cells. Consequently, to a breakthrough in SLE targeted therapies, by accomplishing the production of a human monoclonal antibody that targets the B cell activating factor, Beimumab, an FDA approved SLE drug, instead of depleting B cells, like Rituximab (Stohl & Hilbert 2012)

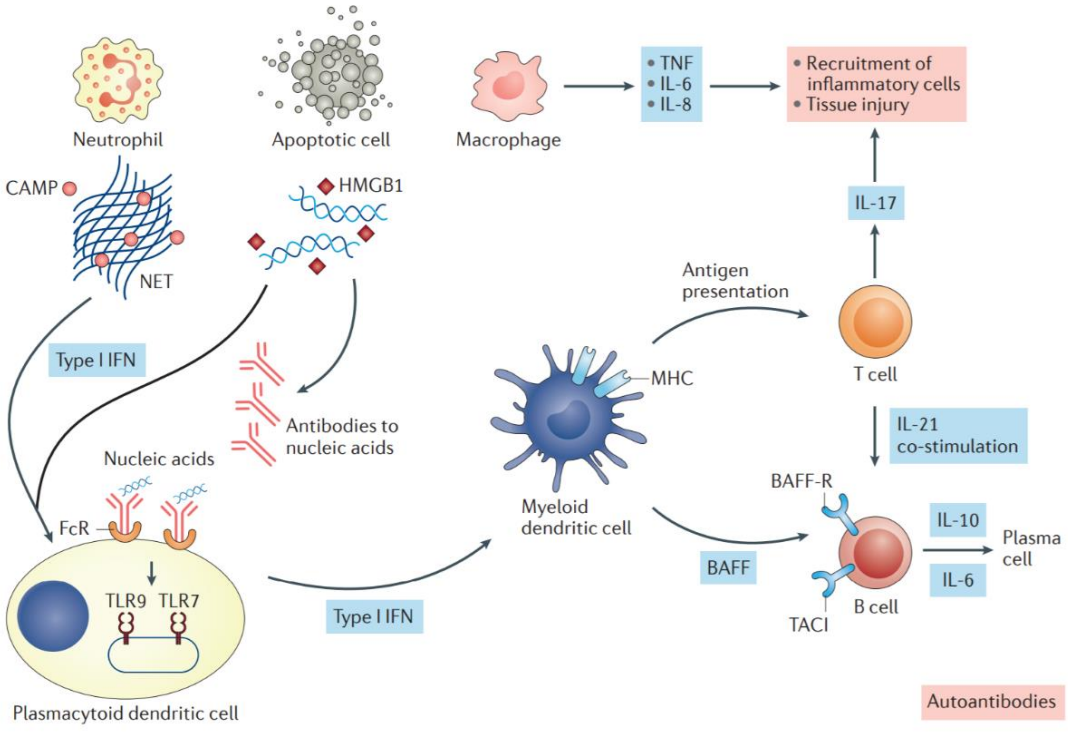


Figure 5. Cellular contributions to the development of SLE (Tsokos et al. 2016).

Pathogenic role of IFNα in SLE

A great number of cells has the capability to produce IFNα. The official major producers are plasmatocytoid dendritic cells (pDCs) also known as type I IFN producing cells (IPCs). Type I interferons and particularly IFNα are considered to play a central role in SLE etiopathogenesis. Several observations led to the identification of IFNα as a central player in SL pathogenesis. IFN α was found to be elevated in lupus sera. In addition, IFNα regulated gene transcripts were shown to be significantly upregulated in peripheral blood of pediatric and adult SLE patients upon gene expression profiling (Bennett et al. 2003). In various studies serum IFNα concentrations and IFN-induced genes positively correlate with SLE disease activity (measured by SLEDAI) (Bennett et al. 2003). Thus, it is well known that IFNα serum levels and expression of IFNα inducible genes consistently increased in SLE patients and usually correlates with disease activity and clinical manifestations (Crow 2014). PDCs have been implicated in the pathogenesis of autoimmune diseases that are characterized by type I IFN signature, such as systemic lupus erythematosus. Moreover, IFNα from SLE sera can differentiate monocytes into activated dendritic cells (DCs), able to present self- antigen supporting that this pleiotropic cytokine could be responsible for initiating development of systemic autoimmunity (Blanco et al. 2001). Binding of IFNα to the two chain type interferon receptor (IFNAR) initiates a signal transduction pathway that results in the expression of IFN-induced genes most of them with immunoregulatory functions on B, T and NK lymphocytes. Consequently, another functioning of type I IFN signaling could be an early event in lupus pathogenesis (López et al. 2016). In addition, it is well established that IFNα induces BAFF, thereby providing support for B cell differentiation and supports immunoglobulin class switching to generate potentially pathogenic autoantibodies (Litinskiy et al. 2015). Several observations indicate that interferons induce BAFF expression: IFNα upregulates BAFF expression by mouse macrophages (Panchanathan & Choubey 2013). IFNα and IFNγ both upregulate BAFF and APRIL expression by human DCs (Litinskiy et al. 2015) and IFNα and IFNγ can upregulate BAFF expression by human monocytes. In a phase I clinical trial of patients with SLE treated with an anti-IFNα monoclonal, BAFF mRNA expression in whole blood was suppressed.

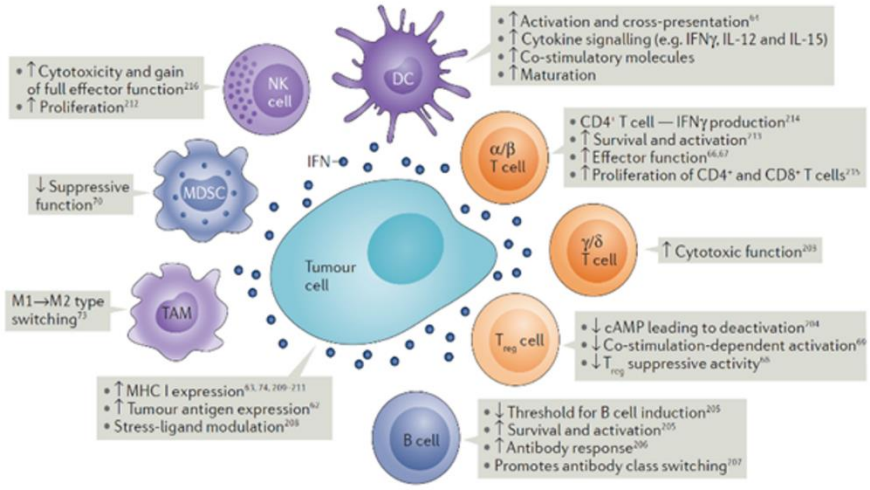


Figure 6. Pleiotropic effect of IFNα in SLE (Parker et al. 2016)

DC-like phenotype of CD14+ Monocytes in lupus

IFNα signaling and activation of nucleic acid sensing and clearance pathways are intertwined orchestrating antiviral immunity such as the differentiation of monocytes to potent antigen presenting dendritic cells with increased capacity to drive and B cell responses (Blanco et al. 2001; Pascual et al. 2006; Banchereau & Pascual 2006).In SLE circulating monocytes are already differentiated into DCs with their function shaped by IFNα (Blanco et al. 2001; Pascual et al. 2006). Neutralizing anti IFNα antibodies have demonstrated that IFNα is the key differentiation factor of monocytes to DCs in SLE serum. These IFNα-DCs exhibit increased HLA-DR, CD86 and BAFF expression, activate autoreactive T cells and promote the expansion and differentiation of autoreactive B cells, therefore bridging innate danger sensing with adaptive autoimmune responses (Blanco et al. 2001). However, the molecular mechanism(s) driving IFNα mediated auto reactivity in SLE APCs remain(s) elusive.

A recent study in our laboratory highlighted that lupus characteristic IFNα signature in monocytes of active, untreated SLE patients is linked to aberrant mitochondrial metabolism, altered lysosomal PH and defective autophagic degradation (Gkirtzimanaki et al. 2018).

Autophagy in autoimmunity and SLE

Dysfunction in autophagy underlies many human diseases. This is to be expected, given the fundamental role of autophagy in protein and organelle quality control metabolic homeostasis and stress response (Choi et al. 2013). Autophagy has been implicated in many physiological and pathological processes. Moreover, autophagic failures appeared to occur in multiple systemic disorders and organ- specific pathologies. As we already mentioned, autophagy drives cell fate decisions (including T cell selection, cell differentiation and apoptosis) and has an important role in immune cell function and signaling. Emerging literature implicates autophagy in intracellular danger signaling, antigen processing and delivers for presentation in APCs (Alissafi et al. 2017; Pascual et al. 2006). Furthermore, LC3-II molecules have been shown to be co-localized with lysosomal compartments in which MHC II complexes are formed, thus implying that autophagy could intersect the MHC class II presentation pathway and consequently play a crucial role in presenting self- antigens to immature T cells in the thymus (Crotzer & Blum 2010). Under normal circumstances, the intracellular process of autophagy is tightly controlled. However, in autoimmune and degenerative joint diseases such as SLE, RA and SSC dysregulated autophagy contributes substantially to pathology. Thus, the process of autophagy implicates a fundamental role in keeping balance of the system.

Focusing on systemic lupus erythematosus disease, a complex autoimmune disease with strong genetic component and specifically into the GWAS and other replicated studies, we can observe a pivotal correlation of the disease with autophagy. In particular, SNPs in several autophagy related gens are associated with SLE susceptibility, as for example, ATG5 and ATG7. In addition, m TOR signaling has been implicated as a major integration of signals related to cellular nutrient and energy status, paying in this way a crucial role in the regulation of cellular metabolism. Activation of m TOR signaling has recently emerged as a key factor in abnormal activation of lymphocytes in SLE (Oaks et al. 2017). Moreover, emerging evidence indicates

that autophagy is upregulated in B cells during plasma cell differentiation in patients with SLE (Rockel & Kapoor 2016). Although not traditionally viewed as autophagy-modulating signaling through BAFF and its receptors BAFF-R, TACI and BCMA could contribute dysregulation on B cell autophagy by modulating multiple signaling pathways.

In the life span of autophagosome formation, the first complex that can be targeted by drugs is the signaling complex that interacts directly with the m TOR pathway. Rapamycin is a lipophilic macrolide antibiotic used in renal transplantation as an immunosuppressant (H Huang 2011). As already mentioned, rapamycin is a potent inhibitor of the Ser /Thr protein kinase m TORC1. Extensive studies have revealed that Rapamycin can also inhibit m TORC2 mediated Akt phosphorylation depending on cell lines or treatment, time and dose. Rapamycin analogs, such as Everolimus or Torin 1 recently documented to be effective in treatment of certain autoimmune diseases. In addition, Rapamycin attenuates excessive BAFF induced cell proliferation and survival which underscores a role of Rapamycin as a potential agent for presenting excessive BAFF-evoked aggressive B cell malignancies and autoimmune diseases (Rockel & Kapoor 2016).

The final destination of macromolecules targeted for degradation through endocytosis, phagocytosis and autophagy are the lysosomes. Lysosomotropic agents accumulate inside the lysosome because of their protonation and result in an increase in lysosomal PH. Among these compounds, the antimalarial hydroxycloroquine (CQ) is the effective treatment of mild SLE manifestation and it is also used in preventing the occurrence of new mild SLE manifestation. Hydroxycloroquine inhibits lysosome function thereby inhibiting TLR activation leading to a down-regulation of IFNα and decreasing the antigen processing necessary for autoantigen presentation. Thus, the lysosome is validated target of therapeutic intervention (Rockel & Kapoor 2016). In addition, IFNα impairs autophagic degradation of mtDNA promoting auto reactivity of SLE monocytes (Gkirtzimanaki et al. 2018). These observations suggest that the link between autophagy dysregulation and SLE pathogenesis is biologically plausible and that targeted modulation of autophagy represent a possible and promising therapeutic direction in SLE.

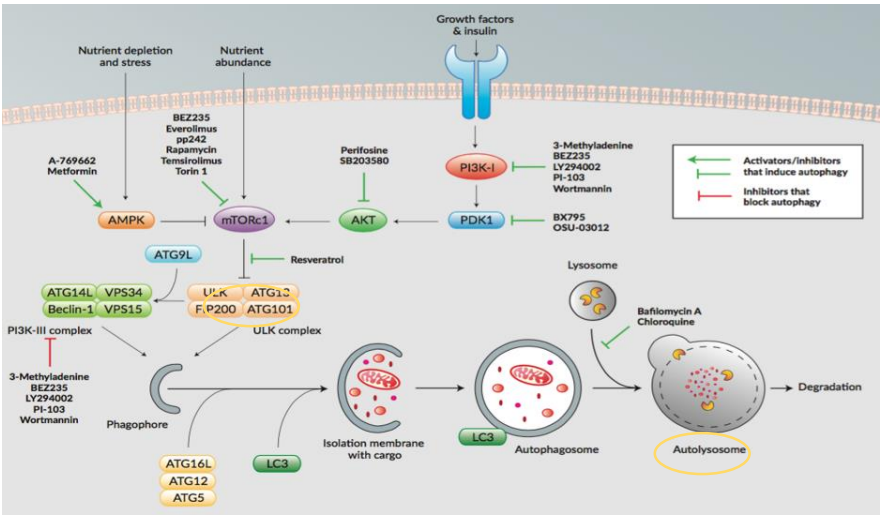


Figure 7. Activators/ inhibitors that induce autophagy, inhibitors that block autophagy

BAFF in SLE pathogenesis.

B cell activating factor of the TNF family (BAFF) is thought to be involved in the pathogenesis of SLE. BAFF is manly secreted by myeloid cells and plays a crucial role in B-cell survival and differentiation, as mentioned above (Vincent et al. 2012; Nardelli et al. 2001). BAFF contributes to the development of SLE by mediating the survival of autoreactive B cells through escape from negative selection (Vincent et al. 2013). In line with these data BAFF transgenic mice have been shown to display an SLE-like phenotype with high titers of anti-ds DNA antibodies and the development of glomerulonephritis. In human high levels of BAFF have been reported in patients suffering from SLE and other autoimmune disorders like Sjogrens syndrome (Groom et al. 2002). In addition, BAFF was found to be elevated in lupus patients with renal and central nervous system and in patients received B-cell depleting therapy (Vincent et al. 2012; Parodis et al. 2015). However, the correlation of serum BAFF levels with SLE disease activity is controversial (Vincent et al. 2012). Clinical studies have confirmed the presence of increased serum levels of BAFF in SLE patients correlating with the activity of disease. BAFF mRNA expression in SLE blood leukocytes correlates better with disease severity than BAFF concentration in blood (Birney et al. 2007). This blood leukocyte may be a major source of BAFF in SLE. Transgenic mice overexpressing BAFF cytokine develop autoimmunity with lupus- like syndrome. Moreover, monocyte BAFF induction and mobilization from intra to extracellular compartments seems to be influenced by IFNα disease activity or anti-ds DNA levels (López et al. 2014; López et al. 2016). Finally, it has also been reported that mDCs- B cells through BAFF contributes to the pathogenesis of SLE indicates BAFF as a key player of the disease. Thus, BAFF represents an important therapeutic target: indeed, Belimumab, an anti-BAFF monoclonal antibody provided clinical benefit in SLE, resulting FDA approval (Stohl & Hilbert 2012; Furie R, Petri M, Zamani O 2015). However, better pharmacologic understanding of the inhibition capability of BAFF antagonists against all forms of BAFF has the potential to impact the therapeutic success of these antibodies in treatment of lupus.

Recent studies have revealed that Belimumab targets only the trimeric BAFF and delineate an aspect concerning the molecular basis of BAFF neutralization by Belimumab (Vigolo et al. 2018; Shin et al. 2018). Moreover, Belimumab is efficacious in achieving low disease activity over 40% of unselected protein (Fanouriakis et al. 2018). However, major differences between BAFF trimer and 60mer forms have been investigated using mouse models, but in humans these forms of BAFF have not been studied in detailed and 60mer which might be important in regulating autoimmune disease, has not been described yet.

One of the major unanswered questions is whether human BAFF exists in both trimer and 60mer forms and what functions these different forms might have in disease. Indeed, if BAFF 60mer functions as the active form in humans, thereby active-form specific –anti-BAFF-therapies might be developed by neutralizing only the active BAFF 60mer or by therapeutically disrupting active 60mer into less active trimer form.

Although, SLE is a clinically heterogeneous disease patients are universally characterized by the presence of autoantibodies particularly those directed against nuclear antigens. Completed deletion of B cells resulted in nullification of the disease. Loss of tolerance and altered B cell differentiation might be genetically determined by variants present from birth. Tolerance can be broken for example by B cell stimulation via cytokines. In particular, BAFF has been implicated in this process. BAFF antagonism in mice clearly leads to improved self-

tolerance and conversely BAFF overexpression leads to autoimmunity. B cells contribute to SLE through their response to antigens, reproduction of other cells and autoantibody production. Autoantibodies contribute to SE through the formation of immune complexes, direct agonist or antagonist action and by interference with intracellular functions. B cells in lupus have been reported to how certain abnormalities such as increased memory and plasma cell expansion, decreased naïve B cell numbers and suppression of inhibitory receptor FcRIIb in memory and plasma cells. Overall, in SLE it is believed that antibody- nucleic acid immune complexes (such as ssRNA or DNA from dead cells) are bound by FcγRIIα, activating TLRs and IFNα production. IFNα increases BAFF production by monocytes or DCs. BAFF interacts with specific receptors on B cells and excess of BAFF can increase autoreactive B-cell survival driving autoimmunity. The significance of B cells in SLE pathogenesis has made B cells an immerging target for therapy development. Thus, BAFF for the unformentioned reasons represents an important therapeutic target. The finest example of the important role of B cells and BAFF in SLE pathogenesis is the human monoclonal antibody against BAFF (Belimumab), as already mentioned.

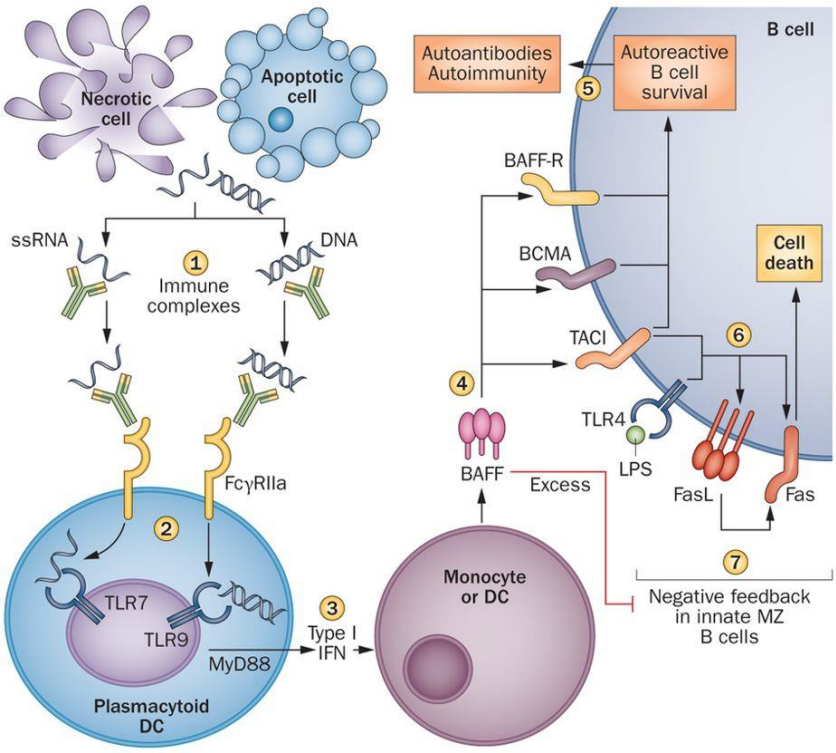


Figure 8. Role of BAFF in the pathogenesis of SLE (Vincent et al. 2012)

Aim of this study

The aim of this study was to investigate the role of autophagy in SLE monocytes, presenting an IFNα-DC like phenotype, in the regulation and secretion of BAFF, contributing to the maintenance and differentiation of auto-reactive B cells through class-switch-recombination. Unraveling a possible mechanism regulating BAFF differential function upon IFNα signaling and a possible mechanism of "production" and maintenance of autoreactive B cells, provides a wider armamentarium for SLE targeting-therapies

MATERIALS AND METHODS

Human Subjects

Peripheral blood samples were obtained from eight SLE patients diagnosed according to the American College of Rheumatology 1982 classification criteria were studied (median age=42.5 yrs.; range: 16 to 70yrs). Active SLE was defined as an SLE Disease Activity Index 2000 score (SLEDAI-2K) higher than 8 at the day of blood draw. Patients were abstained from their medications - including steroids and CQ - for at least 24 hours prior to blood sampling. Moreover, n=8 patients were completely off therapy at the time of enrolment to the study. Healthy age- and sex-matched volunteers from the Department of Transfusion Medicine of the University Hospital of Heraklion served as controls. The study was approved by the Ethics Committee of the University Hospital of Heraklion and all subjects gave written informed consent prior to study.

Serum collection

Healthy and SLE peripheral blood sera were collected in SST vacutainers and centrifuged at 2500 rpm for 15minutes, RT. Serum was collected under sterile conditions and stored in aliquots at -80°C.

Reagents

RPMI-1640 (Gibco), fetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (100μg/ml), were all from Gibco, Carlsbad, CA. For culture of monocytes recombinant Universal Type, I IFN (Hu-IFN-αA/D[Bg/II] – 200u/ml working concentration, was purchased from PBL. Plaquenil (hydroxycloroquine-CQ: 46.1μM) was purchased from Sanofi Aventis. Rapamycin (Rapam.:1 μM) was from Sigma-Aldrich. Ficol Histopaque 1077 was from Sigma-Aldrich. Fluorochrome-conjugated monoclonal antibodies to BAFF-CD257 (PE conjugated), HLA DR (PerCP conjugated), CD80 (APC conjugated) and CD86(PE conjugated) were all from e Biosciences. Western Blot primary monoclonal antibodies rabbit monoclonal anti-BAFF, mouse monoclonal anti-LC3 and anti-Actin were from Ebioscience. Secondary monoclonal antibodies anti-mouse HRP by Millipore and anti-rabbit HRP by Cell signaling. Immunofluorescent primary antibodies mouse anti-LC3 by Nanotools (1:20 dilution), rabbit anti-BAFF by Thermo-Scientific (1:100 dilution) were used. Immunofluorescent secondary antibodies Alexa fluor® 555 anti-mouse IgG (1:500) and Alexa fluor® 488 anti-rabbit IgG (1:500) were by Molecular Probes. For visualization of the nuclei DAPI (300n M) from Sigma-Aldrich was used.

Peripheral blood monocyte isolation.

PBMCs were isolated from heparinized human peripheral blood or Buffy coats by Ficoll-Histopaque-1077 (GE-Healthcare) density centrifugation. Erythrocytes were eliminated by hypotonic lysis (1ml ddH2O for 35s and 1ml 1.8% NaCl). Viability was measured 99% by trypan blue dye exclusion. Monocytes were magnetically isolated (MACS, Miltenyi Biotec) and purity

was evaluated by flow cytometry (CD14+ cells/total live cells). Preparations of ≥ 94% purity were used in all experiments.

Cell culture

Purified $CD14^+$ monocytes were cultured in RPMI-1640/L- glutamine, supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin and 100μg/mL streptomycin for up to 18 hours. $CD14^+$ monocytes were stimulated with Universal Type I IFN (Hu-IFN-αA/D[Bg/II] – 200u/ml working concentration, PBL), IFN γ (50 u/ml) Rapamycin(1μM), mitoTempo (2nM), LPS (5μg/ml), chloroquine (CQ: 46.1μM) or heterologous healthy or SLE serum (10% v/v in serum free medium).

Flow cytometry

Cells were stained with fluorochrome-conjugated antibodies, extracellular markers, (anti-CD14 clone HCD14, anti-BAFF, anti-CD86, anti-HLADR, anti-CD86) for 20 min at 4°C in PBS/5% FBS. Monoclonal antibodies specific for CD14 were used to identify monocyte population. Monoclonal antibodies specific for HLA-DR, CD80, CD86 were used as maturation-activation markers. Additionally, a monoclonal antibody specific for BAFF was used for extracellular staining. Apoptosis and cell death was obtained with staining with Annexin V (BD Pharmingen) and 7AAD (Biolegend). Samples were acquired on a FACS Calibur (BD Biosciences) and analyzed using the FlowJo software (Tree Star).

Immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris·HCl at pH 8, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS) supplemented with protease and phosphatase inhibitors (Complete EDTA Free; Roche Applied Science) and equal amounts of proteins were subjected to SDS-PAGE on 12% gels and then transferred to Immobilon-Psq membrane (Millipore). Membranes were blocked with 5% or 3% skimmed milk, 1% BSA in T-BST and then incubated with anti-LC3B, anti-BAFF, anti-actin and anti-mouse HRP, anti-rabbit according to manufacturer's suggestions. The image was resolved by ECL system (Pierce and Millipore) and detected by Image Blot (BIORAD). Relative intensity of bands was calculated with Fiji software.

Immunofluorescence (IF)

For IF microscopy cells were plated on Poly-L-lysine coverslips (1,5-2 x105 cells /slide). When the appropriate treatments were finished, slides were washed once with PBS, fixed with 4% PFA for 20mins (RT) re-washed twice with PBS, refixed and permeabilized with 100% ice cold MetOH for 10mins (RT) and blocked with 2% BSA in 0,1% saponin (BS). Primary antibodies were left for 1hr at RT or O/N at 4°C. Slides were washed several times with BS and the secondary antibodies were incubated for 1hr at RT. Antibodies used were mouse anti-LC3 (nanotools, 1:20 dilution), rabbit anti-BAFF (Thermo-Scientific, 1:100 dilution), followed by incubation with Alexa fluor® 555 anti-mouse IgG (1:500) and Alexa fluor® 488 anti-rabbit IgG (1:500). For visualization of the nuclei DAPI (Sigma-Aldrich) was used. Samples were cover slipped with mowiol and visualized using inverted confocal live cell imaging system Leica SP8.

Real time-PCR (RT-PCR)

Total RNA from cultured monocytes was collected using the TRIZOL (Invitrogen) extraction protocol with Turbo DNAse (Ambion) treatment in order to eliminate any genomic DNA concentrations, according to manufacturer's instructions.

cDNA was prepared from isolated RNA using Perfect Real time cDNA Synthesis Kit (Takara) according to manufacturer's protocol. 50ng of RNA was used as a template for every reaction. RNAse H (1U/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 20μl. PCR primers used were as follows:

Primers	Forward	Reverse
GAPDH	5'CATGTTCCAATATGATTCCACC3'	5'GATGGGATTTCCATTGATGAC3'
BAFF	5'CCTCACGGTGGTGTCTTTCTA3'	5'AACGGCACGCTTATTTCTGCT3'
CXCL10	5'GTGGCATTCAAGGAGTACCTC3'	5'TGATGGCCTTCGATTCTGGATT3'

Expression was normalized to GAPDH and calculated by the change-in-threshold method [2^(-ΔΔCT)].

Enzyme-linked Immunosorbent Assay (ELISA)

Detection of human BAFF and CXCL10 (e Bioscience) in human sera and culture supernatants harvested at the indicated time points, were performed by sandwich ELISA, following the manufacturer's recommendations. Light absorbance at 450 nm was measured using the ELx800 Biotek plate reader. Background signal was subtracted. All samples were assessed in duplicates.

Statistical Analysis

Statistical analysis was performed with the Prism Software (GraphPad) by nonparametric Mann-Whitney test or paired t test. p values less than 0.05 were considered significant. ns, not significant

RESULTS

SLE serum can induce BAFF mRNA expression on healthy CD14+ monocytes

For the experimental monitoring of BAFF expression, CD14+ monocytes from healthy donors and SLE patients were collected from freshly isolated peripheral blood and then were subjected to real time PCR analysis. BAFF mRNA was expressed at higher levels in healthy donors compared with SLE patients (Figure 9A). For this reason, we also proceeded to PCR analysis, from CD14+ monocytes incubated with either normal (healthy) or SLE serum. It was noticed that there is a portion of monocytes plus SLE serum which can induce BAFF expression in healthy CD14+ monocytes in contrast with monocytes plus healthy serum (Figure 9B). Important to note, the SLE sera which induced high BAFF mRNA levels were found to have high IFNα levels (data not shown).

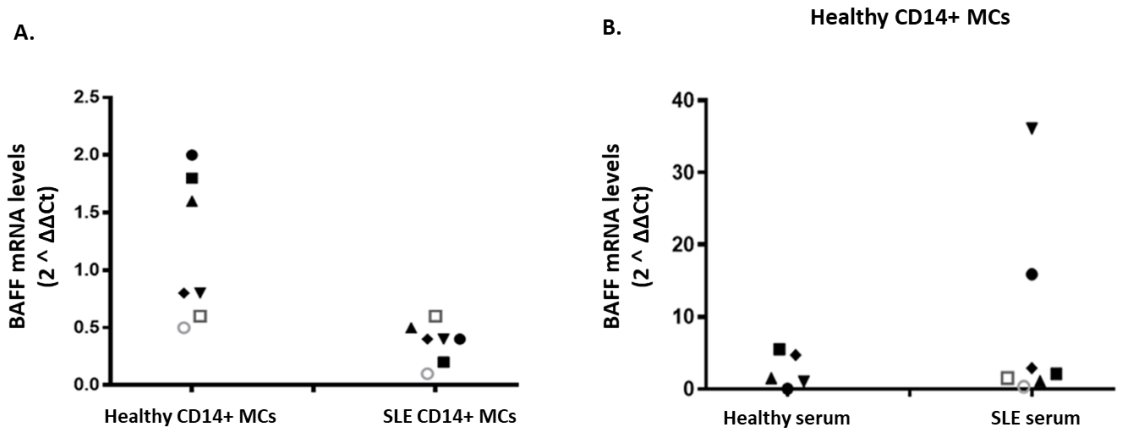


Figure 9. SLE serum can induce BAFF mRNA expression in healthy CD14+ monocytes

(A) Relative mRNA expression of BAFF compared to GAPDH in $CD14^{+}$ monocytes from healthy donors (n = 7) and SLE patients (n = 7), (B) Relative mRNA expression of BAFF compared to GAPDH in CD14+ monocytes from healthy donors stimulated with healthy (n=5) or SLE serum (n=7).

IFNα signaling Induce BAFF membrane expression on healthy CD14+ monocytes

Monocytes exposed to SLE serum acquire a DC-like phenotype with increased antigen presenting capacity (Blanco et al. 2001). IFNα is a crucial cytokine that has been documented to drive this DC-like phenotype in SLE monocytes (Blanco et al. 2001). In line with this, Cxcl 10 mRNA, a known IFNα- inducible gene, was upregulated in SLE serum- treated monocytes (data not shown). In addition, it is well established that levels of BAFF have been found elevated on monocytes from SLE patients compared to healthy donors (López et al. 2016; López et al. 2014). Moreover, soluble and membrane- bound BAFF found up-regulated in CD14+ monocytes upon IFNα stimulation for 3 days (Litinskiy et al. 2015) and IFNα stimulation can

induce BAFF expression in healthy monocytes even in shorter time points of 6 or 12hrs (López et al. 2014). This prompted us to examine if type I IFN signaling directly affects BAFF expression of CD14+ monocytes from healthy donors. Previous work in our lab indicated the functional importance of IFNα- mediated monocyte hyperactivity in SLE, with a working concentration of IFNα 10.000 u/ml in 18hr stimulation time point. We tested *in vitro* BAFF membrane expression and MHCII antigen presenting capacity of CD14+ monocytes isolated form peripheral blood of healthy donors following 18hr stimulation with IFNα in a working concentration of 10.000 u/ml and in a more physiologically relevant working concentration of IFNα 200 u/ml. Using flow cytometry, we observed an important increase of BAFF membrane expression on CD14+ monocytes treated with IFNα 200 u/ml compared to untreated or treated with IFNα 10.000 u/ml derived from healthy donors (Figure 10A), followed by a significant increase of HLA-DR membrane expression. Using flow cytometry, levels of BAFF and HLA-DR membrane expression of BAFF+ monocytes from healthy donors (n=8) were analyzed based on their geometric mean fluorescence intensity (Geo MFI) and revealed a significant induction of BAFF membrane expression, in parallel with a more significant induction on their HLA-DR membrane expression (Figure 10B). Thus, we concluded that our working concentration of IFNα stimulation from now on will be assessed to 200 u/ml.

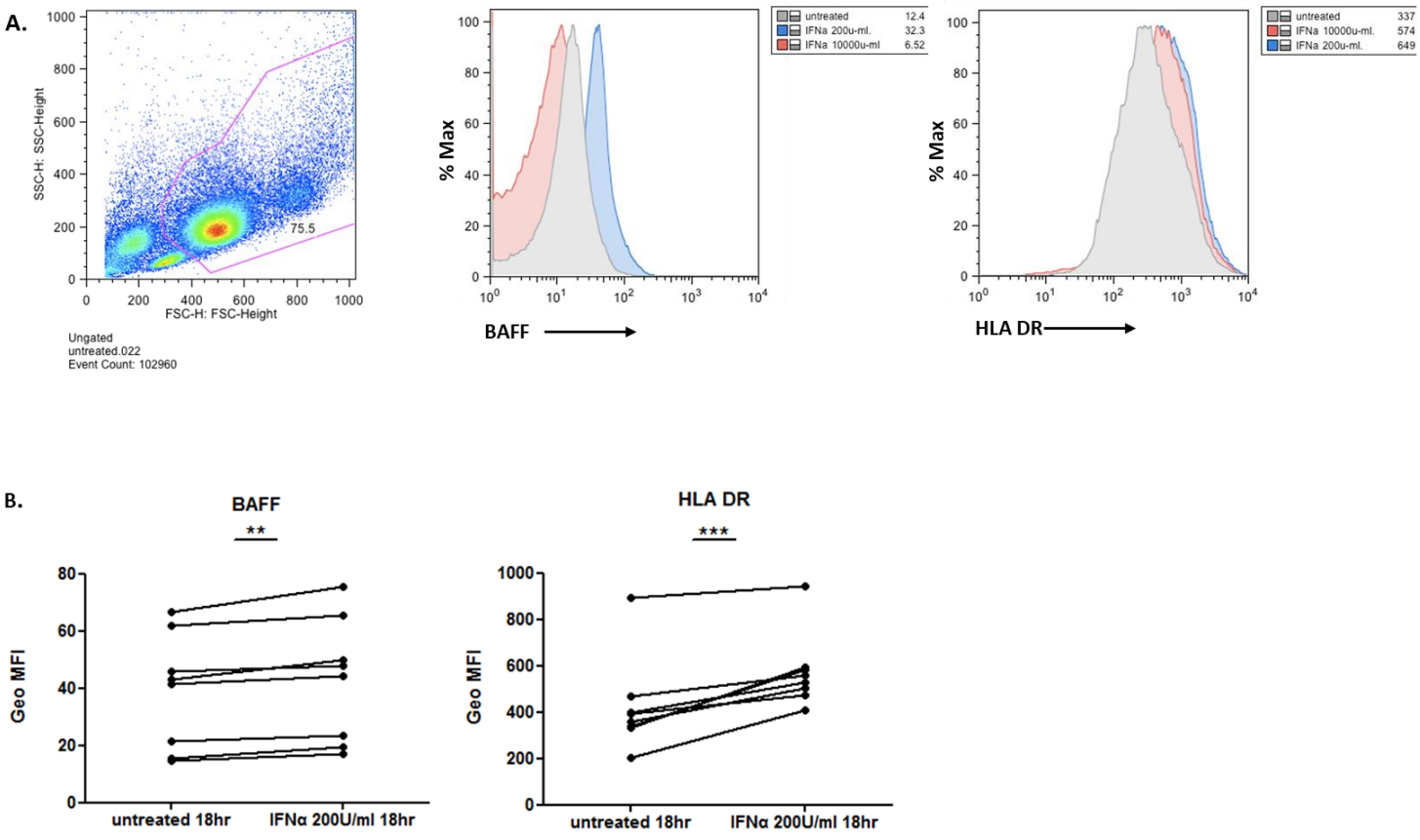
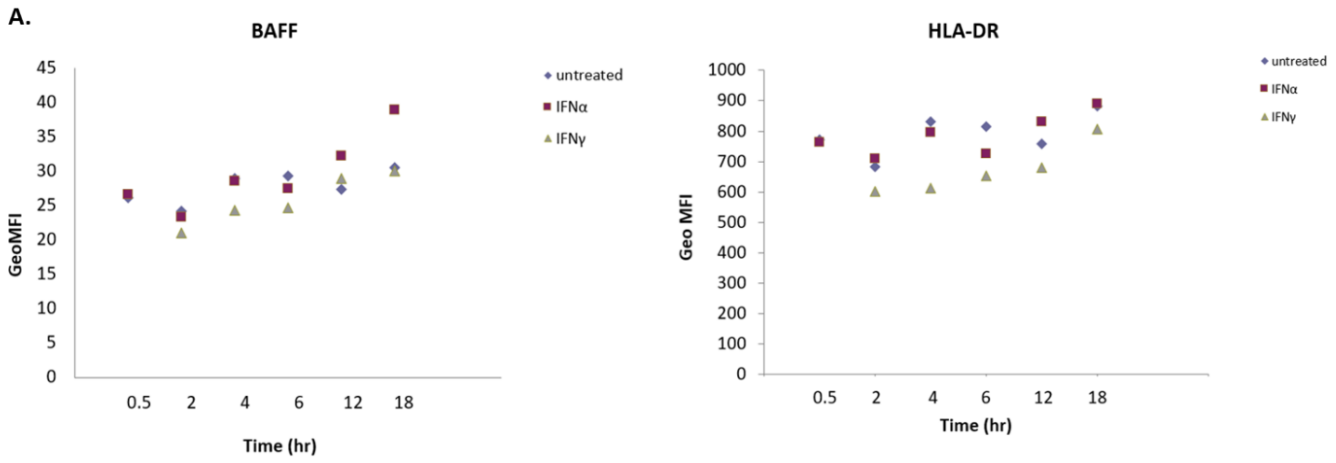


Figure 10. Type I IFNα signaling induces BAFF membrane expression in healthy CD14 + monocytes

CD14⁺ monocytes from healthy donors were cultured for 18hr ± r IFNα 200 u/ml, 10.000 u/ml as depicted. (A) Levels of BAFF and HLA-DR membrane expression were analyzed by flow cytometry. Geometric mean fluorescence intensity (Geo MFI) of gated live cells (CD14+) are listed on right side of histograms. Representative histograms are depicted concerning the difference in BAFF and HLA-DR membrane expression in healthy monocytes cultured with different IFNα concentrations. (B) Levels of BAFF and HLA-DR membrane expression were analyzed by flow cytometry. Geometric mean fluorescence intensity (Geo MFI) averages of BAFF + cells are plotted (n=8) and analyzed using paired t test.

IFNα signaling Induces BAFF mRNA and protein in 18hr stimulation of healthy CD14+ monocytes

In order to establish the kinetics of IFNα stimulation that results in increased of BAFF expression on CD14+ monocytes from healthy donor, we performed a time course experiment of 0,5hr, 2hr, 4hr, 6hr, 12hr and 18hr time points. CD14+ monocytes were isolated from the peripheral blood of healthy donor and cultured in the presence or absence of exogenous IFNα or IFNγ (used as control). Cells were harvested at different times and surface- bound (extracellular) and intracellular BAFF expression were determined separately by flow cytometry. Our results, established that 18hr stimulation with IFNα, indeed results in significant increase of BAFF extra and intracellular expression compared with other shorter time points (Figure 11A,11B). Using flow cytometry, levels of BAFF+ monocytes from healthy donors were analyzed based on their Geo MFI and revealed significant increase of membrane BAFF expression in 18hr stimulation with IFNα (Figure 11A, Table 1). This significant increase of membrane BAFF expression in 18hr was followed by a relative significant increase of HLA-DR expression (Figure 11A). Following the same frame work of analysis, similar results observed for the intracellular BAFF expression (Figure 11B). In accordance with these results, assessment of BAFF mRNA levels of CD14+ monocytes of healthy donor in different time points concluded with the same detectable difference between these conditions (Figure 11C). Thus, until now, 18hr stimulation of CD14+ monocytes with IFNα (200u/ml) reveals a significant difference on BAFF and HLA-DR expression compared with shorter time points.



	untreated	IFNα	IFNγ
extracellular			
0.5 hr	26,1	26,6	
2hr	24,2	23,3	21,06
4hr	28,9	28,6	24,3
6hr	29,3	27,5	24,7
12hr	27,4	32,2	29
18hr	30,5	38,9	30,1

Table 1. GeoMFIs of BAFF from BAFF+ cells

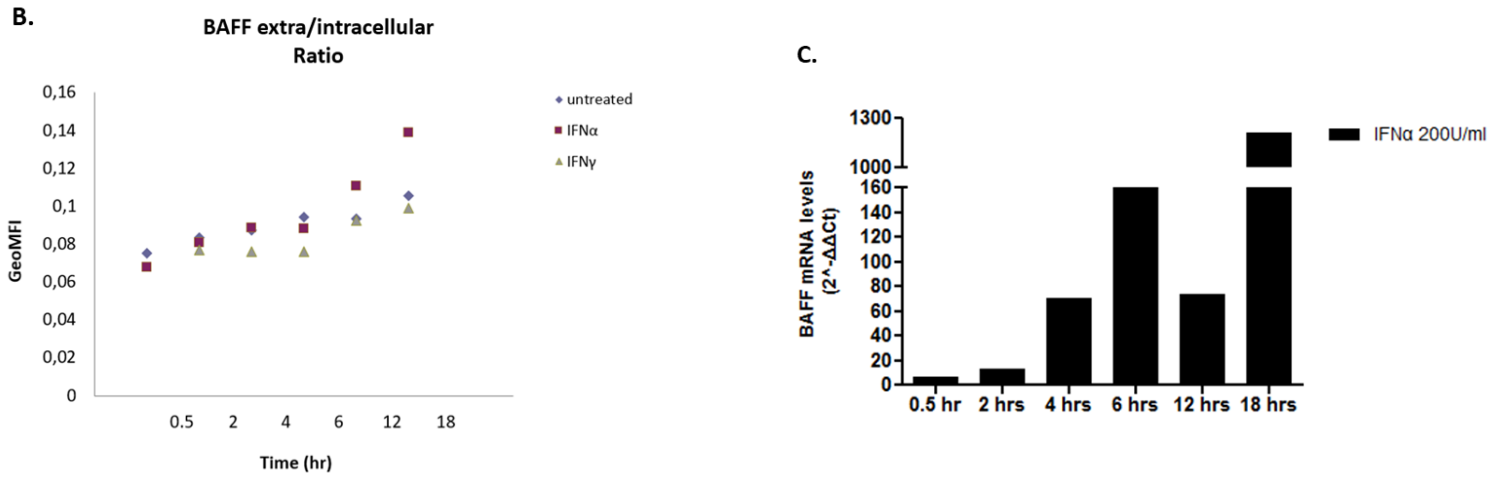


Figure 11. Type I IFNα signaling induces BAFF expression in 18 hr stimulation of healthy CD14+ monocytes

CD14 $^{+}$ monocytes from healthy donor were treated ± r IFNα 200 u/ml or IFNγ for different time points as depicted.
(A) Levels of BAFF and HLA-DR surface expression were analyzed by flow cytometry. A representative result is depicted.
Geometric mean fluorescence intensity (Geo MFI) averages of BAFF+ cells are graphed and data presented in Table1.

(B) Kinetics of surface: intracellular BAFF GeoMFIs of BAFF+ cells are graphed. (C) Relative mRNA expression of BAFF compared to GAPDH in CD14^{+} monocytes from healthy donor.

Based on the last result, we sought to decipher if 18hr IFNα stimulation could induce a similar induction of BAFF protein levels and secretion by CD14+ monocytes from healthy donors. Western blot assayed BAFF protein levels were increased upon IFNα stimulation on CD14+ monocytes compared with the unstimulated condition (untreated) (Figure 12A). In addition, by the use of immunofluorescence microscopy for BAFF ^{488} in CD14+ monocytes, we observed that BAFF protein levels are increased upon IFNα stimulation compared with unstimulated monocytes (Figure 12B). In contrast with these results, IFNα stimulation had no effect on soluble BAFF secretion by CD14+ monocytes from healthy donors after 18hrs (Figure 12C). BAFF ELISA measurement in the culture supernatants of CD14+ monocytes in presence or absence of IFNα for 18hrs from healthy donors (n=3) revealed no appearance of secreted form of BAFF (Figure 12C). In order to verify that no appearance of BAFF secretion was not a result of not working well culture supernatants and that our cell culture supernatants are activated we proceeded to CXC10 ELISA measurement in the same culture supernatants as for BAFF. CXCL10 secretion by CD14+ monocytes used as a cumulative indicator of IFNα signaling. Indeed, these same culture supernatants revealed a difference in CXC10 secretion upon IFNα stimulation compared with unstimulated condition (Figure 12D).

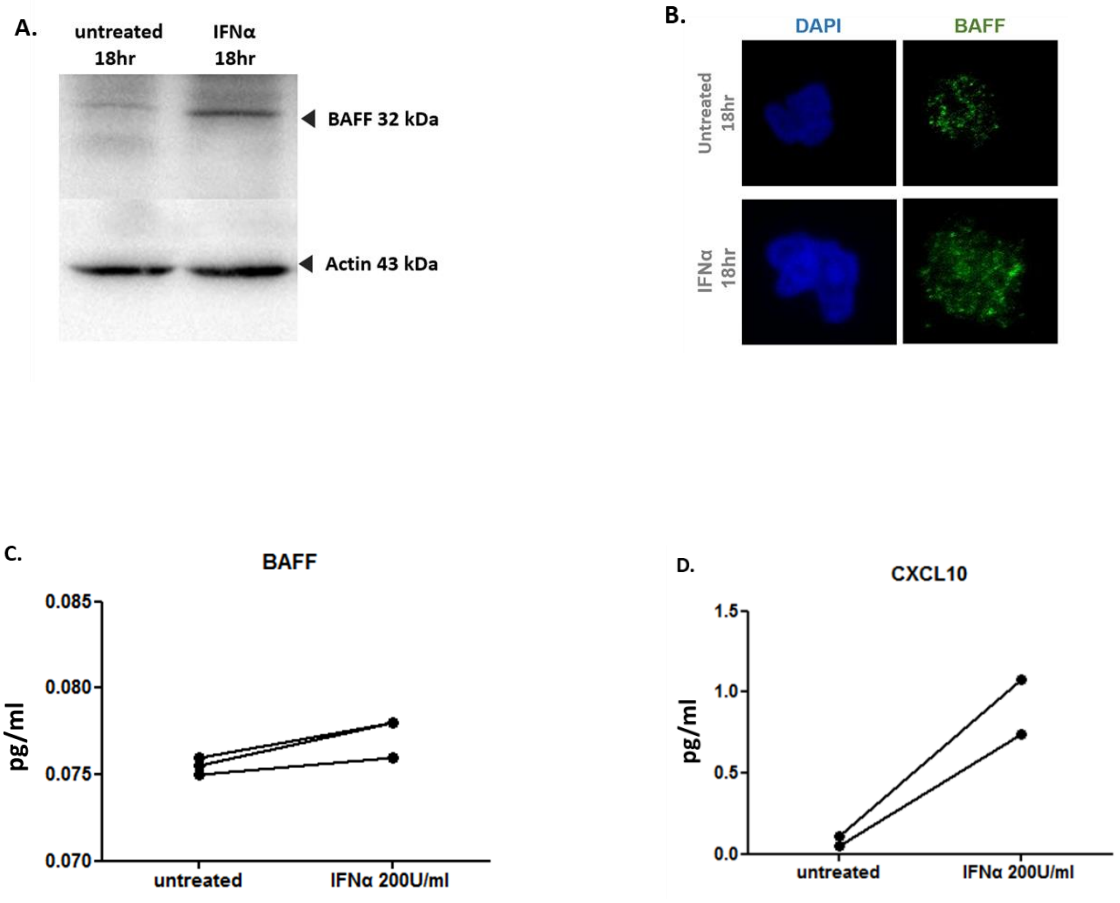


Figure 12. Type I IFNα signaling affects BAFF protein levels in 18 hr stimulation of healthy CD14+ monocytes but has no affection on BAFF secretion.

$CD14^+$ monocytes from healthy donors were treated ± r IFNα 200 u/ml for 18 hr as depicted.
(A) Representative western blot analysis of BAFF protein levels upon IFNα signaling. Relative intensities and molecular weights of BAFF to actin are shown.
(B) Confocal microscopy for DAPI and BAFF ^{488} in $CD14^+$ monocytes from healthy donors treated as indicated.
One representative result is depicted respectively. (C) Fold change of secreted BAFF measured by ELISA in culture supernatants from 18hr stimulated or not monocytes (n = 3). (D) pg./ml of secreted CXCL10 measured by ELISA in same culture supernatants as (C) (n = 2) and analyzed using paired t test.

IFNα signaling Induces BAFF expression through autophagic modulation in healthy CD14+ monocytes

SLE monocytes display increased autophagosome formation but defective autolysosomal degradation. IFNα signaling impairs the completion of autophagy and enhances the immunogenic potential of monocytes. Moreover, IFNα signaling impairs autophagosomal degradation in healthy CD14+ monocytes (Gkirtzimanaki et al. 2018). By considering the recent publication of our laboratory we wanted to decipher whether IFNα signaling has the capacity to drive BAFF protein increase through autophagic modulation in healthy CD14+ monocytes. In order to investigate, if and how BAFF expression or protein levels are affected in an autophagy dependent manner, we used CD14+ monocytes from the peripheral blood

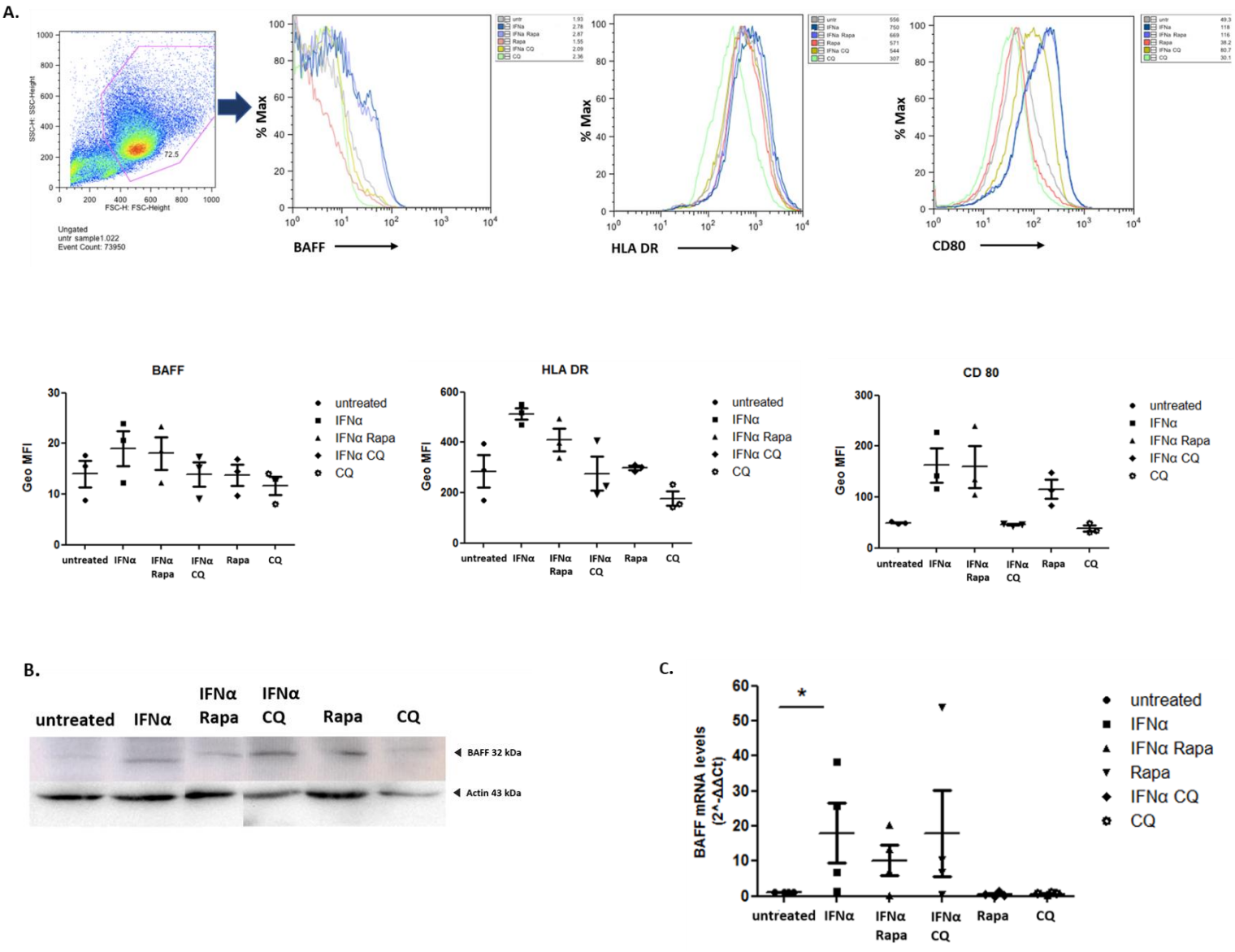
of healthy donors in the presence or absence of IFNα, ± Rapamycin (Rapa), ± hydroxycloroquine (CQ) for 18hrs. Rapamycin is an inhibitor of mechanistic target of rapamycin (m TOR) signaling and potent enhancer of autophagosome formation and autolysosomal degradation. Using flow cytometry, we observed that autophagy modulation is implicated in BAFF membrane expression (Figure 13A). Levels of BAFF membrane expression indicates an increase upon IFNα stimulation as we already mentioned and this increase changes by the presence of Rapamycin and CQ. Specifically, the addition of Rapamycin in IFNα stimulated CD14+ monocytes appeared to have a minor decrease of BAFF membrane levels compared to IFNα alone (Figure 13A). Moreover, the presence of Rapamycin alone significant decreased BAFF membrane levels compared to IFNα alone stimulation and restored BAFF membrane levels even more from the basal (untreated) (Figure 13A). This decrease of BAFF membrane levels to basal upon the presence of Rapamycin followed by a similar decrease on HLA-DR and CD80 levels (Figure 13A). In addition, minor decrease of BAFF membrane levels upon IFNα Rapamycin compared with IFNα lone revealed to increase on HLA-DR levels. However, concerning CD80 levels between these two conditions there is no change (Figure 13A). Hydroxycloroquine (CQ) inhibits lysosome function thereby inhibiting TLR activation leading to a down-regulation of IFNα and decreasing the antigen processing necessary for autoantigen presentation. Indeed, in the presence of CQ in IFNα stimulated CD14+ monocytes appeared to decrease significantly HA-DR and CD80 but also BAFF membrane expression levels compared to IFNα alone (Figure 13A). Moreover, the presence of CQ alone significant decreased BAFF membrane levels compared to IFNα alone stimulation and restored BAFF membrane levels to basal (untreated) (Figure 13A). Taken together, these results represent our first strong evidence that BAFF membrane expression upon IFNα signaling is affected in an autophagy dependent manner in CD14+ monocytes from healthy donors (n=3) (Figure 13A).

For further observation of these results we wanted to decipher how BAFF protein levels impaired in the presence or absence of these autophagy modulators (Rapamycin and CQ) upon or not IFNα signaling in CD14+ monocytes from peripheral blood of healthy donor. Western Blot analysis revealed that IFNα affects BAFF protein levels (already mentioned Figure 12A, Figure 13B) and this affection follows again an autophagy dependent manner (Figure 13B). Quantification of BAFF protein levels with respect to actin levels confirmed these result as IFNα BAFF protein levels (IFNα BAFF/actin=0,65) were induced compared to untreated (untreated BAFF/actin= 0,31). Concerning the presence of autophagy modulators, Rapamycin addition to IFNα restored BAFF protein levels to basal (IFNα Rapa BAFF/actin= 0,39) and CQ addition to IFNα increased BAFF protein levels compared to IFNα alone (IFNαCQ BAFF/actin= 0,8). Rapamycin and CQ without addition of IFNα seem to induce BAFF protein levels in comparison with untreated condition or IFNα alone (Rapa BAFF/actin= 0,8, CQ BAFF/actin=0,9).

In accordance with these results, assessment of BAFF mRNA levels of CD14+ monocytes from healthy donor stimulated with or without IFNα in absence or presence of autophagy modulators concluded to similar detectable differences between these conditions (Figure 13C).

During autophagy cytoplasmic form LC3I processed and recruited to autophagic vesicles in autophagosomes, where LC3II generated by site specific proteolysis. In order to identify whether autophagy is implicated in regulation of BAFF protein expression, we proceeded to CD14+ monocyte stimulation with IFNα in presence or absence of Rapamycin and CQ in

healthy donors and examine BAFF ^{488} and LC3^{555} expression by Immunofluorescence microscopy and the possibility of BAFF ^{488} - LC3 ^{555} co localization. Immunofluorescence staining of CD14+ monocytes with this monoclonal antibody reveals the specific punctae distribution of endogenous LC3 as a hallmark of autophagic activity. In agreement with our previous results IFNα stimulation revealed high protein levels of BAFF compared to untreated condition and interestingly leads to a significant co localization of BAFF ^{488} with LC3 $^{555.}$ (Figure 13D). In the presence of Rapamycin with or without IFNα stimulation BAFF proteins levels observed again lower compared with IFNα alone or with untreated. In fact, Rapamycin revert co localization of BAFF ^{488} with LC3 $^{555.}$ observed upon IFNα signaling (Figure 13D). On contrary, CQ alone stimulation showed an obvious increase of BAFF protein levels in a correlation with the Western Blot result (Figure 13B) and a detectable co localization of BAFF ^{488} with LC3 $^{555.}$ (Figure 13D).



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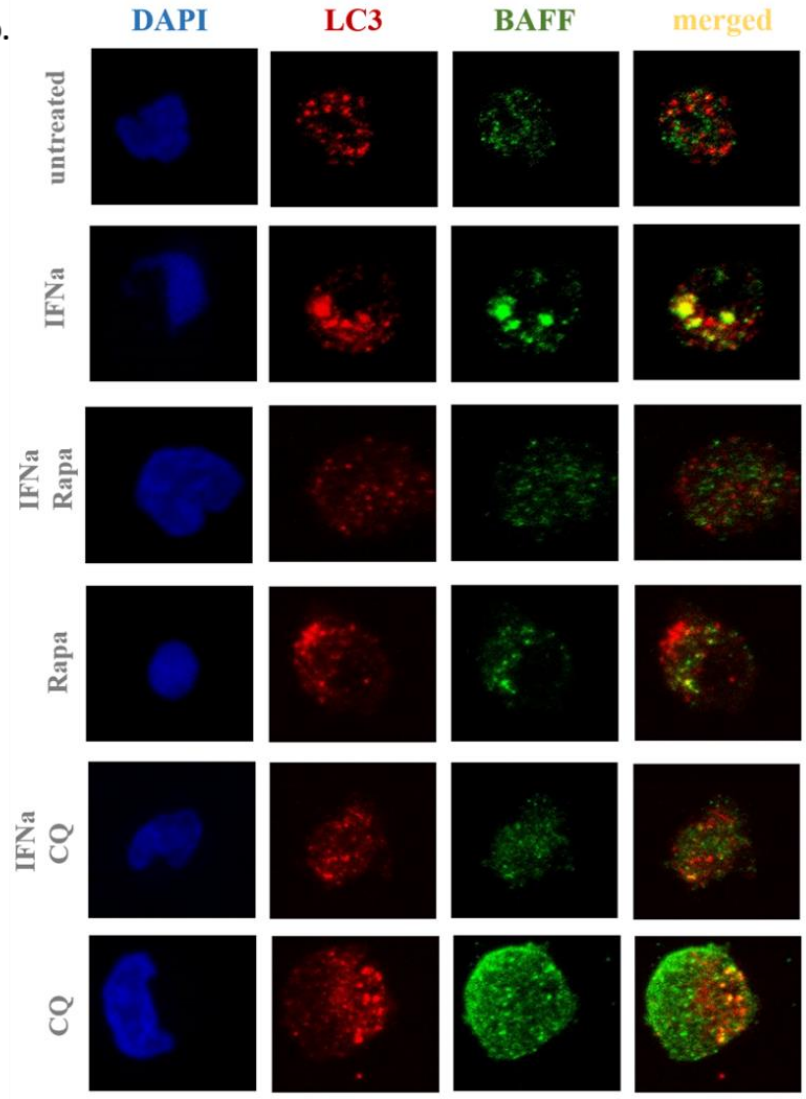

Figure 13. IFNα signaling impairs BAFF expression through autophagic modulation in healthy CD14+ monocytes

$CD14^+$ monocytes from healthy donors were treated ± r IFNα in the presence or absence of autophagy modulators, Rapamycin and CQ for 18 hr as depicted. (A) Levels of BAFF, HLA-DR AND CD80 surface expression were analyzed by flow cytometry. Representative histograms are depicted. Geometric mean fluorescence intensity (Geo MFI) averages of BAFF+ cells for BAFF, HLA-DR and CD80 are graphed (n=3). (B) Representative western blot analysis of BAFF protein levels upon IFNα stimulation ± Rapa/CQ. Relative intensities and molecular weights of BAFF to actin are shown. (C) Relative mRNA expression of BAFF compared to GAPDH in $CD14^+$ monocytes from healthy donor (n=4) treated or not with IFNα ± Rapa/ CQ. (D) Confocal microscopy for DAPI, BAFF ^{488} and LC3 ^{555} in $CD14^+$ monocytes from healthy donors treated as indicated. One representative result is depicted.

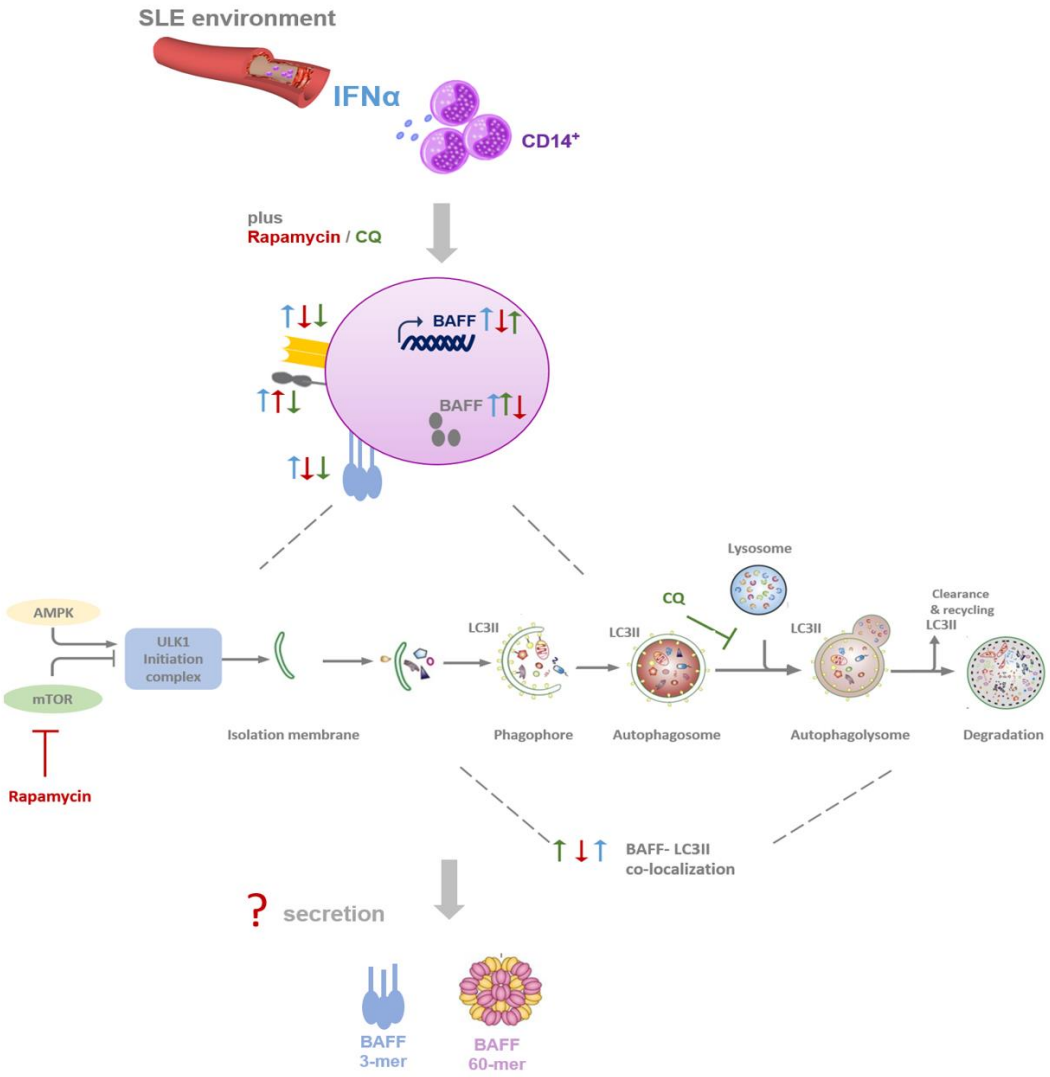

Figure 14. Graphical abstract of how IFNα signaling Induces BAFF expression through autophagic modulation in healthy CD14+ monocytes.
IFN α induces BAFF membrane expression as well as protein and transcription levels in CD14+ monocytes. Upon IFNα stimulation with addition of Rapamycin (inducer of autophagy process), BAFF membrane expression is reduced as well as protein and transcription levels. In contrast, IFNα stimulation with addition of CQ (inhibitor of autophagy process) reduces BAFF membrane expression but increases protein and transcription levels of BAFF. These autophagy modulators seem to involve in BAFF regulation upon IFNα stimulation in CD14+ monocytes of healthy individuals. Secretion of BAFF by monocytes, remains to be elusive.

	Conditions	Untreated	IFNα	IFNα Rapa	IFNα CQ	Rapa	CQ
BAFF	Membrane expression	—	↑	↓	↓	↓	↓
	Protein levels	—	↑	↓	↑	↑	↑
	mRNA levels	—	↑	↓	↑	↓	↓
	LC3 colocalization	—	↑	↓	↑	↓	↑

Table 2: Changes of BAFF expression upon IFNα through autophagic modulation in healthy CD14+ monocytes.

Rapamycin and CQ prevent IFNα mediated membrane BAFF increase in HLADR+ monocytes and abrogate IFNα induced CSR in BAFFR+ B cells

It is well established that monocytes up-regulate both membrane-bound and soluble BAFF after stimulation with IFNα which contribute to B cell differentiation and Ig class switching (Litinskiy et al. 2015). Thus, we wanted to assess whether pretreated CD14+ monocytes with IFNα and autophagic modulators can influence the differentiation status of B cells. In order to assess this, co-culture of 18hrs pre-treated CD14+ monocytes and naïve B cells proceeded for 6 days. Using flow cytometry, we observed that IFNα pretreated CD14+ monocytes expressed higher levels of membrane- bound BAFF in contrast with untreated condition. The addition of Rapamycin and CQ to the culture reduce these levels (Figure 15). Corresponding results from the B cell analysis was also performed. IFNα pretreated monocytes which expressed high levels of membrane bound BAFF tend to induced the highest levels of differentiation and Ig class switch recombination (evidenced as reduction in IgD positive B cells) of naïve B cells, whereas these effects found to be ameliorated when Rapamycin and CQ were present into the cell culture, indicating respective changes (Figure 16).

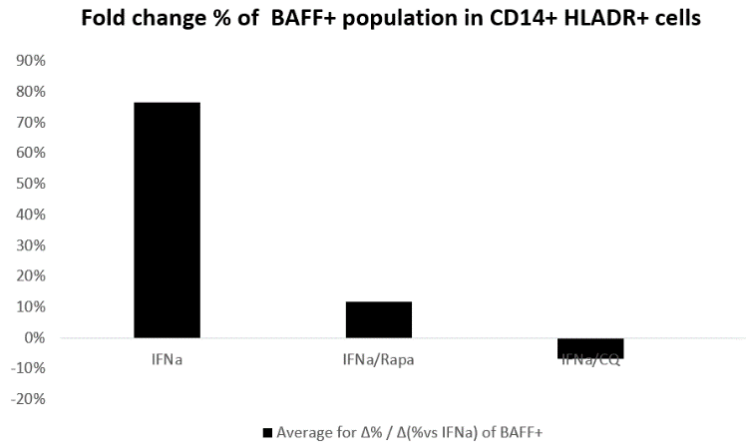

Figure 15. Rapamycin and CQ prevent IFNα mediated membrane BAFF increase in HLA DR+ monocytes

Six days MLR with 18hrs pretreated CD14+ healthy monocytes. Percentages of Δ% of BAFFR+ IGD+ B cells at the end of co-culture. CD14+ monocytes from healthy donors were cultured with IFNα alone or/with autophagy modulators Rapamycin/CQ, after treatment they were pooled with naïve B cells

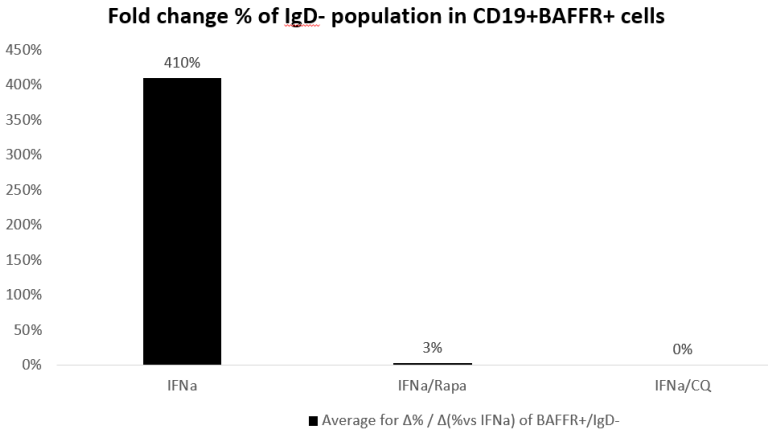

Figure 16. IFNα pre-treated healthy monocytes induce high levels of naïve B cell differentiation and class-switching. Rapamycin and CQ abrogate IFNα induced CSR in BAFFR+ B cells

Six days MLR with 18hrs pretreated CD14+ healthy monocytes. Percentages of Δ% of BAFFR+ IGD- B cells at the end of co-culture.CD14+ monocytes from healthy donors were cultured with IFNα alone or/with autophagy modulators Rapamycin/CQ, after treatment they were pooled with naïve B cells

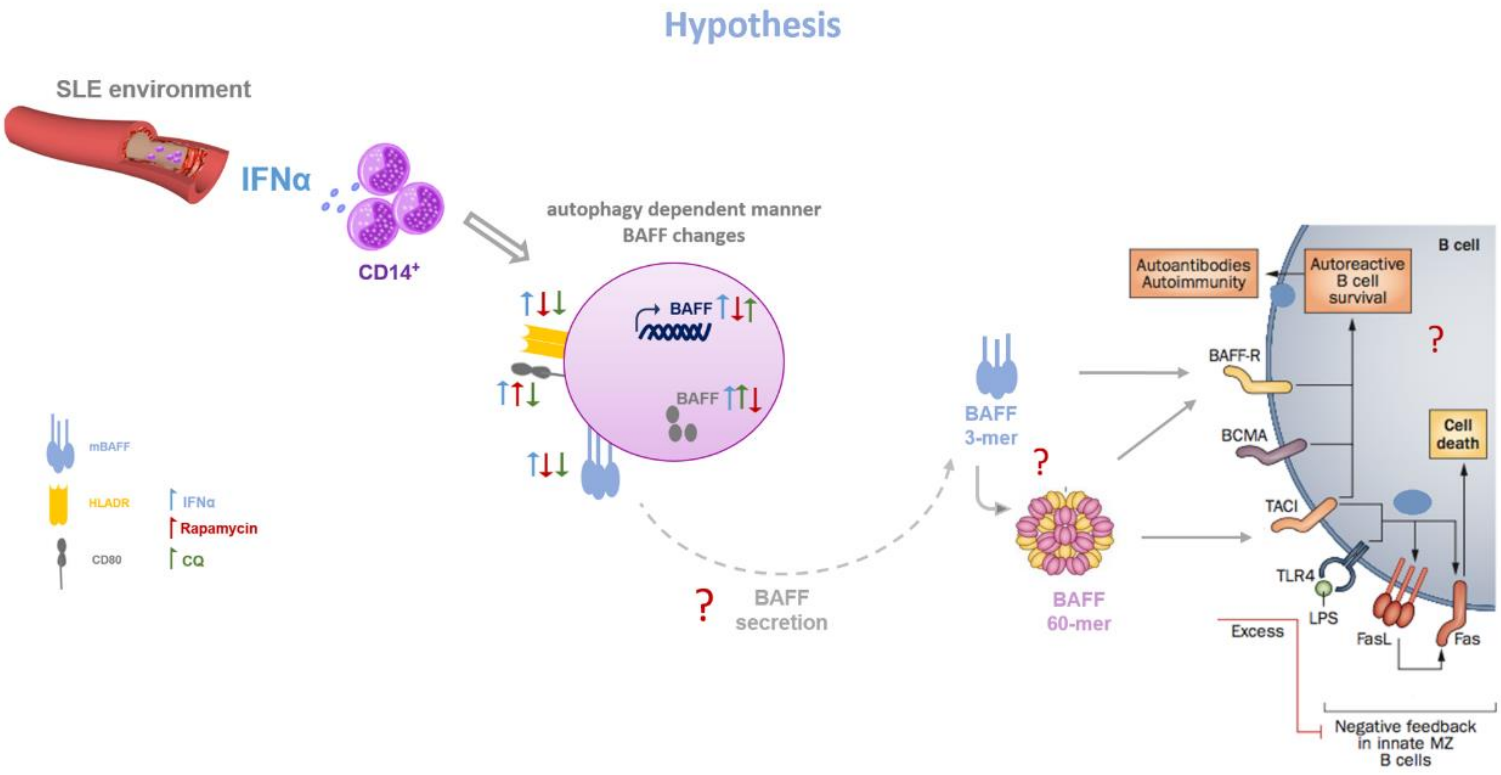

Figure 17: Hypothesis of our study

IFNα signaling induces BAFF membrane expression, protein levels and transcription in CD14+ monocytes, but there is a question concerning the secretion of BAFF by monocytes. In addition, IFNα signaling induces BAFF expression trough autophagic modulation, as Rapamycin and CQ (an inducer and an inhibitor of autophagy respectively) changes BAFF expression upon IFNα signaling. Remains unclear how BAFF secretion by monocytes upon IFNα signaling and through autophagic modulation could influence B cells.

Discussion

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by heterogeneous clinical manifestation and the presence of multiple cellular and molecular abnormalities in the immune system, including leukocyte activation and cytokine dysregulation. Type I IFNs, particularly IFNα, considered playing a pivotal role in SLE pathogenesis. IFNα serum levels as well as the expression of IFNα-inducible genes are consistently increased in SLE patients, usually correlated with disease activity and clinical manifestations. Furthermore, IFNα from SLE sera can differentiate monocytes into activated dendritic cells (DCs) able to present self-antigens (Blanco et al. 2001) supporting that this pleiotropic cytokine could be responsible for initiating development of systemic autoimmunity (López et al. 2016). Binding of IFNα to the two-chain type I interferon receptor (IFNAR) initiates a signal transduction pathway that results in the expression of IFN-induced genes, most of them with immunoregulatory functions on B, T and NK lymphocytes, monocytes/ macrophages, DCs and neutrophils (José M. González-Navajas et al., 2013). Consequently, anomalous functioning of type I IFN signaling could be an early event in lupus pathogenesis. In addition, recent study in our laboratory highlighted an important role for aberrant autophagy in IFNα- mediated monocyte hyper activity in SLE monocytes (Gkirtzimanaki et al. 2018).

The detrimental role of B lymphocytes in systemic lupus erythematosus is evident from the high levels of pathogenic antinuclear autoantibodies (ANAs) found in SLE patients. Affirming this causative role, additional antibody-independent roles of B cells in SLE were appreciated. In recent years, many defects in B cell selection and activation have been identified in murine lupus models and SLE patients that explain the increased emergence and persistence of autoreactive B cells and their lowered activation threshold. Therefore, clinical trials with B cell depletion regimens in SLE patients were initiated but disappointingly the efficacy of B cell depleting agents proved to be limited. Remarkably however, a major breakthrough in SLE therapy was accomplished by blocking B cell survival factors.

Belumimab, a human monoclonal antibody widely used until today does not deplete B cells but instead targets the B cell survival cytokine BAFF (B-cell Activating Factors, also known as BLyS [B-lymphocyte stimulator]) rather than eliminating B cells. This surprising finding indicates that although SLE is a B cell-driven disease, the amplifying crosstalk between B cells and other cells of the immune system likely evokes the observed tolerance breakdown in B cells. Moreover, this implies that intelligent interception of pro-inflammatory loops rather than selectively silencing B cells will be a key to the development of new SLE therapies (Laurens P. Kil et al., 2013).

Furthermore, since IFNα has been identified as an efficient inducer of Blys expression (López et al. 2014) and taking into consideration data from recent published work of our laboratory (Gkirtzimanaki et al. 2018), we wanted to decipher whether aberrant autophagy in lupus monocytes participates in the secretion/membrane expression and protein induction of BAFF, which is vital for the maturation and class switch recombination of B cells, and as a result a key player in the pathogenesis of the disease.

Consequently, from our results, CD14+ monocytes from healthy donors revealed an up-regulation of BAFF mRNA levels in addition of SLE serums, characterized of high levels of IFNα,

indicating that IFNα has a positive effect on translational expression of BAFF gene in monocytes (Figure 9). We decipher that a more physiologically relevant concentration of IFNα (200 u/ml) has a significant increase concerning BAFF membrane expression compared to higher concentrations of IFNα and the untreated condition (Figure 10A) in parallel with obvious increase of their HLA DR expression. Moreover, this important induction of BAFF expression (membrane- bound levels, mRNA and protein levels) upon IFNα stimulation observed specifically in 18hr stimulated CD14+ heathy monocytes (Figure 11 and 12A, B). As a result, this finding indicates that IFNα influences BAFF expression in healthy monocytes, and consequently, considering that also IFNα drives to aberrant autophagy (Gkirtzimanaki et al. 2018), this increase may be due to autophagic mechanism. Thus, concerning our wonder about the affection of BAFF expression upon IFNα in an autophagy dependent manner and in order to identify that autophagic flux participates in the BAFF protein upregulation, BAFF increased membrane expression and BAFF increased transcriptional levels, we used autophagic modulators. We delineate that BAFF membrane expression changes obviously in the presence of Rapamycin and CQ (Figure 13A) alone compared to IFNα alone and with regard to these changes HLA DR and CD80 are similarly affected (Figure 13A). However, IFNα Rapamycin did not affect BAFF membrane expression compared to IFN α (Figure 13A). Indeed, Rapamycin an inhibitor of mechanistic target of rapamycin (m TOR) signaling and potent enhancer of autophagosome formation and autolysosomal degradation, a known inducer of autophagy, induced the BAFF protein levels in contrast to untreated or IFNα stimulated monocytes (Figure 13B). Respectively, CQ which is a suppressor of autophagy induced also BAFF protein levels compared with untreated and IFNα conditions (Figure 13B). These observations were followed by same changes regarding the mRNA levels of BAFF (Figure 13C).

These results revealed for the first time that IFNα dysregulated autophagic machinery resulting in increased BAFF expression (increase membrane expression, protein levels, mRNA levels) and prevent its autophagic degradation, resulting to its accumulation. These observations come to an agreement with the fact that IFNα impairs the completion of autophagy, autophagolysosomal degradation in healthy CD14+ monocytes and enhances the immunogenic potential of monocytes (Gkirtzimanaki et al. 2018).

In addition, the fact that Rapamycin and CQ also influence BAFF expression in a corresponding way, with respect to control indicates that *indeed autophagy takes part in BAFF protein regulation, BAFF transcriptional regulation and BAFF membrane expression in CD14+ monocytes*. In agreement with these findings, immunofluorescence microscopy revealed a significant co localization of BAFF with LC3 protein, a marker of autophagosomes, and an induction of BAFF protein levels upon IFNα signaling and CQ signaling alone, whereas Rapamycin reverted this observation, suggesting that inducing autophagy in presence of IFNα leads to a possible completion of autophagy and consequently to BAFF degradation from lysosomal hydrolases (Figure 13D). Finally, IFNα pre-treated monocytes induce higher levels of B cell class switching, in comparison to control whereas in presence of autophagic modulators, such as Rapamycin and CQ these percentages were reduced with respect to IFNα alone (Figure 14, Figure 15).

In conclusion, our results revealed that IFNα plays an important role in BAFF mRNA expression, since SLE serum identified with high levels of IFNα, upregulate BAFF translational expression in healthy CD14+ monocytes in contrast to healthy serums. Moreover, IFNα has the potential to induce BAFF membrane expression, protein levels as well as transcriptional levels of BAFF in CD14+ monocytes in contrast to control conditions. However, in presence of Rapamycin

and CQ these levels are significantly reduced indicating that autophagic machinery is a key player in BAFF regulation. Finally, the co-culture results demonstrate that autophagic regulation of BAFF in IFNα-stimulated monocytes have functional significance, since they cause corresponding variations in the modulation level of B lymphocytes.

SLE therapies switch from traditional to more sophisticated strategies and B cells have become a major target. As the search for new therapies continues, important players of B cell activation will be targeted for therapy. Autophagy is an evolutionary conserved membrane-trafficking mechanism by which cells deliver cytoplasmic substrates to lysosomes for their recycling to maintain cellular metabolic equilibrium and homeostasis (Klionsky et al. 2011). Emerging literature implicates autophagy in intracellular danger sensing antigen processing, and delivery for presentation in APCs (Alissafi et al. 2017; Pascual et al. 2006).

Rescue of autophagic homeostasis, could be an emerging target for SLE therapy, since it seems that sustained BAFF signals by SLE monocytes results in maturation and maintained of Auto-IgG producing B cells, contributing to SLE pathogenesis. understanding (one of) the molecular mechanism (s) regulating BAFF differential function upon IFNα signaling will provide us with a wider armamentarium for SLE targeting therapies.

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