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Estrogen immunomodulation in systemic autoimmunity: evidences in *in vitro* models on a human myeloid cell line

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ABSTRACT

Background: Estrogens have an important role in determining immune system development, regulation and response to stimuli. Estrogens also influence pathogenesis and progression of autoimmune diseases, including Systemic Lupus Erythematosus (SLE), where there is a strong sex bias: female to male prevalence ratio is about 9:1. Estrogens, and in particular 17-β estradiol (E2), act as transcription factor through the binding to its specific receptors, ERα and ERβ. Among genes regulated by E2, Interferon α (IFNα) and B Lymphocyte Stimulator (BLyS) seem to be affected, and BLyS seems to be an IFN-inducible gene. Both cytokines are increased in SLE patients, indicating a link between hormones and cytokines induction in autoimmunity; BLyS over-expression can represent also a sign of the so called "IFN signature" characterizing systemic autoimmune diseases.

Objective: The aim of the present study was to evaluate the effects of E2 on BLyS mRNA and protein expression in a human myeloid cell line, in order to propose an *in vitro* model of estrogen systemic immunomodulation, also considering IFNα as a possible mediator of this signaling pathway.

Materials and Methods: U937 monocytes and U937-derived macrophages (treated with 50ng/mL Phorbol 12-Myristate 13-Acetate for 72 hours, Sigma-Aldrich) were exposed to E2 (Sigma-Aldrich) 100nM, 10nM, 1nM or 0.1nM. Cell viability was evaluated with Trypan Blue and Burker Chamber cell counting. Total RNA was extracted after 6, 24 and 48 hour administration, and culture medium was collected for protein quantification. Quantitative PCR was performed for *BLyS* and *IFNα* genes. *GAPDH* was used as reference gene. Primer concentration was 100nM and cDNA quantity was 25ng. Data were analyzed with $2^{-\Delta\Delta Ct}$ method and statistical analysis was performed with REST-384© version 2 using Pair Wise Fixed Reallocation Randomisation Test©. ELISA assay, using a commercially available kit "Quantikine® ELISA" for Human BAFF/BLyS/TNFSF13B (R&D System) was performed for BLyS protein detection in culture supernatants. Data were normalized for total protein and differences in protein levels between treated and control cells during times were performed with multivariate analysis for repeated measures. The same protocols were used for treatment with exogenous human recombinant INFα (hr-IFNα, 1000IU/mL, Enzo Life Sciences),

investigating *BLyS* mRNA expression at 6, 10, 24 and 48 hour and BLyS protein release at 6, 24 and 48 hour administration.

Results: E2 did not induce any modulation of *BLyS* gene expression at any time or concentration used, in both monocytes and derived-macrophages. BLyS protein release was increased during time, and the highest E2 doses induced BLyS protein mobilization in monocytes, while a BLyS release was noticed in derived-macrophages starting from 1nM E2. In monocytes, E2 induced a time-dependent *IFNα* up-regulation especially at doses 10nM and 1nM, while in differentiated macrophages E2 induced a significant *IFNα* down-regulation at 24 hours at doses 100nM and 10nM. IFNα treatment induced a significant up-regulation of *BLyS*, both in monocytes and in derived-macrophages at any time of treatment, but the effect was higher and faster in differentiated cells than in steady-state monocytes. Regarding BLyS protein release, IFNα treatment did not induce a significant BLyS increase compared with that observed in untreated cells.

Conclusion: Within 48-hour treatment E2 dose-dependently induces *IFNα*, but not *BLyS* expression, while exogenous IFNα affects *BLyS* transcription but not protein release. These findings suggest that estrogens could primarily have a role in the modulation of cytokines belonging to innate immunity, such as IFNα, with different effects depending on target cell phenotype and milieu. Estrogen modulation of adaptive immune response, here exemplified by BLyS, seems to be the result of estrogen-induced IFNα up-regulation, thus confirming the link between innate and adaptive immune activation in systemic autoimmunity.

RIASSUNTO

Background: Gli estrogeni svolgono un ruolo importante nel determinare lo sviluppo, la regolazione e la risposta agli stimoli del sistema immunitario. Essi sono implicati anche nella patogenesi e nella progressione delle malattie autoimmuni, come il Lupus Eritematoso Sistemico (LES), dove la prevalenza delle donne sugli uomini risulta essere di 9 a 1. Gli estrogeni, ed in particolare il 17-β estradiolo (E2), agiscono come fattori di trascrizione attraverso il legame con i loro specifici recettori, ERα ed ERβ. Tra i geni regolati da E2 troviamo l'Interferone α (IFNα) e B Lymphocyte Stimulator (BLyS); BLyS sembra essere un gene indotto anche da IFNα. Entrambe queste citochine si trovano ad alti livelli nei sieri dei pazienti con LES, indicando un possibile collegamento tra ormoni e induzione citochinica nell'autoimmunità; l'over-espressione di BLyS può anche rappresentare un segnale della cosiddetta "IFN signature", che caratterizza le malattie autoimmuni sistemiche.

Obiettivo: Lo scopo del presente studio è stato quello di valutare gli effetti di E2 sull'espressione di BLyS mRNA e proteina in una linea cellulare mieloide umana, così da proporre un modello *in vitro* di immunomodulazione sistemica indotta da estrogeni, considerando IFNα come un possibile mediatore di questa via di segnale.

Materiali e Metodi: Cellule umane U937, monocitarie e simil-macrofagiche (trattati con 50ng/mL di Forbolo 12-Miristato 13-Acetato, Sigma-Aldrich), sono state trattate con E2 (Sigma-Aldrich) alle concentrazioni 100nM, 10nM, 1nM, 0.1nM. La vitalità cellulare è stata valutata mediante conta cellulare con il colorante vitale Trypan Blue e Camera di Burker. L'RNA totale è stato estratto dopo 6, 24 e 48 ore dal trattamento, e il medium di coltura è stato raccolto per la quantificazione proteica. La PCR quantitativa (qPCR) è stata eseguita per i geni *BLyS* e *IFNα*. *GAPDH* è stato utilizzato come gene reference. Sono stati utilizzati i primer alla concentrazione 100nM e cDNA nella quantità di 25ng. I dati sono stati analizzati con il metodo $2^{-\Delta\Delta Ct}$ e l'analisi statistica è stata eseguita con REST-384© version 2 using Pair Wise Fixed Reallocation Randomisation Test©. Per determinare la quantità di proteine nel surnatante di coltura è stato utilizzato il kit ELISA commerciale "Quantikine® ELISA" for Human BAFF/BLyS/TNFSF13B (R&D System). I livelli di BLyS proteina sono stati normalizzati per i livelli di proteine totali e le differenze nei livelli di proteina tra cellule trattate e di controllo

sono stati valutati con un'analisi multivariata per misure ripetute. Gli stessi protocolli sono stati utilizzati per il trattamento con IFNα ricombinante umano esogeno (hr-IFNα, 1000IU/mL, Enzo Life Sciences), indagando l'espressione dell'mRNA di *BLyS* a 6, 10, 24 e 48 ore e il rilascio di BLyS proteina a 6, 24 e 48 ore dal trattamento.

Risultati: E2 non induce modulazione dell'espressione genica di *BLyS* ai tempi e alle concentrazioni testate, sia nei monociti che nei macrofagi-derivati. I livelli di BLyS proteina nel surnatante di coltura aumentano nel tempo, le più alte dosi di E2 inducono la mobilizzazione e il rilascio di BLyS in coltura nei monociti, mentre nei macrofagi-derivati si è notato un rilascio di BLyS partendo dalla concentrazione di E2 1nM. Nei monociti E2 induce un'up-regolazione tempo-dipendente di *IFNα,* specialmente alle dosi 10nM e 1nM, mentre nei macrofagi differenziati E2 induce una significativa down-regolazione di *IFNα* a 24 ore, alle dosi 100nM e 10nM. Il trattamento con IFNα induce una significativa up-regolazione di *BLyS*, sia nei monociti che nei macrofagi-derivati ad ogni tempo di trattamento, ma gli effetti sono risultati più rapidi e più marcati nei macrofagi rispetto ai monociti. Per quanto riguarda il rilascio di BLyS proteina, il trattamento con IFNα non ha indotto un significativo aumento di BLyS rispetto alle cellule non trattate.

Conclusioni: Entro 48 ore, il trattamento con E2 modifica in maniera dose-dipendente l'espressione di *IFNα*, ma non quella di *BLyS*, mentre IFNα esogeno influisce sulla trascrizione di *BLyS*, ma non sul rilascio della proteina stessa. Queste evidenze suggeriscono che gli estrogeni possono avere primariamente un ruolo nella modulazione di citochine appartenenti all'immunità innata, come l'IFNα, con effetti differenti dipendenti dal fenotipo cellulare e dal milieu citochinico. La modulazione estrogenica della risposta immunitaria adattativa, qui esemplificata da BLyS, sembra essere il risultato dell'up-regolazione di IFNα indotta da estrogeni, a conferma della stretta inter-relazione tra immunità innata e adattativa nell'autoimmunità sistemica.

INTRODUCTION

1.1 Estrogens and systemic autoimmune diseases

Immunity is the balanced state of our immune system that realizes adequate biological defenses and responses against pathogens and non-self particles. Immune system has specific properties in order to induce protection: recognition, specificity, production of antibodies, cell-mediated reaction and memory. When there is a breakdown in this balance, immune system can undergo different pathological states:

- Autoimmunity: inability to discriminate between self and non-self
- Immunodeficiency: absent/inadequate immune response
- Hypersensitivity: extreme/inappropriate immune response

In immunity and pathological immune conditions, sex differences play an important role in determining immune system development, regulation and response to stimuli. In particular, females are more resistant to infections than males, because of their heightened immune response, but at the same time they are more prone to develop autoimmune disorders and inflammatory diseases [1]. Sex hormones are key effectors of sexual dimorphism in immunity and autoimmunity: in Systemic Lupus Erythematosus (SLE), the prototype systemic autoimmune disease, female to male prevalence ratio is around 9:1 [2]. Estrogens are female sex hormones deeply involved in development, progression and maintenance of autoimmune diseases, including SLE.

1.1.1 Estrogens

Estrogens are primarily produced by ovaries and to a lesser extent by adrenal gland and testis. Estrogens, like other steroid hormones, originate by cholesterol through the so called steroidogenesis. The biosynthesis of steroids is mediated by enzymes. Estrogens derived from steroidogenesis are: estradiol, estrone and estriol. 17-β estradiol (E2), primarily produced by ovaries, is the major estrogenic metabolite. E2 derives from testosterone through the action of the enzyme aromatase (Figure 1.1). Due to their hydrophobic nature, all steroids circulate in blood system complexed to proteins, aspecific (albumin) or specific ones. Circulating E2 binds sex

hormones binding globulin (SHGB) with high affinity, and albumin with low affinity. Estrogen concentrations vary during different physiological states in women, ranging from 0-30pg/mL during menopause to 188-7192pg/mL during pregnancy.

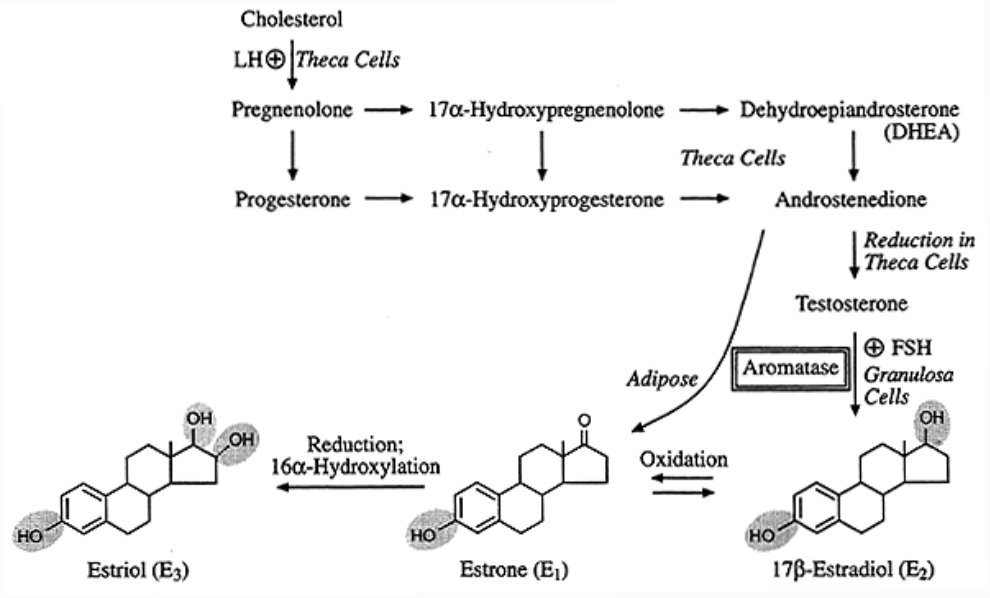


Figure 1.1 Estrogen biosynthesis
[http://chemistry.gravitywaves.com/CHE452/23_Sex%20Steroid%20Horm.htm]

Estrogens perform their activity through the binding to specific receptors. Estrogen receptors (ERs) are dimetric nuclear receptors, which are inhibited by heat shock proteins (Hsp) binding: when estrogens bind receptors, Hsp is unbound and the complex estrogen-receptor acts as a transcription factor for estrogen specific target genes (Figure 1.2).

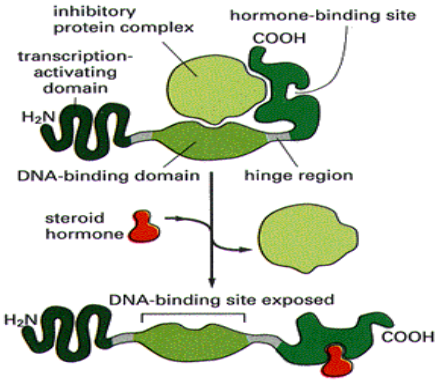


Figure 1.2: Estrogen receptor structure
[http://farmacia.unich.it/fisiologia/didattica/fisioc/lez/AA_2010-11/complementi/complementi_di_fisiologia_del_28-marzo-5aprile-2011.pdf]

Gene expression regulation by E2 and ER is a multifactorial process, involving both genomic and non genomic actions. The genomic pathway is activated by the direct binding of E2-ER to estrogen responsive elements (ERE) on the target gene's promoter, or by the interaction of E2-ER with other transcription factors (like AP-1 or SP-1), that indirectly bind promoter regions. The non genomic pathway has a rapid effect, because E2 binds membrane ERs, activates kinases and phosphatases and increases the ion flux across the membranes, causing physiological effects. The last pathway is the ligand-indipendent one, where growth factor signaling leads to activation of kinases that may phosphorylate and activate transcription factor activity of ERs [3] (Figure 1.3).

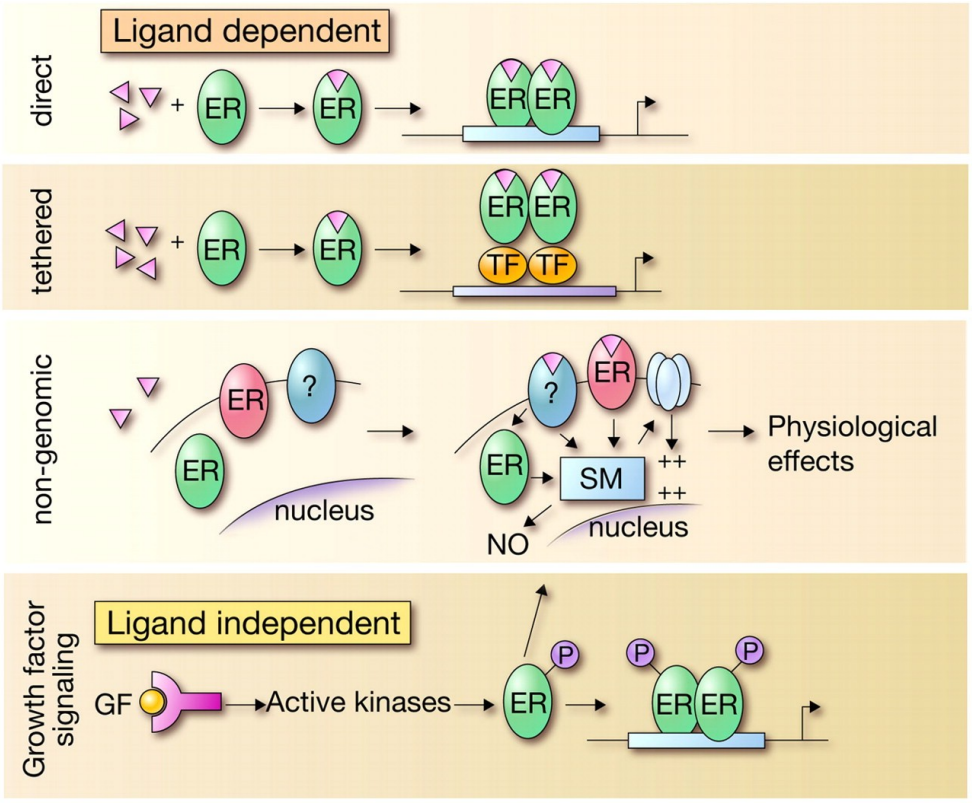


Figure 1.3: Models of pathways involved in ERs activation [3].

ERs possess highly conserved functional domains: the central and most conserved one, the DNA-binding domain (DBD), is involved in DNA recognition and binding, whereas the ligand-binding domain (LBD) is the COOH-terminal multifunctional region. At the NH_2-terminus, there is the transcription-activating function 1 (AF-1) domain, at the COOH-terminus LBD, there is the transcription-activating function 2 (AF-2) domain. Both AF

domains recruit a range of co-regulatory protein complexes to the DNA-bound receptor [3] (Figure 1.2 and Figure 1.4). There are two distinct estrogen receptors, ERα (NR3A1) and ERβ (NR3A2), both belonging to the nuclear receptor (NR) family of transcription factors and with high sequence affinity (Figure 1.4). They are encoded by different genes located on chromosome 6 (6q25.1) and chromosome 14 (14q22-24), respectively. They have different tissue distribution, transcriptional effect and ligand-binding affinity. ERα, when activated, promotes cellular proliferation and differentiation pathways, while ERβ seems to act as a transcription repressor, promoting an anti-proliferative effect.

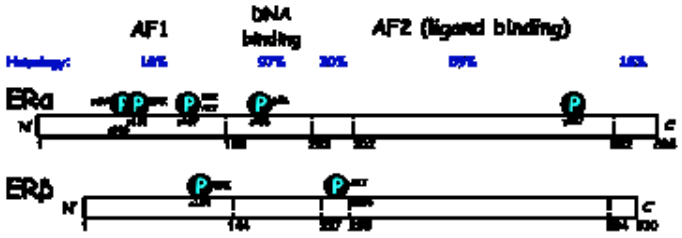


Figure 1.4 Estrogen receptor isoforms: ERα and ERβ
[https://en.wikipedia.org/wiki/Estrogen_receptor]

E2 could use different signaling pathways depending both on the cellular type and on the physiological status of the cell. In this way, E2 evokes distinct gene responses in different types of target cells.

1.1.2 Estrogens and Systemic Lupus Erythematosus

SLE is a systemic autoimmune disease, in which pathogenesis is complex, multifactorial, and not fully understood. Both genetic and environmental factors are involved, and autoantibody production and immune-complex deposition are key effector factors. SLE primarily affects females of childbearing age, with a female:male ratio of about 9:1 [2], suggesting a role for sex hormones in disease pathogenesis. Fluctuations of endogenous estrogens' concentrations, corresponds to fluctuations in the disease activity: on this regard, pregnancy represents a good physiological model in which a T-helper 2 (Th2)-guided humoral immune response is established, and high estrogen levels could be responsible for Th1-Th2 shift [4]. In SLE, humoral

immune response is aberrantly activated, and pregnancy state could induce a flare of the disease [5]. Also hormonal replacement therapy (HRT) and the use of oral contraceptives are linked to an increased risk of SLE, indicating that also exogenous estrogens could affect lupus disease activity [6,7]. SLE patients also show an altered steroid hormone metabolism, leading to an increased concentration of estrogen metabolites in plasma [8]. Studies on animal models confirm the role of estrogens in promoting the development of SLE. New Zeland Black/White F1 (NZB/W F1) mice is a lupus-prone murine model that spontaneously develops glomerulonephritis (GLN) within 20-28 weeks of age [9]. Ovariectomy [10] or genetic ablation of ERα signaling [11] in this mouse model, showed decreased autoantibody production and mortality, while 17-β estradiol treatment accelerates GLN and disease progression [12]. Regarding these evidences, both endogenous and exogenous estrogens could modulate disease activity, probably via ERα activation: another study in NZB/W F1 mice has demonstrated that ERα activation exerts an immune-stimulatory effect in lupus, while ERβ activation has a poor immunosuppressive effect on the disease [11,13]. ERs are present in specific immunocompetent cells: monocytes-macrophages, and T and B cells [8]. Usually, estrogens enhance cell proliferation and reduce cell apoptosis [14], but it was also demonstrated that high doses (10^{-4}M – 10^{-7}M), similar to those achieved during pregnancy, induce cell cycle arrest and apoptosis in monocyte cell lines [15]. This evidence suggests the role of estrogens in pregnancy in reducing cell-mediate immune response, thus favoring humoral response. Concerning cells of the adaptive immune system, estrogens can alter survival and activation of B cells in a B cell-autonomous fashion, inducing auto-reactivity and proliferation [16]; moreover, estrogens promote survival of self-reactive B cells at peripheral check-point, evading immune tolerance mechanism, being this a fundamental step in autoimmunity development [17]. Self-reactive B cells can survive also by the increased production, by myeloid cells, of the B-cell survival factor tumor necrosis factor ligand superfamily member 13B (TNFSF13B), also known as B Lymphocyte Stimulator (BLyS) or B-cell activating factor (BAFF), which seems to be stimulated by E2 [12,18]. Estrogens can also modulate the

production of pathogenic IgG autoantibodies, influencing IgG subclass selection [19,20]. A recent study demonstrated that ERs bind directly to key regulatory elements in the immunoglobulin heavy chain locus in activated B cells, influencing antibody expression and class switching recombination, thus altering B cell responses to self- and non-self-antigens [21]. So, estrogens can affect autoantibodies repertoire, influencing both the risk and the severity of the disease. In autoimmune disorders autoantibodies bind autoantigens, derived mainly from impaired clearance of apoptotic debris, forming immune-complexes (ICs) that can deposit in specific organ tissues (i.e. the kidney). Autoimmunity can be promoted also by an imbalance in different subsets of $CD4^+$ cells. Estrogens reduce the number of immature thymic lymphocytes ($CD4^+/CD8^+$) and thymic stromal tissue, through thymic involution, thereby interfering with T-negative selection and tolerance induction, while enhancing hepatic T cell lymphopoiesis [22,23]. The production of auto-reactive T helper cells is involved in the breakdown of B cell self-tolerance, inducing production of autoantibodies, favoring the development of autoimmunity [24,25]. Estrogens can regulate pathways leading to the production of Interleukin-2 (IL-2), an important determinant for T-cell tolerance and differentiation [26]. Moreover, timing and dose of estrogen influence T cell response: a continuous treatment of low-dose E2 enhances antigenic-specific $CD4^+$ T cell responses and strongly promotes Th1 cell development, with production of IFNγ (an important inducer of Ig-class switching in B cells), and this effect requires the expression of ERα in hematopoietic cells [27]. Despite this last finding, pregnancy induces a shift from Th1 to Th2 humoral response, due to the higher levels of hormones, and in particular estrogens: this Th1-Th2 shift could explain why Th2-mediated autoimmune disease, such as SLE, tends to develop and worsen during pregnancy [28]. Estrogens can influence also $CD4^+$ regulatory T (Treg) cells development and function, fundamental cells that prevent autoimmunity, controlling self-reactive B and T cells at the periphery [29]. In pregnant mice, expansion of maternal Treg populations specific for fetal antigens helps to protect developing fetuses against immune attack [30]. Major estrogen immunomodulatory effects are depicted in Figure 1.5.

An important cytokine, highly expresses in SLE patients, and strongly enhanced by estrogens, is type I Interferon (IFN).

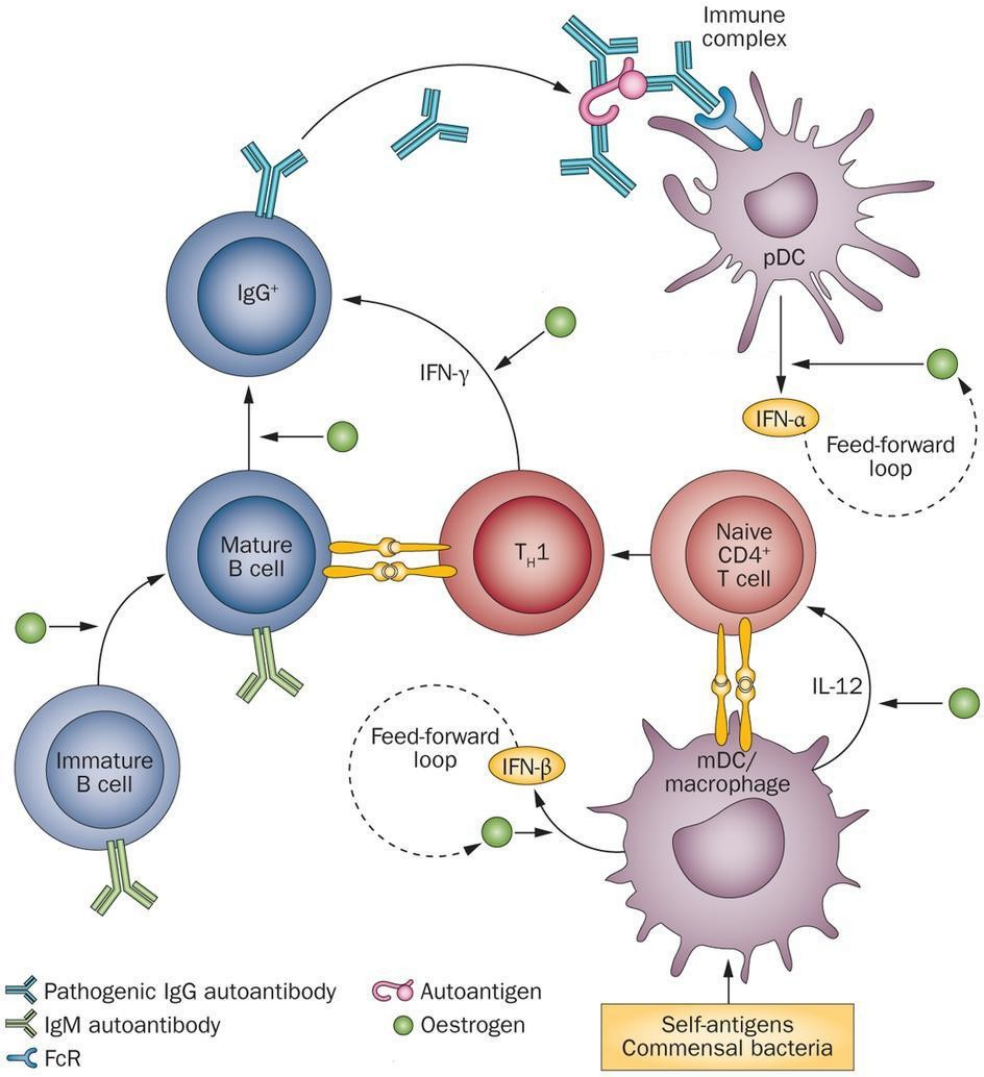


Figure 1.5: Potential mechanisms through which estrogen might modulate the loss of tolerance and regulate the production of pathogenic IgG autoantibodies in SLE. Modified from [31].

1.2 Type I Interferon system

1.2.1 Type I Interferon in SLE

Interferons (IFNs) are a group of cytokines, produced and released by host cells in response to the presence of pathogens or tumor cells. IFNs are so named for their ability to "interfere" with viral replication, protecting cells from virus infection. IFNs are typically divided into three different groups:

type I, type II and type III IFNs. The type I IFN family is the largest one and has a pivotal role in SLE pathogenesis: it includes 12 different IFNα subtypes, and one IFNβ, IFNω, IFNε and IFNκ subtype each [32]. Type I IFNs are produced by all leucocytes, but the most potent producer is the plasmacytoid dendritic cell (pDC). Pattern recognition receptors (PRRs), including Toll Like Receptors (TLRs), localize in the cytosol or in the endosome of different immune cells, and recognize viruses, bacteria or microbial nucleic acids, inducing the pathway of IFN production. In particular TLRs recognize double stranded (ds)RNA (TLR3), single stranded (ss)RNA (TLR7) or dsDNA (TLR8 and TLR9) [33]. All type I IFNs bind the ubiquitously expressed heterodimeric IFNα/β receptor (IFNAR), activating the canonical signaling pathway JAK/STAT. The transcription factor complex STAT1-STAT2-IRF9 formed, binds interferon-stimulated response elements (ISREs) in promoters of IFN-regulated genes, and induces the transcription of genes that act in preventing virus replication and expansion (antiviral properties) (Figure 1.6) [34].

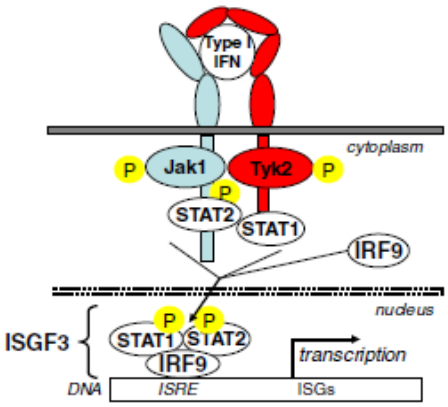


Figure 1.6: Canonical JAK/STAT signaling pathway of type I IFNs [35].

Type I IFNs modulate also the innate and the adaptive immune system, in order to induce an efficient clearance of viruses and develop a long-lasting immunity. Type I IFNs enhance maturation, activation and chemokine release by cells of innate immunity, modulate the polarization and the differentiation of T cells, and activate B cells, increasing TLR7 and TLR9 expression on their surface and BLyS production, and inducing differentiation into antibody-producing plasma cells [36]. An autoimmune disease can occur

when there is an inappropriate regulation of type I IFN system, leading to a loss of peripheral tolerance. Evidences indicate that IFNα administration in genetically predisposed patients, can cause an increase occurrence of autoantibodies and autoimmune diseases [37] and can worsen the disease [38]. SLE patients present increased serum levels of type I IFNs: ICs, containing nucleic acids derived by impaired clearance of apoptotic or necrotic cells [39,40], could act as endogenous inducers of IFNs, activating DCs through TLR7 or TLR9 [41,42]. Evidences in pediatric lupus patients [43], suggest the role of type I IFN system in initiating the disease process. Studies in murine models confirm the role of IFNα in exacerbating GLN, augmenting BLyS production and reducing survival in NZB/W F1 mice compared to wild-type mice [44]; the inhibition of both TLR7 and TLR9 in the same lupus mouse model, leads to the improvement of proteinuria, GLN and reduction of autoantibody production and amelioration of the disease [45], indicating an important role for TLR in inducing type I IFN pathway in SLE. An etiopathogenic model of SLE could include an initial infection by a virus that induces type I IFN production and release of cellular material from dying cells. Impaired clearance of apoptotic cells is a source of autoantigens: self-nucleic acids are recognized by TLRs, present on the surface of DCs, that internalize them and start the production of IFNα, stimulating their maturation and autoantigen presentation. B and T cells are recruited by DCs and B lymphocytes are activated to produce autoantibodies [46]. TLRs, present on the surface of auto-reactive B cells, can co-stimulate B cells, together with autoantigens, and can lead to the activation and differentiation into antibody-producing plasma cells [47]. Nucleic acids binding autoantibodies form ICs that can be internalized by plasmacytoid DCs (pDCs), producing IFNα, which stimulates and activates DCs and T cells, leading to the chronic activation of type I IFN and the self perpetuation of antibody production and inflammation [48] (Figure 1.7).

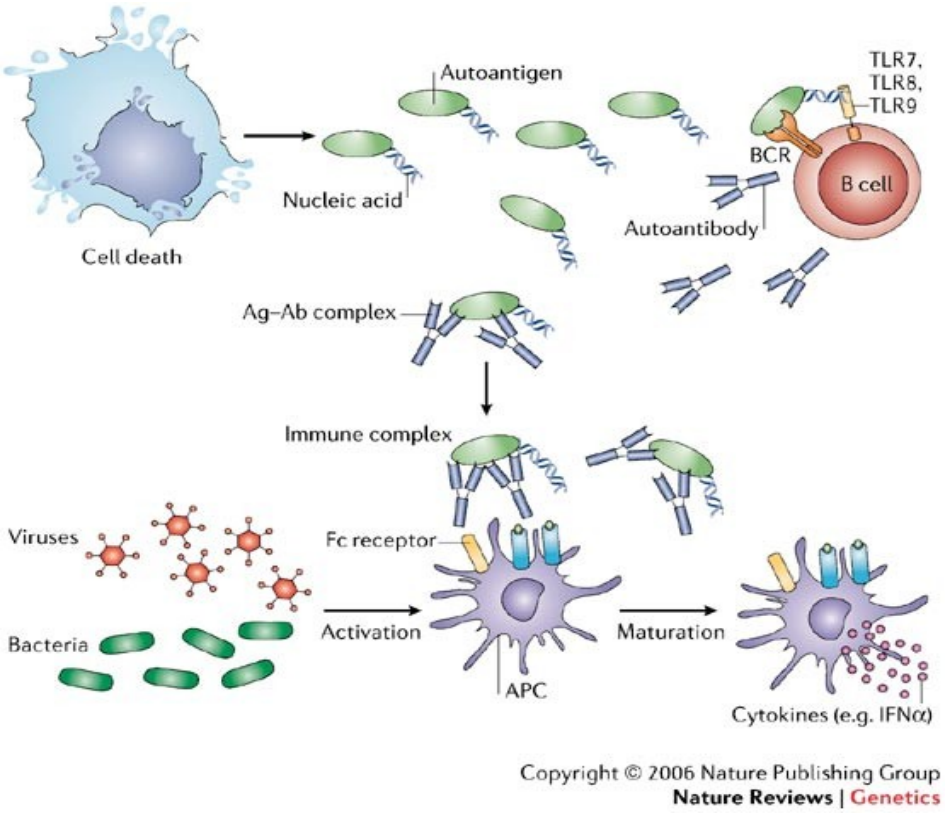


Figure 1.7: Model of innate immune responses in autoimmunity [49].

Type I IFN production is controlled by a network of cytokines, surface receptors and immune cells, but also hormones have an important role in IFN modulation.

1.2.2 Estrogen and type I IFN in SLE

Estrogen enhances type I IFN production, inducing a sexual dimorphic response: women produce a stronger type I IFN response than men, when vaccinated [50] or when pDCs are activated [51]. E2 administration, through ERα activation, seems to stimulate IFNα production by pDCs of post-menopausal women, stimulated ex vivo with SLE-related immune complexes [52]. Experiments on mouse models demonstrate that the increased production of IFNα by pDCs via ERα, mediates up-regulation of genes involved in type I IFN production; such gene over-expression induces the development of autoimmune kidney disease via IFNα activation. IFNα can also produce a feed-forward loop, inducing expression of ERα, amplifying E2 signaling in innate immune cells [31] (Figure 1.5). A recent study

demonstrates that also environmental estrogen, bisphenol A (BPA), stimulates type I IFN signaling, through the up-regulation of both ERα and IFNs in murine and human myeloid cells, augmenting IFN-inducible proteins that regulate innate immune responses (activating inflammasome activity) and modifying lupus susceptibility [53]. As in innate immune cells, ER activity may regulate also the function of adaptive immune cells. A recent study highlighted the role of E2 in inducing IFNα activation in B cells: E2 via ERα down-regulates the expression of microRNA (miRNA), promoting IKKε expression that phosphorylate STAT1, inducing the transcription of IFNα inducible genes, affecting B cell activity [54] (Figure 1.8).

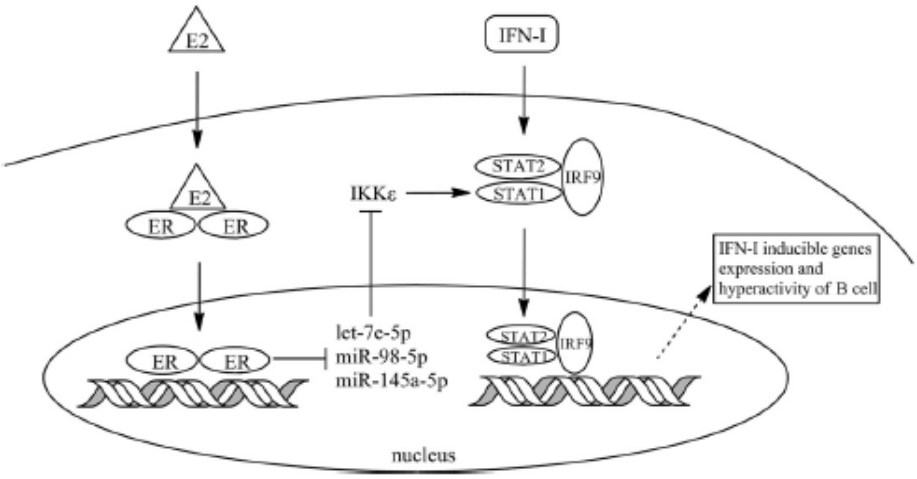


Figure 1.8: Proposed effective mechanism of E2 on amplifying IFNα signaling activation in B cells [54].

1.3 B Lymphocyte Stimulator (BLyS)

1.3.1 BLyS and SLE

B lymphocyte stimulator (BLyS), also known as B cell-activating factor (BAFF) or tumor necrosis factor ligand superfamily member 13B (TNFSF13B) was discovered in 1999 [55], and more recently, it was identified as a key cytokine in SLE pathogenesis [56]. BLyS is a type II membrane protein member of the TNF family, highly conserved during evolution; its gene is located on chromosome 13q34 and encodes a protein of 285 aminoacids [57]. Membrane BLyS can be cleaved at a furin protease site,

obtaining the soluble cytokine [55,57]; evidences in neutrophils suggest that cells can also produce and store soluble BLyS and release it under specific stimuli [58,59]. BLyS is mainly produced by primary myeloid cells (macrophages, neutrophils and DCs), and also produced by myeloid cell lines, such as K-562, HL-60, THP-1 and U937 [57]. BLyS can be bound by three receptors: TNF receptor superfamily member 13C (BAFF receptor [BAFF-R] or BLyS receptor 3 [BR3]), TNF receptor superfamily member 17 (B-cell maturation antigen [BCMA]) and TNF receptor superfamily member 13B (transmembrane activator and cyclophilin ligand interactor [TACI]). BCMA and TACI can bind also a proliferation-inducing ligand (APRIL), a related cytokine member of the TNF superfamily member, while BR3 specifically recognizes BLyS [60]. BR3 and TACI are expressed on B cells, while BCMA is expressed on plasmablasts and plasma cells: BLyS-BR3 binding promotes survival and maturation of immature B cells, BLyS-TACI binding induces T-cell-independent responses of B cells to type I and type II antigens, negative regulation of B-cell compartment and class-switch recombination of B cells, BLyS-BCMA binding promotes plasma cell survival [61] (Figure 1.9). BLyS-mediated B-cell maturation consists into two steps: at first, soluble BLyS binds BR3 on B cells, promoting B-cell survival; at second, membrane BLyS-TACI binding modulates B-cell phenotype [62].

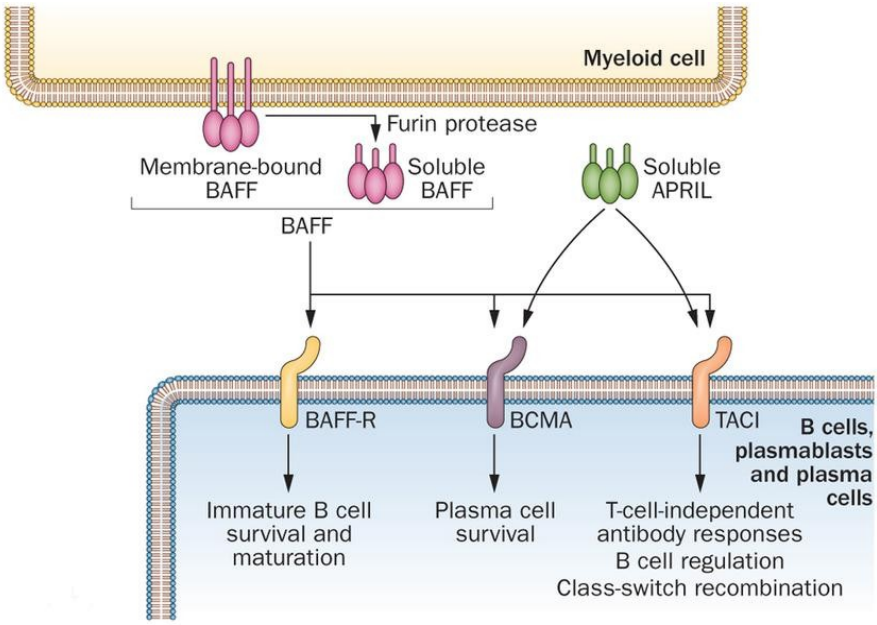


Figure 1.9: Soluble BAFF and APRIL signaling. Modified from [61].

BLyS exerts fundamental roles in maturation, survival and differentiation of B cells: BLyS promotes B cell maturation in the spleen at the T1-T2 immune tolerance check-point [63], and its excess may lead to expansion of low-affinity self-reactive B cells, mainly marginal zone (MZ) B cells [64]. BLyS exerts a regulatory effect also on T cell function [65,66]. BLyS over-expression in mice (BLyS transgenic mice), can induce an impaired production of mature B cells as well as effector T cells, and consequently autoantibodies, leading to the development of an autoimmune disease, similar to human SLE [67,68], while BLyS blockade reduces SLE flares in mouse models [69] and BLyS knock-out mice become immunodeficient [70]. Evidences of BLyS-substained autoimmunity in mouse models are strengthened by the observation that BLyS serum levels are higher in SLE patients than in healthy individuals and correlate with disease activity, modifying the threshold for negative selection and favoring the survival of auto-reactive B cells [71,72]. All these findings suggest BLyS as an effective target for the treatment of SLE. Belimumab is the first biological target therapy approved for SLE treatment: it is a fully human recombinant monoclonal $IgG_1\lambda$ antibody, which targets soluble human BLyS [73].

1.3.2 Estrogen and BLyS in SLE

As previously mentioned, estrogen induces BLyS expression and production: a recent study from our group in NZB/W F1 lupus-prone mice suggests the effect of exogenous estrogen in inducing BLyS release and disease exacerbation: the direct correlation found between BLyS and anti-C1q or anti-dsDNA production indicates that BLyS over-production influences self-B cell repertoire, increasing levels of nephritogenic autoantibodies [12]. In addition, a model of BLyS up-regulation by E2 and IFNα in primary myeloid murine cells has been described [18]. Studies performed in primary human myeloid cells confirm the role of type I IFN in inducing SLE disease, also through the up-regulation of BLyS: IFNα influences the mobilization from intra- to extracellular compartments of BLyS in primary human monocytes and this mechanism is accelerated more in myeloid cells of active

SLE patients than in cells of healthy people [74]. These evidences suggest BLyS as an E2- and an IFN-inducible gene (Figure 1.10).

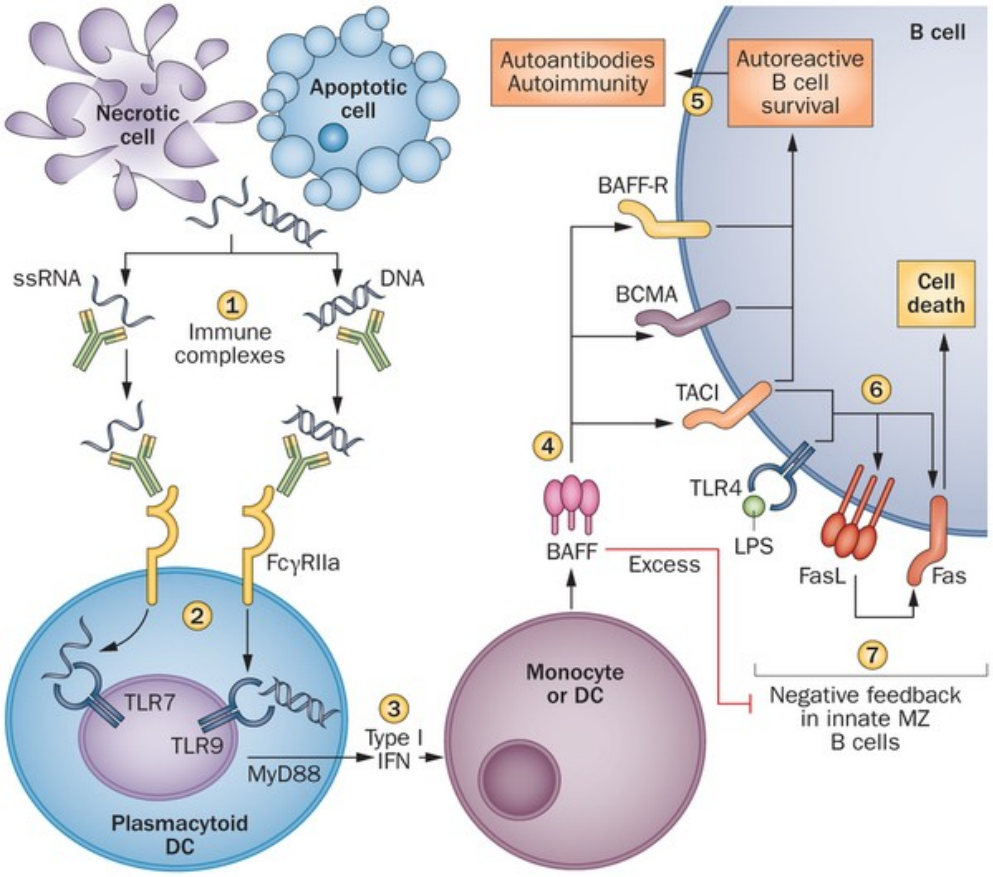


Figure 1.10: Role of BLyS in the pathogenesis of SLE [61].

AIM OF THE STUDY

Sex dimorphism is strictly related to the pathogenesis of systemic autoimmune diseases, including Systemic Lupus Erythematosus (SLE). In this context hormones, and estrogens in particular, play an important role in molecular mechanisms sustaining autoimmunity. Recent *in vivo* studies in lupus-prone mice, highlighted the role of 17-β estradiol in the modulation of cytokines, including B Lymphocyte Stimulator (BLyS). BLyS is produced by myeloid cells and affects maturation and survival of B cells. Its over-expression in SLE patients suggests its pivotal role in SLE pathogenesis and progression. Despite evidences, the molecular mechanisms leading to autoimmunity by estrogen and BLyS interaction remain poorly understood, also because results from *ex vivo* studies in humans are inconsistent, heterogeneous and scarcely reproducible. Monocyte/macrophage cells are implicated in initiation, maintenance and resolution of inflammatory response through signal molecules, which can also affect adaptive immune cell functions. U937 is a human myeloid cell line that can respond to estrogen signaling and constitutively expresses BLyS.

The aim of the present study was to evaluate the effects of 17-β estradiol on BLyS mRNA and protein expression in a human myeloid cell line, in order to propose an *in vitro* model of estrogen systemic immunomodulation, also considering IFNα as a possible mediator in this signaling pathway.

MATERIALS AND METHODS

2.1 Reagents

2.1.1 Phorbol 12-Myristate 13-Acetate

Phorbol 12-Myristate 13-Acetate (PMA) (Sigma-Aldrich, Saint Louis, MO, United States) was diluted in Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich) to the final concentration of 12.5mg/mL (20mM). Stock solution was maintained at -80°C. Further dilutions with DMSO were performed to obtain a final working concentration of 50µg/mL (80µM). Aliquots of diluted solution were maintained at -20°C.

2.1.2 Estrogen

17-β estradiol (E2) (Sigma-Aldrich) was diluted in sterile Absolute Ethanol Anhydrous (EtOH) (Carlo Erba Reagents, Milan, Italy) to the final concentration of 1mM. Work aliquots of stock solution were maintained at -20°C in glass vials, protected from light, as indicated in manufacturer's instruction. Dilutions from stock solution were performed with culture medium just before cell culture treatment.

2.1.3 Interferon α

Human recombinant Interferon α-2b (hr-IFNα) (Enzo Life Sciences, Farmingdale, NYC, USA) was reconstituted with sterile distilled water to the final concentration of 0.1mg/mL, corresponding to a total activity of $500 \cdot 10^3$IU/mL. Stock solution was maintained in aliquots at -80°C.

2.2 Human myeloid cell line

U937 is a monocytic cell line derived from 37 years old man with a histiocytic lymphoma. Cells grow in suspension and present a big round shape with cytoplasmatic inclusions visible at the phase contrast microscope Telaval 31 (Zeiss, Oberkochen, Germany) (Figure 2.1). U937 cell line was used for all experiments. Cells were maintained in Roswell Park Memorial Institute medium (RPMI 1640) (Life Technologies, Carlsbad, CA, USA)

supplemented with 10% Fetal Bovine Serum (FBS) (Life Technologies), 1% Penicillin/Streptavidin (100µg/mL) (Life Technologies) and 1% Glutamine (200mM) (Life Technologies). U937 cells were cultured in vertical 75cm^2 flasks (Corning Incorporated, BD Falcon^{TM}, Corning, NY, USA) at 37°C under normoxia (air plus 5% CO_2) in a humidified atmosphere (Incubator CO_2 Model Series 6000, Heraeus Instruments, Hanau, Germany). Medium was changed every 3-4 days to maintain cell density from 100.000cells/mL up to 1.000.000cells/mL. Cell count, to define cell density, was performed using Burker chamber and Trypan Blue vital staining (Sigma-Aldrich).

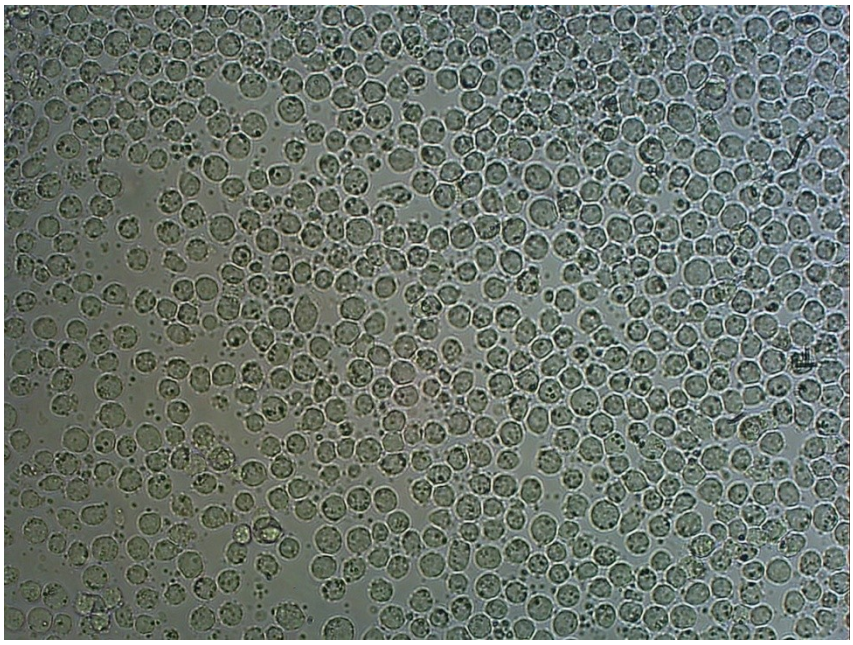


Figure 2.1:U937 cells at phase contrast microscope. Magnification 200x.

2.2.1 U937 PMA differentiation

U937 cells were induced to differentiate in macrophage-like cells using PMA. Several cell densities, PMA concentrations and times of treatment were tested. Cell differentiation was evaluated for cell adhesiveness, presence of pseudopodia and increased cell dimensions. 1.000.000 cells treated with 50ng/mL PMA after 72 hours at dark, showed marked adhesiveness to the plate, formed cell-aggregates (Figure 2.2A), augmented dimensions and presented pseudopodia (Figure 2.2B).

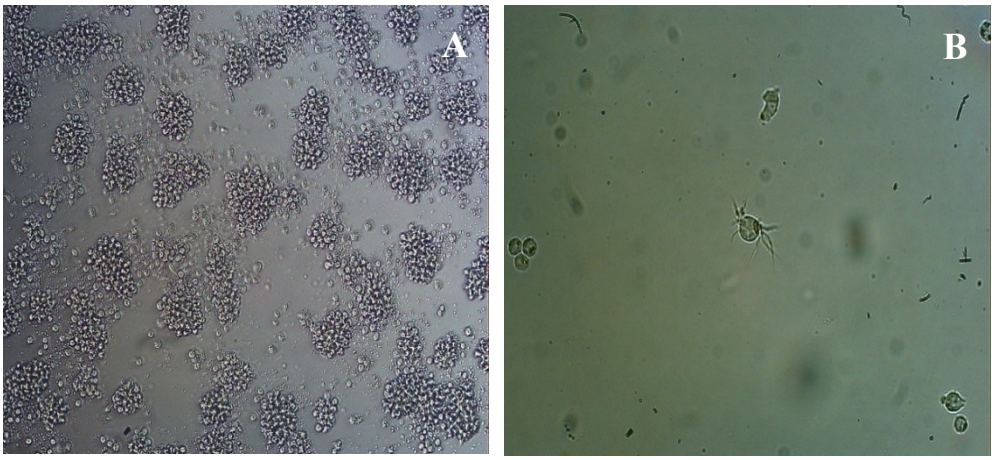


Figure 2.2: U937-derived macrophages after PMA treatment at phase contrast microscope. A: cells aggregates derived from PMA differentiation. Magnification 100x. B: Cells with pseudopodia derived from PMA differentiation. Magnification 200x.

2.3 *In vitro* treatment protocols

2.3.1 Estrogen

U937 cells were seeded at 500.000cells/mL in 12-wells plate. E2 was added at different concentrations: 100nM, 10nM, 1nM, 0.1nM. EtOH (E2 vehicle) was added in control wells, to reach a final concentration of 0.01%. In U937 derived-macrophages, medium containing PMA was replaced with medium containing E2 or EtOH. Cells were harvested after 6, 24 and 48 hours. Three independent experiments, for each cell line, were performed.

2.3.2 IFNα

U937 cells were seeded at 500.000cells/mL in 12-wells plate. hr-IFNα was added at the final concentration of 1000IU/mL. In U937 derived-macrophages, medium containing PMA was replaced with medium containing hr-IFNα or without treatment (control wells). Cells were harvested after 6, 10, 24 and 48 hours. Three independent experiments, for each cell line, were performed.

2.4 Real-time reverse transcription polymerase chain reaction

2.4.1 RNA extraction

Total RNA was isolated from cells using TRIzol® Reagent (Life Technologies). U937 cells were harvested by centrifugation at 2000rpm (revolutions per minute) for 2 minutes and washed with 1mL sterile Phosphate-Buffered Saline (PBS). Growth medium was collected from each sample and stored at -80°C for protein quantification. Cell pellets were treated with 300µL TRIzol® and incubated 5 minutes at room temperature (RT); chloroform (Sigma-Aldrich) was added in ratio 1:5 chloroform:TRIzol®. Samples were shacked vigorously by hand for 15 seconds and then incubated 5 minutes at RT. Samples were then centrifuged at 12000*g* for 15 minutes at 4°C. The aqueous phase, containing RNA (about 50% of total volume), was removed and placed into a new tube. 1µL of glycogen (Life Technologies) and then 0.5mL 100% Isopropanol (Sigma-Aldrich) per 1mL TRIzol® used, were added. Samples were stored at -20°C for 30 minutes and then centrifuged 12000*g* for 20 minutes at 4°C. After centrifugation, RNA formed a gel-like pellet on the side and bottom of the tube. Supernatants were removed from the tubes with glass Pasteur pipette and RNA pellets were washed with 75% EtOH (ratio 1:1 ethanol:TRIzol®). Samples were centrifuged at 12000*g* for 10 minutes at 4°C and then EtOH was removed. RNA pellets were dried at air for about 5 minutes and then resuspended with DEPC treated water pyrogen free DNase/RNase free (Invitrogen, Carlsbad, CA, USA).

For U937 derived-macrophages, RNA extraction protocol differentiated in cell harvesting. Growth medium was removed from culture wells, centrifuged to pellet suspension cells, and then placed in a new tube and stocked at -80°C for protein quantification. TRIzol® reagent was added directly to wells, and adherent cells were lysed pipetting them up and down several times.

Total RNA was quantified at Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA quantity starting from 250ng/µL and with ratio 260/280 between 1,8 and 2, and ratio 260/230 near 2, was considered a good sample for further experiments.

2.4.2 Retrotranscription

RNA was retrotranscribed in cDNA (complementary DNA) using Reverse Transcription System Kit (Promega, Madison, WI, USA). 1µg RNA was incubated at 70°C for 10 minutes in thermo-block (Elettrofor, Rovigo, Italy); the sample was then briefly spun (13000rpm for 1 minute) and then placed on ice. Master mix reaction was prepared following this scheme:

Reagents	**final concentration**
$MgCl_2$, 25mM	5mM
Buffer 10X	1X
dNTP, 10mM	1mM each dNTP
RNasin, 40u/µL	1u/µL
Oligo(dT), 0.5µg/µL	0.5µg
AMV Reverse Transcriptase, 25u/µL	0.75u/µL

Mix was added to each sample, to reach the final volume of 20µL. Sample was incubated in 2720 Thermal Cycler (Applied Biosystem, Foster City, CA, USA) using the following program: 42°C for 15 minutes, 95°C for 5 minutes and 4°C for 5 minutes. cDNA samples, 50ng/µL concentrated, were stored at -20°C till used.

2.4.3 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

GoTaq®qPCR Master Mix (Promega) was used to perform qPCR for genes of interest.

Primer efficiency

To determine the best cDNA quantity and primer concentration to be used both in U937 monocytes and in U937-derived macrophages, primer efficiency curve was performed. Scalar quantity of cDNA was tested (100ng, 20ng, 4ng, 0.8ng and 0.16ng) with 100nM or 200nM primers. Threshold Cycle (Ct) obtained was interpolated on y-axis, while the log(base5) of cDNA quantity (5 because each cDNA quantity is 1/5 of the previous) was interpolated on x-axis. In the equation obtained (y=mx+q), the coefficient was used in the efficiency formula $E=5^{-(1/m)}$. The percentage of efficiency was evaluated with this formula: E%=(1-E)·100. E values between 1.95 and 2.05 (95%-105%) were considered as good amplification efficiencies.

Primer sequences were obtained from the software primer-BLAST by NCBI [http://www.ncbi.nlm.nih.gov/tools/primer-blast/]. Gene sequence was entered in the software and the following primer parameters included: PCR product size of 100-150 base pairs (bp), annealing temperature of 60°C (range from 57°C to 63°C) and spanning an exon-exon junction (to elude genomic contaminations). Because *BLyS* gene generates two transcripts, *BLyS* primers were created in order to include both mRNA isoforms. In the table below the primer sequences are reported:

Gene	**Primer forward**	**Primer reverse**	**Melting Temperature**
GAPDH	5'-AAT GGA AAT CCC ATC ACC ATC T-3'	5'-CGC CCC ACT TGA TTT TGG-3'	78.5°C
BLyS	5'-GAC TGA AAA TCT TTG AAC-3'	5'-TAT TTC TGC TGT TCT GAC-3'	78.0°C
IFNα	5'-CCT GAT GAA TGC GGA CTC CA-3'	5'-TTC TGC TCT GAC AAC CTC CC-3'	80.5°C

Relative gene expression

25ng cDNA was incubated with the following components:

Components	Initial Concentration	Final Concentration
GoTaq®qPCR Master Mix	2x	1x
Primer F	10 µM or 20 µM	100 nM
Primer R	10 µM or 20 µM	100 nM
H_2O	To reach a final volume of 10 µL	

96-well plate with components and cDNA was sealed and it was centrifuged at 1100rpm for 1 minute to bring all reaction components together and eliminate air bubbles. qPCR reaction was performed with Real time PCR platform Bio-Rad CFX96 Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA), following fast cycling program: 95°C for 2 minutes (first activation), 50 cycles of 95°C for 3 seconds (denaturation) and 62°C for 30 seconds (annealing/extension). Dissociation/melting curve was obtained with 0.5°C increasing steps, each of 5 seconds, from 62°C to 95°C. The presence of a single pick in the melting curve indicated no contamination in samples and the presence of a single amplicon. Melting temperatures for each gene are indicated in the Table above. In each experimental plate also proper negative controls were added to exclude any contamination. Quantification of PCR products was normalized according to the amount of *GAPDH* cDNA (reference gene). Each sample for each experiment was loaded in triplicate. Relative quantification of gene expression was calculated by using a ΔΔCt (Ct, threshold cycle of real-time PCR) method based on signal intensity of the PCR, according to the following formula: $\Delta Ct = Ct_{target\ gene} - Ct_{GAPDH}$; $\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$; $Ratio = 2^{-\Delta\Delta Ct}$ [75].

2.5 Protein quantification

2.5.1 Enzyme-linked immunosorbent assay (ELISA) for soluble human BLyS levels determination in culture supernatants

Cell culture supernatants were tested for BLyS protein using a commercially available kit "Quantikine® ELISA" for Human BAFF/BLyS/TNFSF13B (R&D System, Minneapolis, MN, USA), following the manufacturer's instructions. Optical densities (OD) were read at 450nm and 540nm (to correct value) at microplate reader Multiskan EX (Labsystems Diagnostics, Vantaa, Finland). Levels of BLyS were expressed as pg/mL; the range of detection was 4000pg/mL-62.5pg/mL and the sensitivity was 2.68pg/mL.

2.5.2 Bradford assay

Total protein concentration in supernatants was quantified using the colorimetric Bradford's protein assay [76]. Bradford colorant (Bio-Rad) was added to scalar dilutions of Bovine Serum Albumin (BSA), from 70.50µg to 4.41µg, to create the standard curve. Optical Densities (OD) at 595nm of samples diluted in Bradford colorant, were read at DU®730, Life Science UV/Vis Spectrophotometer (Beckman Coulter, Brea, CA, USA). Concentrations were expressed as µg/µL.

2.6 Statistical analyses

Differences in cell densities between treated and control cells were determined using unpaired t-test ($p<0.05$ was considered as statistically significant).

Statistical analyses for qPCR data were performed with Relative Expression Software Tool – 384 (REST-384©) version 2 using Pair Wise Fixed Reallocation Randomisation Test© [77]. Statistical significance was considered at $p<0.01$.

Differences in protein levels between treated and control cells during times were performed with multivariate analysis for repeated measures (ANCOVA), using IBM SPSS Statistics version 22.0 software.

All graphs were designed with GraphPad Prism version 5 software.

RESULTS

3.1 U937 monocytes

3.1.1 E2 treatment: cell growth

U937 cell line was treated with different doses of E2 or EtOH at the highest concentration found in E2 administration (0.01%). Cells were counted after 24, 48 and 72 hours, in order to evaluate whether E2 (100nM) or EtOH could induce cell death or block cell proliferation. Figure 3.1 shows cell density at each time point in the three conditions: there is no significant difference in cell growth between untreated and EtOH- or E2-treated cells at each time point of observation.

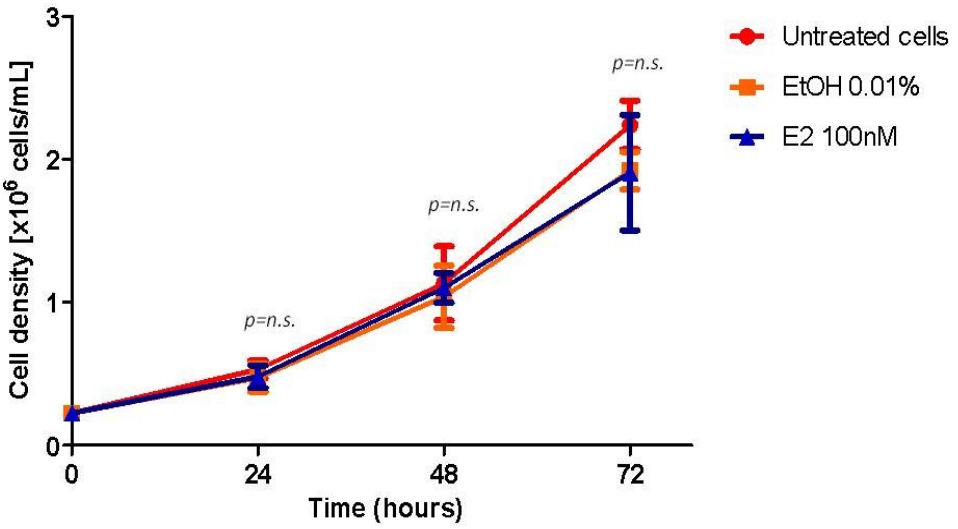


Figure 3.1: U937 cell density in untreated, EtOH-treated and E2-treated cells. Error bars represent standard deviations.

EtOH treated cells were considered as controls in the following experiments. E2 at the highest dose did not affect cell proliferation and morphology: cells maintained round shape and cell inclusions.

3.1.2 Primer efficiency curves

Before evaluating target genes expression, primer's efficiency was performed in U937 cell line. Table 3.1 indicates mean Ct values of *GAPDH*, *BLyS* and *IFNα* genes for each cDNA dilution (from 100ng to 0.16ng), using 100nM primer concentration. LOG represents the log(base5) of cDNA serial dilution. Extreme values, that did not interpolate in a good way the curve,

were excluded. Figure 3.2 shows reference and target genes trends, with the corresponding linear regression equation and the coefficient of determination (R^2).

cDNA (ng)	LOG	Ct *GAPDH*	St.Dev.	Ct *BLyS*	St.Dev.	Ct *IFNα*	St.Dev.
100	2.861353			22.32	0.266		
20	1.861353	21.09	0.214	24.30	0.269	31.34	0.523
4	0.861353	23.58	0.163	26.22	0.007	33.60	0.209
0.8	-0.13865	26.22	0.176	29.46	0.148	36.06	0.126
0.16	-1.13865	28.10	0.106			38.48	0.042

Table 3.1: Mean Ct values of *GAPDH*, *BLyS* and *IFNα* genes, at each cDNA dilution (from 100ng to 0.16ng). Each sample was loaded in triplicate. St.Dev.=Standard Deviation.

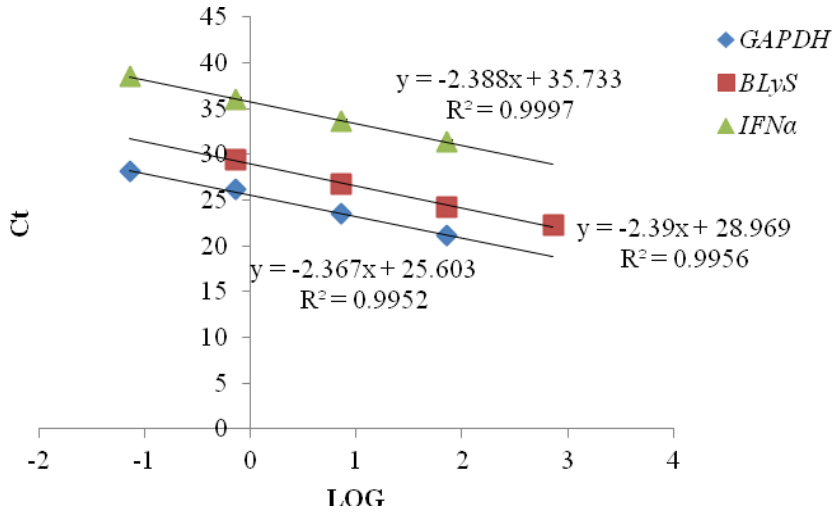


Figure 3.2: Efficiency curves of *GAPDH* (blue), *BLyS* (red) and *IFNα* (green) genes. For each gene the equation of linear regression is indicated. R^2 = coefficient of determination. Ct = Threshold Cycle. LOG = log(base5) of cDNA quantity.

Coefficient of determination values are near 1 and indicate that the line derived from points is similar to the line derived from linear regression. The efficiency for each gene is indicated in the expressions below. E is the efficiency, E% is the percentage of efficiency.

GAPDH: $E = 5^{-(1/-2.367)} = 1.97$ $E\% = (1.97-1) \times 100 = 97\%$

BLyS: $E = 5^{-(1/-2.390)} = 1.96$ $E\% = (1.96-1) \times 100 = 96\%$

IFNα: $E = 5^{-(1/-2.388)} = 1.96$ $E\% = (1.96-1) \times 100 = 96\%$

All efficiencies are similar and comprised between 95% and 105%. Efficiency curves performed with 200nM primers (data not shown) did not satisfy efficiency criteria. Real-time PCR performed afterwards were obtained using 100nM primers and 25ng cDNA.

3.1.3 E2 treatment: *BLyS* gene expression

U937 cells treated with E2 at different doses were evaluated for *BLyS* gene expression. *BLyS* mRNA was firstly evaluated in untreated cells at different time points. Cells used for seeding were considered as internal control, and relative fold change = 1 was attributed to them. There were no statistical differences in *BLyS* mRNA between U937 cells treated with vehicle EtOH 0.01% during time, compared to cells at time zero (Table 3.2): vehicle (EtOH) treated cells could be considered as internal-time control for cells treated with E2 at scalar doses. No statistically significant *BLyS* mRNA modulation was observed at each time point and at each E2-dose administration (Figure 3.3). Also high E2 doses (100nM and 10nM) did not modulate gene expression within 48 hours.

	U937 + EtOH 0.01%	
	***BLyS* Fold Change**	***p***
6 hours	0.71 (0.39 – 1.28)	*n.s*
24 hours	0.99 (0.68 – 1.42)	*n.s*
48 hours	0.85 (0.54 – 1.34)	*n.s*

Table 3.2: Mean (min – max) *BLyS* fold change of U937 cells treated with EtOH 0.01% at different time points. U937 at time zero were considered as internal control with fold change equal to 1.

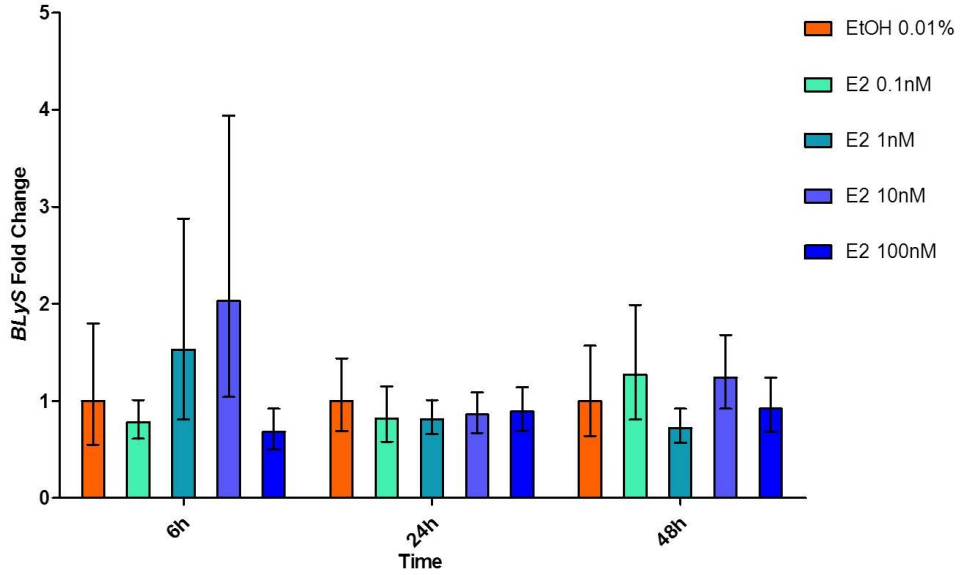
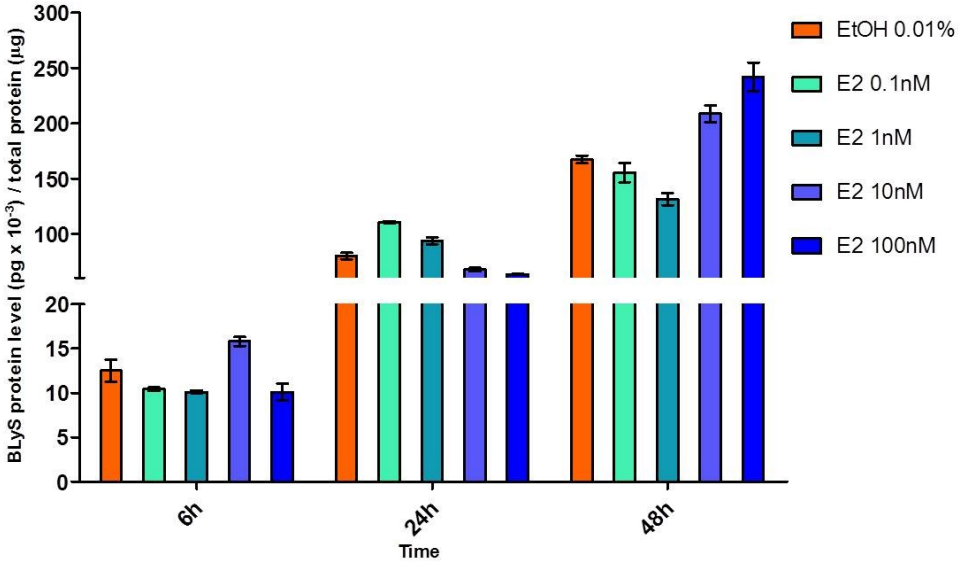


Figure 3.3: Mean (min-max) fold changes of *BLyS* mRNA expression in U937 cells after 6, 24 and 48 hours of E2 treatment. Fold change = 1 was attributed, for each time point, to U937 cells treated with EtOH 0.01% (orange), considered as controls.

3.1.4 E2 treatment: BLyS protein level

Normalized BLyS protein levels (pg/µg total protein) in cell supernatants after E2 treatment is visualized in Figure 3.4. BLyS protein increased during time of treatment (F_T), both in untreated and E2-treated cells. The dose of treatment poorly influences BLyS release in culture medium during time (F_{GxT}). E2 treatment induced a significant increase in BLyS protein release at the highest doses (100nM and 10nM), while 1nM dose influenced in a negative way BLyS releasing by monocyte cells (F_G).



	F_G	*p*	F_T	*p*	F_{GxT}	*p*
E2 0.1nM	8.823	n.s.	1195.735	*0.001****	26.053	*0.036**
E2 1nM	167.003	*0.006***	1203.654	*0.001****	40.200	*0.024**
E2 10nM	61.474	*0.016**	1768.765	*0.001****	43.010	*0.022**
E2 100nM	41.215	*0.023**	1119.304	*0.001****	69.158	*0.014**

Figure 3.4: Mean (± St.Dev.) BLyS protein release, normalized for total proteins, in U937 monocytes after E2 treatment. The table shows the output of the multivariate analysis. F_G = E2 treatment; F_T = Time; F_{GxT} = E2 treatment x Time. *p<0.05; **p<0.01; ***p≤0.001

3.1.5 E2 treatment: *IFNα* gene expression

E2 treatment did not induce *BLyS* mRNA modulation (paragraph 3.1.3), but it could be interesting to explore whether E2 could modulate *IFNα* mRNA within 48 hours. The RNA samples analyzed for *BLyS* mRNA were also evaluated for *IFNα* qPCR. First of all it was looked at *IFNα* expression by untreated cells during time (Table 3.3).

	U937 + EtOH 0.01%	
	***IFNα* Fold Change**	***p***
6 hours	0.61 (0.33 – 1.14)	*n.s.*
24 hours	0.80 (0.60 – 1.06)	*n.s.*
48 hours	0.16 (0.09 – 0.28)	*0.001***

Table 3.3: Mean (min – max) *IFNα* fold change of U937 untreated cells at different time points. U937 at time zero were considered as internal control with fold change = 1. ***p=0.001

After 48 hours, untreated cells showed a relevant statistically significant down-regulation of *IFNα* mRNA expression. E2 treatment induced *IFNα* up-regulation at each dose and at each time point. The highest increase was noticed at 48 hours, where control cells expressed the lowest basal levels of target gene. The physiological E2 concentrations (1nM and 10nM) induced a time-dependent increase in *IFNα* mRNA expression. The other E2 concentrations induced gene expression fluctuations during time, particularly at 24 hours, where it seemed to be a down-regulation of *IFNα* expression (Figure 3.5).

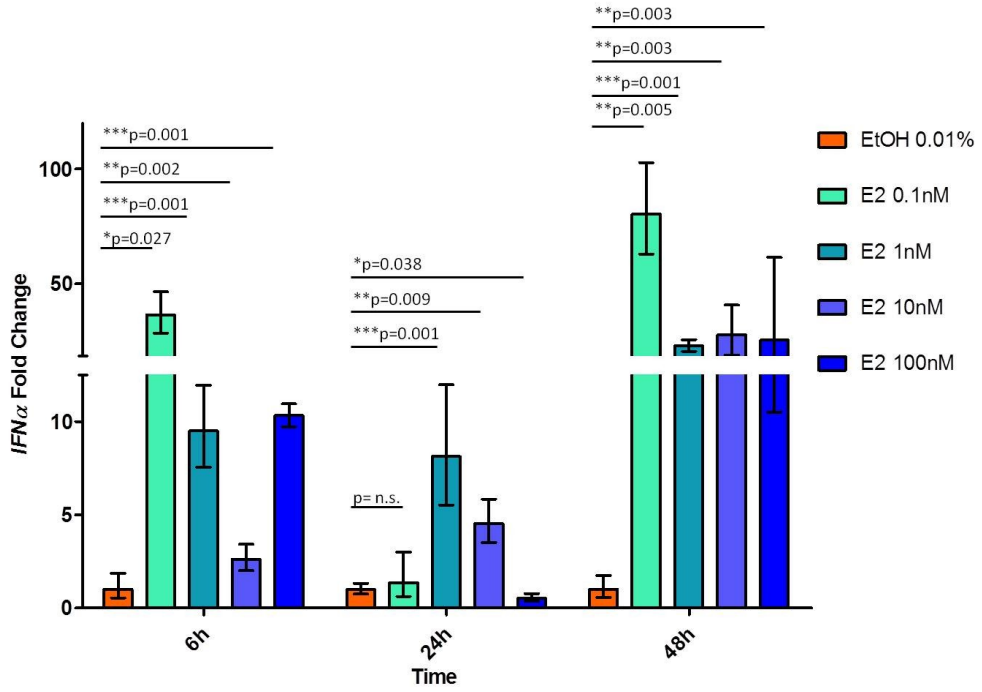


Figure 3.5: Mean (min-max) fold changes of *IFNα* mRNA expression in U937 cells after 6, 24 and 48 hours of E2 treatment. Fold change = 1 was attributed, for each time point, to U937 cells treated with EtOH 0.01% (purple), considered as controls. *p<0.05 **p<0.01 ***p<0.001

3.1.6 hr-IFNα treatment: cell growth

U937 cell growth was evaluated after hr-IFNα administration: starting from 48 hours, cell stopped dividing, and the difference with untreated cells was statistically significant (Figure 3.6). Cell growth retardation was not due to cell apoptosis, but to cell division arrest.

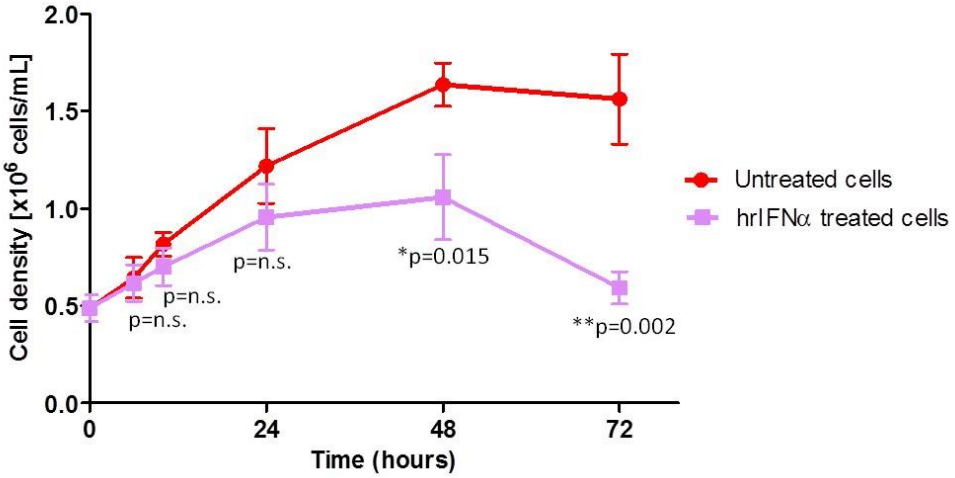


Figure 3.6: U937 cell density of untreated (red) and hr-IFNα-treated (violet) cells. Error bars represent standard deviations. *p<0.05 **p<0.01

3.1.7 hr-IFNα treatment: *BLyS* gene expression

IFNα treatment was performed to evaluate *BLyS* mRNA modulation. As in E2 treatment, U937 control cells (untreated), were considered as for basal *BLyS* gene expression at each time points (Table 3.4).

	U937	
	***BLyS* Fold Change**	***p***
6 hours	0.96 (0.77 – 1.20)	*n.s.*
10 hours	1.00 (0.82 – 1.23)	*n.s.*
24 hours	1.43 (1.32 – 1.56)	*0.002***
48 hours	1.35 (1.03 – 1.77)	*0.004***

Table 3.4: Mean (min – max) *BLyS* fold change of U937 untreated cells at different time points. U937 at time zero were considered as internal control with fold change = 1. **p<0.01

Starting from 24 hours, untreated cells slightly increased *BLyS* mRNA expression, and the difference compared with time zero was statistically significant.

hr-IFNα treatment induced moderate significant *BLyS* up-regulation starting from 10 hours, and the modulation is maintained during time (Figure 3.7).

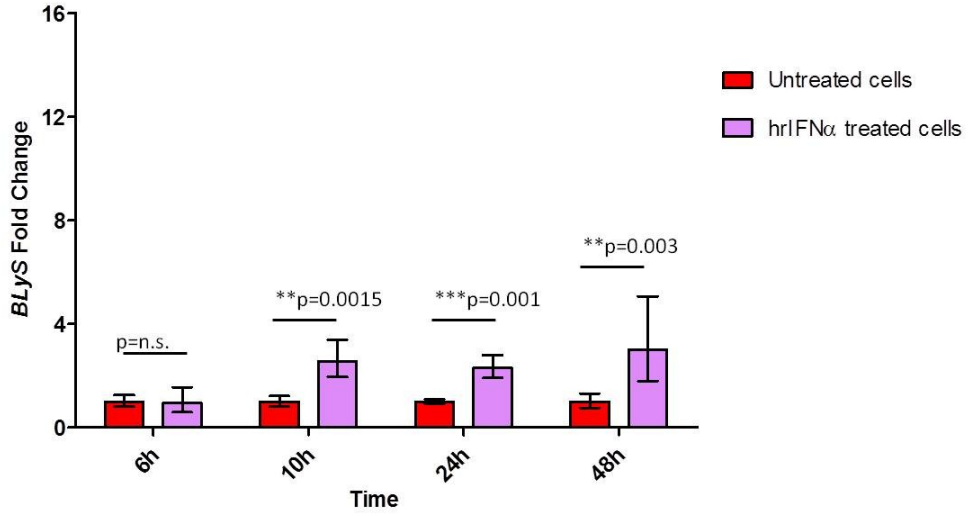


Figure 3.7: Mean (min-max) fold changes of *BLyS* mRNA expression in U937 cells after 6, 10, 24 and 48 hours of hr-IFNα treatment. Fold change = 1 was attributed, for each time point, to U937 untreated cells (red), considered as controls.**p<0.01 ***p=0.001

3.1.8 hr-IFNα treatment: BLyS protein level

Normalized BLyS protein levels (pg/µg total protein) in cell supernatants after IFNα treatment is visualized in Figure 3.8. BLyS protein significantly accumulated during time of treatment (F_T), both in untreated and IFNα-treated cells. The dose of treatment did not influenced BLyS release in medium (F_G), and the treatment did not modify significantly BLyS protein release during time (F_{GxT}).

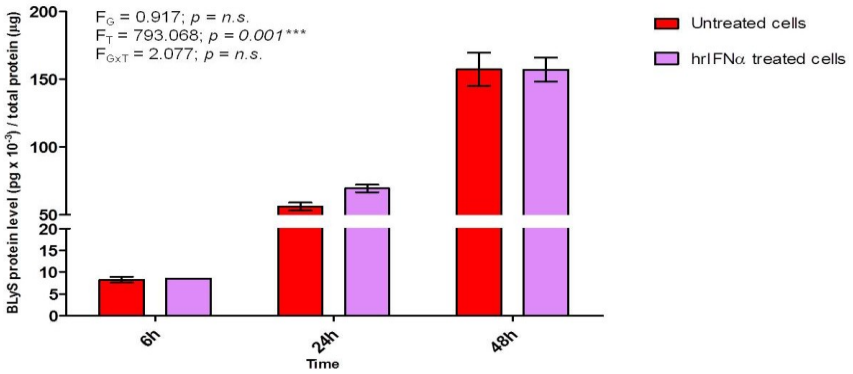


Figure 3.8: Mean (± St.Dev.) BLyS protein release normalized for total proteins in U937 monocytes after IFNα treatment. The table shows the output of the multivariate analysis. F_G = hr-IFNα treatment; F_T = Time; F_{GxT} = hr-IFNα treatment x Time. ***p≤0.001

3.2 U937-derived macrophages

3.2.1 PMA treatment: *BLyS* gene expression

PMA induces cell differentiation from U937 monocytes to macrophage-like cells. PMA treatment blocks cell proliferation, cells changed morphology, but do not increase in number. After medium replacement, macrophage-derived cells undergo apoptosis after 48-72 hours: qPCR experiments were performed within this time lapse. *BLyS* gene basal expression, after PMA differentiation, was not different from that of monocytic untreated cells (Figure 3.9).

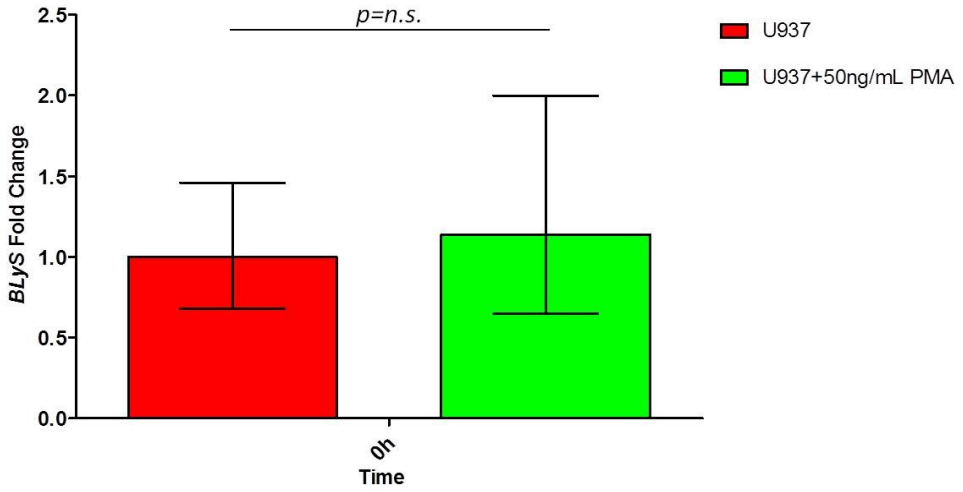


Figure 3.9: Mean (min – max) *BLyS* fold change of U937 untreated and PMA-treated cells.

3.2.2 Primer efficiency curves

Before evaluating target genes expression, primer's efficiency was performed in U937-derived macrophages cell line. Table 3.5 shows mean Ct values of *GAPDH*, *BLyS* and *IFNα* genes for each cDNA dilution (from 100ng to 0.16ng), using 100nM primer concentration. LOG represents the log(base5) of cDNA serial dilution. Extreme values, that did not interpolate in a good way the curve, were excluded. Figure 3.10 shows reference and target genes trends, with the corresponding linear regression equation and the coefficient of determination (R^2).

ng	LOG	*GAPDH*	St.Dev.	*BLyS*	St.Dev.	*IFNα*	St.Dev.
100	2.861353			23.43	0.295		
20	1.861353	22.58	0.262	25.09	0.135	29.32	0.063
4	0.861353	24.93	0.203	27.46	0.231	31.56	0.228
0.8	-0.13865	27.34	0.007	30.23	0.194	33.80	0.211
0.16	-1.13865	29.70	0.158			36.20	0.127

Table 3.5: Ct mean values of *GAPDH*, *BLyS* and *IFNα* genes, for each cDNA dilution (from 100ng to 0.16ng). Each sample was loaded in triplicate. St.Dev.=Standard Deviation.

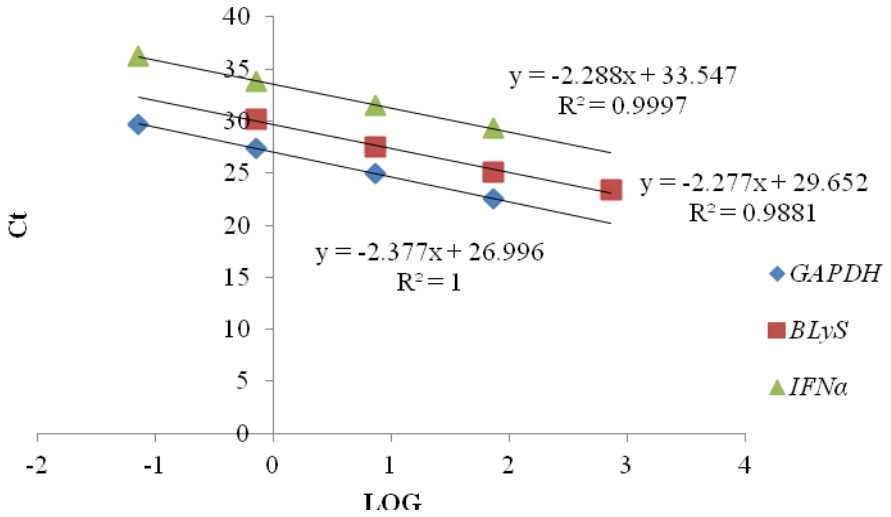


Figure 3.10: Efficiency curves of *GAPDH* (blue), *BLyS* (red) and *IFNα* (green) genes. For each gene linear regression is indicated. R^2 = coefficient of determination. Ct = Threshold Cycle. LOG = log(base5) of cDNA quantity.

Coefficient of determination values are near or equal to 1 and indicate that the line derived from points is similar to the line derived from linear regression. The efficiency for each gene is indicated in the expressions below. E is the efficiency, E% is the percentage of efficiency.

GAPDH: $E = 5^{-(1/-2.377)} = 1.97$ $E\% = (1.97-1) \times 100 = 97\%$

BLyS: $E = 5^{-(1/-2.277)} = 2.03$ $E\% = (2.03-1) \times 100 = 103\%$

IFNα: $E = 5^{-(1/-2.288)} = 2.05$ $E\% = (2.05-1) \times 100 = 105\%$

All primers efficiencies are included between 95% and 105%. Efficiency curves performed with 200nM primers (data not shown) did not satisfy efficiency criteria. Real-time PCR performed afterwards were obtained using 100nM primers and 25ng cDNA, as in U937 monocytes.

3.2.3 E2 treatment: *BLyS* gene expression

U937-derived macrophages treated with E2 at different doses were evaluated for *BLyS* gene expression. *BLyS* mRNA expression was firstly evaluated for untreated cells at different time points. Cells after 72 hours induction with PMA were considered as internal control, and relative fold change equal to 1 was attributed to them. There was no statistical difference between U937-derived macrophages treated with EtOH 0.01% compared with cells at time zero (Table 3.6): EtOH-treated cells could be considered as internal-time control samples for cells treated with scalar concentrations of E2. No statistically significant *BLyS* mRNA modulation was found at each time point and at each E2-dose administration (Figure 3.11). At 6 hours, 1nM E2 induced a slight *BLyS* down-regulation, but $p<0.05$ value was not considered significant. Also high E2 doses (100nM and 10nM) did not modulate gene expression within 48 hours.

	U937 + PMA + EtOH 0.01%	
	***BLyS* Fold Change**	***p***
6 hours	0.63 (0.45 - 0.89)	*n.s.*
24 hours	0.97 (0.78 - 1.20)	*n.s.*
48 hours	0.82 (0.69 - 0.97)	*n.s.*

Table 3.6: Mean (min – max) *BLyS* fold change of U937-derived macrophages treated with EtOH 0.01% at different time points. U937 at time zero were considered as controls with fold change equal to 1.

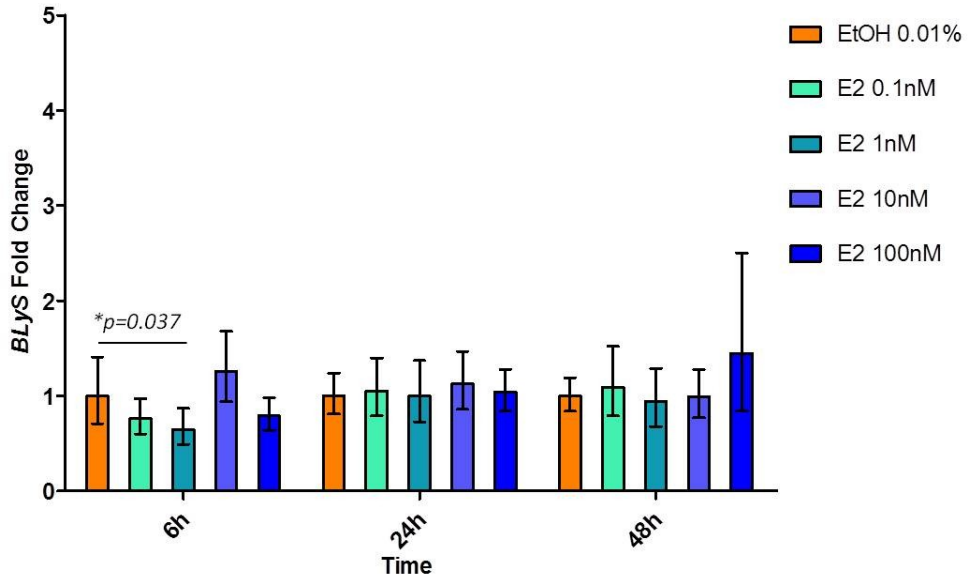
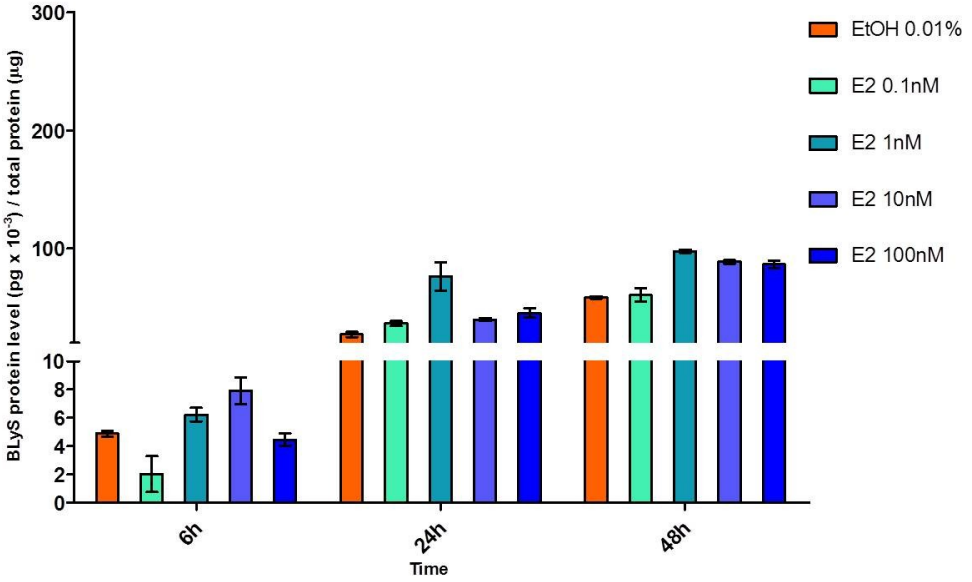


Figure 3.11: Mean (min-max) fold changes of *BLyS* mRNA expression in U937-derived macrophages after 6, 24 and 48 hours of E2 treatment. Fold change = 1 was attributed, for each time point, to U937-derived macrophages treated with EtOH 0.01% (orange), considered as controls. *p<0.05.

3.2.4 E2 treatment: BLyS protein level

Normalized BLyS protein levels (pg/µg total protein) in U937-derived macrophage supernatants after E2 treatment is visualized in Figure 3.12. BLyS protein accumulated during time of treatment (F_T), both in untreated and E2-treated cells. The dose of treatment poorly influenced BLyS release during time (F_{GxT}). E2 treatment induced a significant increase in BLyS release starting from 1nM (F_G).



	F_G	*p*	F_T	*p*	F_{GxT}	*p*
E2 0.1nM	8.670	n.s.	314.100	*0.003***	3.751	n.s.
E2 1nM	121.489	*0.008***	198.657	*0.005***	23.709	*0.040**
E2 10nM	356.646	*0.003***	2420.824	*0.000****	101.036	*0.010***
E2 100nM	59.919	*0.016**	1782.369	*0.001****	81.302	*0.012**

Figure 3.12: Mean (± St.Dev.) BLyS protein release, normalized for total proteins, in U937-derived macrophages after E2 treatment. The table shows the output of the multivariate statistical analysis. F_G = E2 treatment; F_T = Time; F_{GxT} = E2 treatment x Time. *p<0.05; **p<0.01; ***p≤0.001

3.2.5 E2 treatment: *IFNα* gene expression

As for U937 monocytes, *IFNα* gene expression after E2 treatment was investigated. The same RNA samples of *BLyS* mRNA evaluation were used for *IFNα* qPCR. First of all, it was looked at *IFNα* expression by control cells during time (Table 3.7).

	U937 + PMA + EtOH 0.01%	
	***IFNα* Fold Change**	***p***
6 hours	1.25 (1.04 - 1.51)	*n.s.*
24 hours	2.30 (1.42 - 3.72)	*n.s.*
48 hours	1.61 (0.93 - 2.80)	*n.s.*

Table 3.7: Mean (min – max) *IFNα* fold change of untreated U937-derived macrophages at different time points. U937-derived macrophages after 72 hours PMA induction were considered as internal control with fold change = 1.

Untreated derived-macrophages did not showed any *IFNα* mRNA modulation during time. E2 treatment induced a statistically significant *IFNα* down-regulation at 24 hours for the doses 10nM and 100nM. Apart from the dosage 100nM, all the other E2 doses induced a time-dependent decrease of *IFNα* mRNA expression (Figure 3.13).

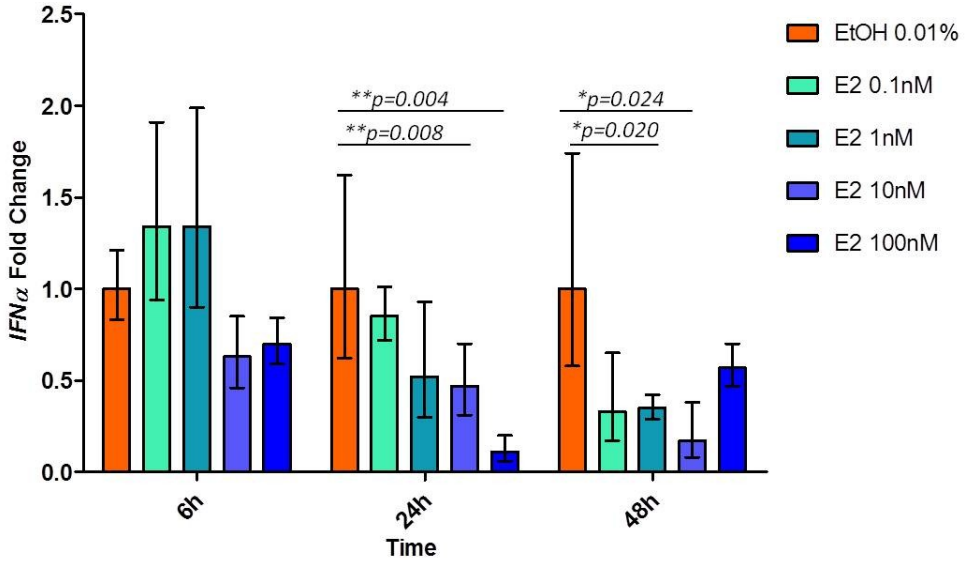


Figure 3.13: Mean (min-max) fold changes of *IFNα* mRNA expression in U937-derived macrophages after 6, 24 and 48 hours of E2 treatment. Fold change = 1 was attributed, for each time point, to U937-derived macrophages treated with EtOH 0.01% (orange), considered as controls. *p<0.05 **p<0.01

3.2.6 hr-IFNα treatment: U937-derived macrophages morphology

During hr-IFNα treatment, U937-derived macrophages underwent apoptosis after 24 hours (Figure 3.14 B), confirming the *in vitro* IFNα anti-proliferative effect. RNA samples derived from 24 and 48 hours were not affected in quantity and quality by this process.

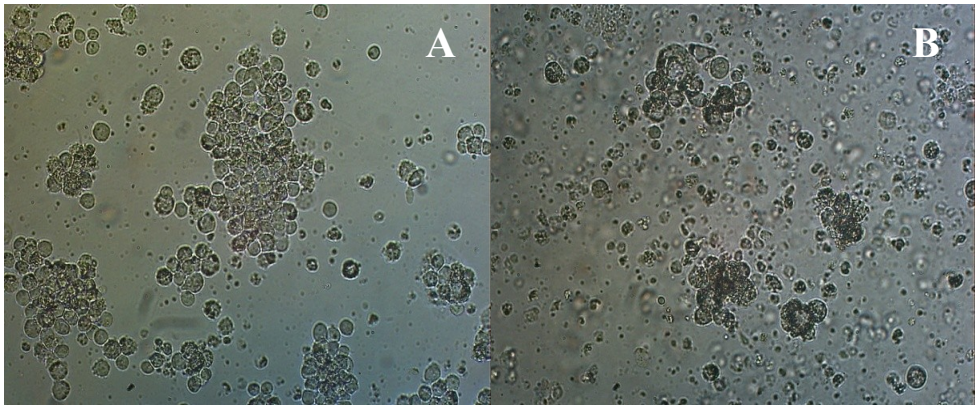


Figure 3.14: Untreated (A) and hr-IFNα treated (B) U937-derived macrophages at phase contrast microscope. Magnification 200x.

3.2.7 hr-IFNα treatment: *BLyS* gene expression

hr-IFNα treatment was performed to evaluate *BLyS* mRNA modulation in macrophage-derived cells. As in E2 treatment, untreated U937-derived macrophages were evaluated for basal *BLyS* gene expression at each time points (Table 3.8).

	U937-derived macrophages	
	***BLyS* Fold Change**	***p***
6 hours	0.57 (0.39 - 0.83)	*n.s.*
10 hours	1.67 (1.17 - 2.39)	*n.s.*
24 hours	0.69 (0.50 - 0.97)	*n.s.*
48 hours	0.58 (0.45 - 0.74)	*0.029**

Table 3.8: Mean (min – max) *BLyS* fold change of untreated U937-derived macrophages at different time points. U937-derived macrophages after 72 hours PMA-induction were considered as internal control with fold change = 1. *p<0.05

At 48 hours, untreated cells slightly decreased *BLyS* mRNA expression. hr-IFNα treatment induced a highly significant *BLyS* up-regulation starting from 6 hours, then the up-regulation decreased, but it was maintained higher than controls during the observed time (Figure 3.15).

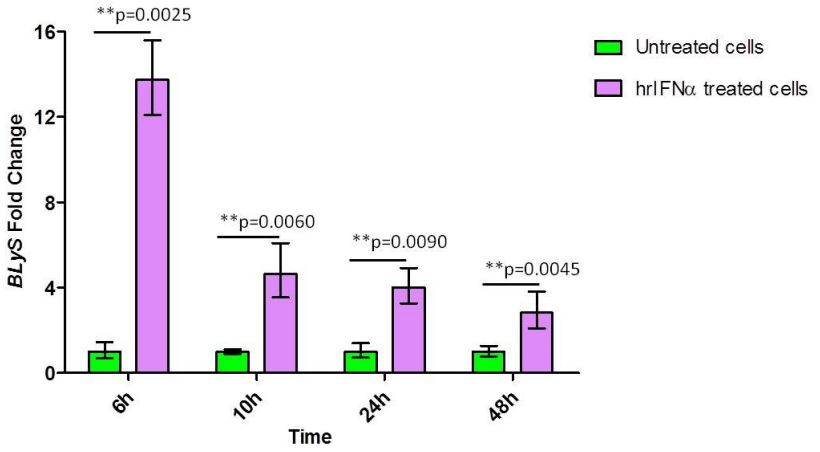


Figure 3.15: Mean (min-max) fold changes of *BLyS* mRNA expression in U937-derived macrophages after 6, 10, 24 and 48 hours of hr-IFNα treatment. Fold change = 1 was attributed, for each time point, to untreated U937-derived macrophages (purple), considered as controls.**p<0.01

3.2.8 hr-IFNα treatment: BLyS protein level

Normalized BLyS protein levels (pg/µg total protein) in cell supernatants after IFNα treatment in U937-derived macrophages is visualized in Figure 3.16. BLyS protein significantly accumulated during time of treatment (F_T), both in untreated and IFNα-treated cells. The dose of treatment did not influenced BLyS release in medium (F_G), but the treatment during time significantly decreased BLyS protein release (F_{GxT}).

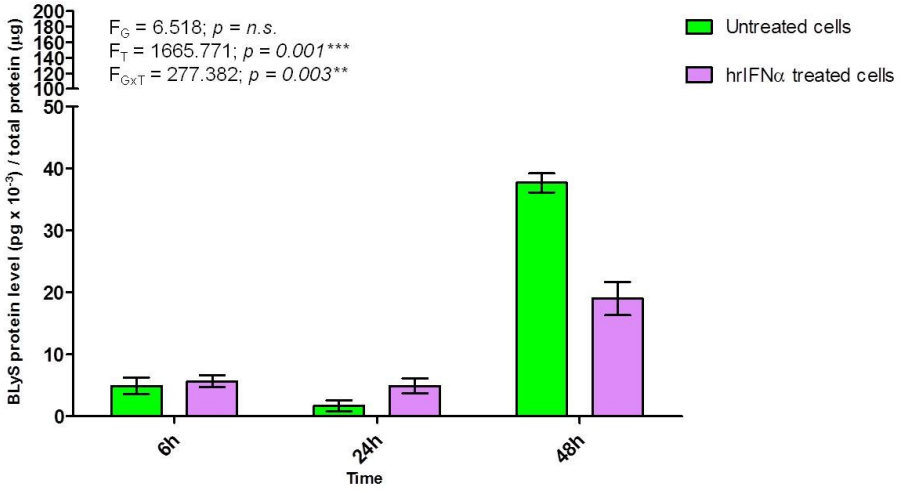


Figure 3.16: Mean (± St.Dev.) BLyS protein release normalized for total proteins in U937-derived macrophages after IFNα treatment. The table shows the output of the multivariate analysis. F_G = hr-IFNα treatment; F_T = Time; F_{GxT} = hr-IFNα treatment x Time. **p<0.01; ***p≤0.001

DISCUSSION
&
CONCLUSIONS

Hormones are important modulators of the immune response, and in autoimmune diseases, such as SLE, women are more affected than men, suggesting a pivotal role of estrogens in the pathogenesis of the disease. During pregnancy, the increase of estrogen levels induces a flare of the disease, and affects the production of pro- and anti-inflammatory cytokines: indeed, steroid hormone and cytokine profiles differ in SLE pregnant patients compared to healthy pregnant subjects [5,28,78]. In this study, it was evaluated the *in vitro* effects of E2 administration on *BLyS* and *IFNα* expression in a human myeloid cell line, as a model of E2-induced effects on key cytokines in the pathogenesis of SLE and other B-cell mediated autoimmune diseases.

The results suggest that in human U937 monocyte macrophage-like cells, a single pulse of E2 for 48 hours did not apparently affect *BLyS* gene expression, also at the highest E2 supra-physiological concentration used (100mM), even if some studies suggest that E2 is capable of inducing *BLyS* expression [18,79]. Very recently, it was reported that E2-induced *BLyS* up-regulation is higher in leukocytes derived from women than in leukocytes from men [79]. U937 as well as other monocytic cell lines, traditionally used as a model of macrophage function, derives from male myeloblastic tumor cells [80]. Otherwise, it has been established that U937 cells express both types of ERs, α and β, although their expression is different between monocytes and macrophages: ERα is prevalent in U937-derived macrophages, while ERβ is expressed mainly on U937 monocytes [81]. Of note, the timing of E2 treatment could influence *BLyS* expression: a work by Calippe *et al.* demonstrated that *in vitro* short-term E2 administration in LPS-activated macrophages does not predict the *in vivo* long-term effect of E2 in inducing mRNA expression of target cytokines [82]. Thus, these considerations could explain the discrepancy between the present study and literature data.

Besides no apparent effects of E2 on *BLyS* gene expression and *de-novo* protein synthesis, in the present study it has been found that E2 at higher doses induces BLyS protein mobilization and release from both monocytes and PMA-derived macrophages, as demonstrated by higher BLyS levels in supernatants from E2-treated cells. Even if BLyS protein level at highest E2 doses is significantly increased in treated than in untreated cells during time, the difference is not so evident. A recent study from our group indicated that the acute increase

of E2 during controlled ovarian stimulation for infertility treatment, does not induce a significant increase in BLyS circulating levels [83]. Conversely, in the experiments performed by Bassi *et al.* [12], the constant daily E2 administration increased BLyS protein levels in lupus-prone mice. These evidences regarding protein levels, in agreement with the findings by Calippe *et al.* [82] at transcription level, suggest that perhaps, a single pulse *in vitro* or a short-term *in vivo* E2 treatment protocol could be necessary but not sufficient to induce BLyS transcription, translation or mobilization.

Regarding E2 effects on *IFNα* gene expression, this study showed that E2 treatment exerts an effect to some extent, which however seems apparently different in monocytes compared to macrophages, suggesting that E2 can evoke distinct gene responses in these two types of cells. E2-induced *IFNα* gene up-regulation was confirmed in monocytes but not in derived macrophages. Looking again at the work by Mor *et al.*, differences in ERs cell type distribution in monocytes and macrophages can induce different and opposite responses to E2 treatment [81]. These findings could also be explained, at least in part, by the recent observation that estrogen-induced *IFNα* up-regulation in primary macrophages is reverted when estrogen is administered after virus infection [84]. Thus, in this *in vitro* model, PMA-induced differentiation before E2 treatment could probably reverse *IFNα* gene response.

Since *IFNα* is modulated by E2, it was investigated whether exogenous IFNα administration could modulate BLyS gene and protein expression. As expected, it was found a strong *BLyS* gene up-regulation, higher in derived-macrophages than in monocytes, probably due to PMA differentiation. Panchanathan *et al.* also demonstrated the IFNα-induced up-regulation of *BAFF* in mouse macrophage cells [18]. Supporting this evidence, there are two other studies, in which exogenously or endogenously induced anti-IFNα antibodies can inhibit both *BLyS* transcription and protein release by blocking IFN signaling: anti-IFN drugs can neutralize over-expression of IFN-inducible genes, such as BLyS, in SLE patients [85,86]. Regarding BLyS protein release after IFNα treatment, these data are not in accordance with those of mRNA expression. BLyS is a trans-membrane protein that can become soluble only after specific furin-cleavage from cell surface. The work by Lopez *et al.*, suggested that, in SLE patients, the release of BLyS from

primary monocytes after IFNα treatment, depends on patient's anti-dsDNA titers and disease activity, suggesting that a peculiar cytokine milieu and/or cell activation could affect BLyS mobilization [74]. Probably, monocytes, and even more macrophages, are induced to express *BLyS* after IFNα induction, but they did not receive any particular stimuli able of promoting the cleavage of membrane-bound protein or the release of the intracellular one.

Taken together all these findings suggest a possible pathway of E2-induced BLyS overexpression in myeloid cells, in which *IFNα* could be an essential mediator. Panchanathan *et al.* proposed that E2 and IFNα could independently induce *BLyS* by acting on a lupus susceptibility modifier protein, named p202, that is up-regulated both by ERα and IFNα [18]. However, this work did not take into account the well-known type I IFN gene activation by E2-ER signaling, that is also been recently described by Dong *et al.* [54]. Another recent paper by Gomez *et al.* [87], put in a causal relationship, in an *ex vivo* study, these three components, highlighting the role of IFNα in inducing BLyS in monocytes, and notably reporting that BLyS secretion has sex-related differences: monocytes from women produced higher basal BLyS levels compared with men, thus confirming a role for estrogen in determining BLyS secretion.

Eventhough this work gives a contribution to elucidating estrogen immunomodulation, there are some limitations concerning the application of the same study design to primary monocyte-macrophage cells from sex-matched healthy subjects and SLE patients. Ex-vivo studies on primary peripheral cell populations could have better explained physiopathological response to E2 or IFNα stimuli; however, a great heterogeneity related to primary cell culture assessment and patient's clinical and treatment characteristics would have been expected. This work tried to propose a simple *in vitro* model of studying estrogen immunomodulatory effects on cytokine's expression in human cell cultures, in order to approach the studies on primary cells in a more standardized and reproducible manner.

In conclusion, cytokines, such as IFNα and BLyS, are key modulators of innate and adaptive immune response, but such responses are exacerbated or attenuated by sex hormones, which have a pivotal role in controlling physiological immunity. In particular, sex hormones are implicated in the pathogenesis of B-cell mediated autoimmune diseases, including SLE.

This study shows that E2 can modulate *IFNα*, and indirectly up-regulate *BLyS* gene expression, suggesting that estrogens could primarily have a role in the modulation of innate immunity, with different effects depending on target cell phenotype and milieu, as observed by comparing monocytes with derived-macrophages. Estrogen effects on *BLyS* up-regulation are probably mediated by IFNα, cytokine of the innate immune response that enhances adaptive immune defenses. This study could represent a model of E2-induced adaptive immune response activation, thus confirming the interrelationship between innate and adaptive immunity in immune system modulation, and try to give a contribution to the elucidation of hormone-induced immune dysregulation in B-cell mediated-systemic autoimmunity.

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