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Abstract

Multiple studies have demonstrated that estrogen (17β Estradiol) affects innate and adaptive immune systems. Its role as protective factor has been implicated in autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus. Estrogen exerts its action on immune cells using estrogen nuclear receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). T helper (Th) cells are key regulators of the adaptive immune system. However, the mechanism how estrogen regulates T helper subset cells is still uncertain. To study the effect of estrogen and molecular mechanism, I silenced the estrogen alpha receptor gene that is predominantly expressed in T cells, by using siRNA approach. I observed that knock down of ERα is affecting Th1 by elevating T-bet, Th2 by increasing GATA3 and Treg by reducing Foxp3 levels. The outcome of this thesis will help in understanding pathophysiological effects of T helper cells and also the role of estrogen receptors in T cells and their effects in cancer immunity and autoimmunity.

Keywords: Estrogen receptor alpha, T helper cells, T helper subsets, Autoimmunity, Estrogen (17β Estradiol).

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Abbreviations

AP-1, activator protein 1

APC, antigen-presenting cell

CD, cluster of differentiation

CREB, cyclic adenosine monophosphate responsive element binding protein

E2, 17β-Estradiol

EAE, experimental autoimmune encephalomyelitis

ESR1, Estrogen alpha receptor

ESR2, Estrogen beta receptor

FOXP3, forkhead box P3

GATA3, GATA binding protein 3

IBD, inflammatory bowel disease

IFN, interferon

Ig, immunoglobulin

IL, interleukin

ILR, IL receptor

iTreg, inducible regulatory T cell

JAK, Janus kinase

JNK, JUN N-terminal kinase

MAPK, mitogen-activated protein kinase

nTreg, natural regulatory T cell

PAGE, polyacrylamide gel electrophoresis

PCR, polymerase chain reaction

PI3K, phosphoinositide-3-kinase

PKC, protein kinase C

RA, rheumatoid arthritis

RORC, RAR-related orphan receptor C

RT, reverse transcriptase

1. Introduction

Estrogen hormone plays an important role in sexual development and reproduction; it is also involved in cell growth, development and differentiation. 17β Estradiol (E2) is the most abundant and potent form of mammalian circulating estrogen that is primarily synthesized in ovaries and placenta. Estrogen affects the target tissues through the interaction with nuclear steroid receptors and also with membrane receptors (Dahlman-Wright et al., 2006) (Figure 1). The physiological effect of E2 is mediated by two specific intracellular nuclear receptors- Estrogen receptor α (ERα, *ESR1*) and Estrogen receptor β (ERβ, *ESR2*) (Kuiper et al., 1996). ERs are ligand–activated transcription factors. The ERα and ERβ are products of the genes *ESR1* and *ESR2*. In humans, *ESR1* is 140 kb long (Ponglikitmongkol et al., 1988) having 8 exons with gene location on chromosome 6q25.1 and the size of product is 595 amino acids with molecular size of 66 kDa. In mouse, the location of the gene was mapped on chromosome 10 (Justice et al., 1990) encoding a protein containing 599 amino acids as protein.

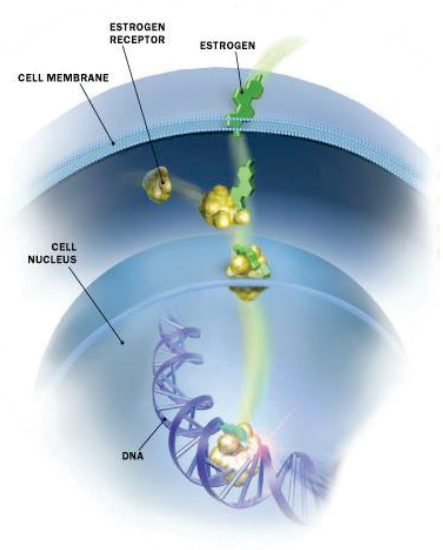


Figure 1: Estrogen signaling in a cell. Estrogen hormone stimulates the estrogen receptor dimer formation. Then estrogen receptor translocates into the nucleus to mediate the regulation of gene expression at transcription level (Illustration by Pam Curry, Adapted from Cure's Illustrated Guide to Cancer book).

1.1 ER α domains

The ER sequence contains 5 functional units (A/B, C, D, E and F) (Figure 2). Among these, the three independent but interacting functional domains are NH2–terminal transcription AF-1 (Activation function-1) domain (A/B), DNA-binding domain (C) and ligand-binding domain containing ligand-dependent transcriptional AF2 (Activation function-2) domain (E). Other structural domains are flexible hinge region (D) and carboxyl terminus (F)(Ascenzi et al., 2006).

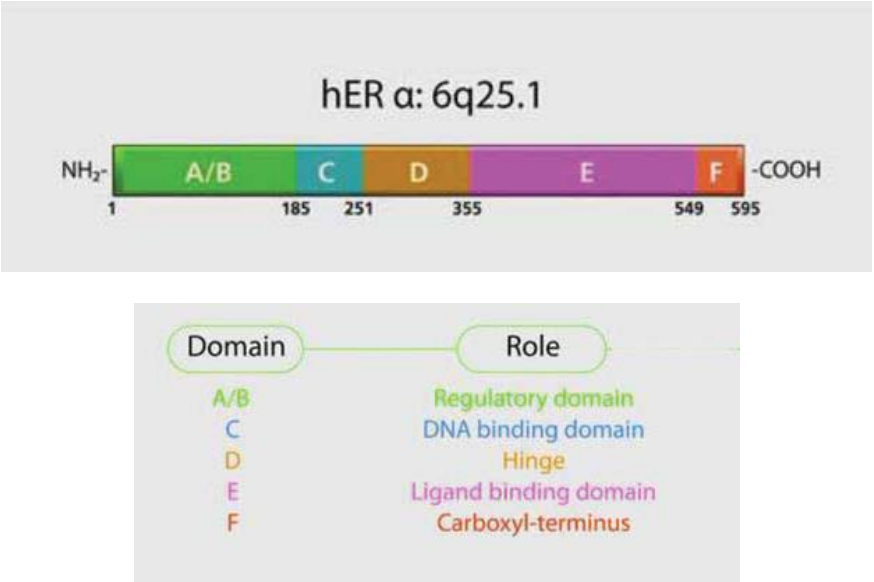


Figure 2: Functional units of Estrogen Receptor protein. Estrogen receptor consists of 595 amino acids in the form 5 subunits (Charitidi et al., 2009, With permission from Elsevier).

AF1 region mediates the transcriptional activation of target genes by recruiting co-regulatory proteins. DBD region is responsible for sequence specific binding of ER's to specific elements in DNA called estrogen-responsive elements (EREs). LBD initiates the gene transcription only in the presence of bound ligand (Delaunay et al., 2000). It contains ligand binding cavity and also binding sites for co-activator and co-repressor proteins.

1.2 ER α splice variants

ER alpha has been reported to show more than 20 alternative splice variants in breast cancer tissues (Poola et al., 2000). Functionally characterized ERα isoforms are of 66, 46 and 36 kDa (Ascenzi et al., 2006) (Figure 3).

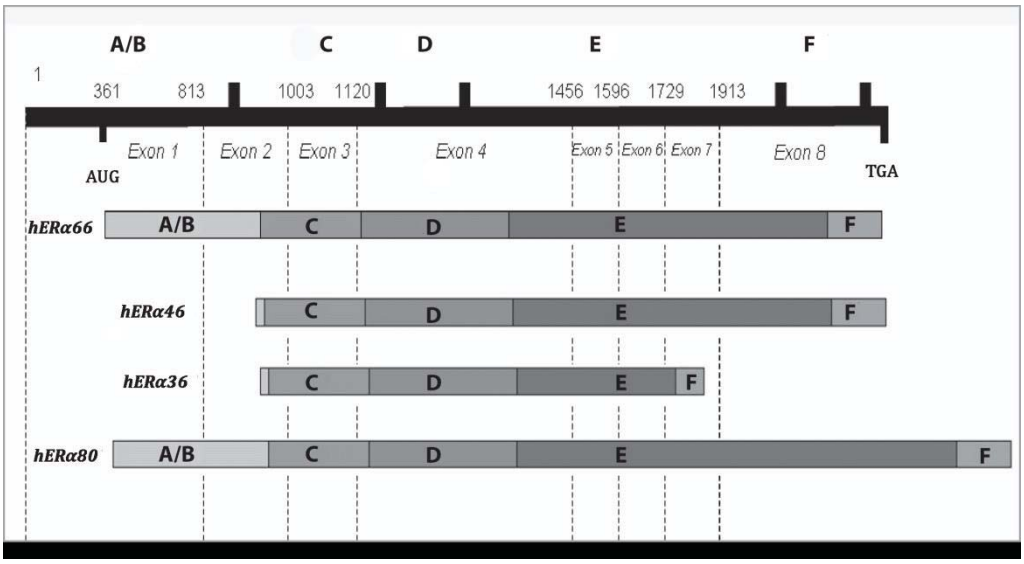


Figure 3: Different splice variants of Estrogen receptor. ERα 80 is full length isoform observed in cancer cells and ERα 66, 46 and 36 are most studied truncated isoforms (Image from Sotoca et al., 2012, Usage under Creative Commons (CC by NC)).

ERα 66 is a well characterized isoform with six domains. Of these, two domains, NH2-terminal A/B domain and COOH-terminal E domain of receptor protein possesses activation function AF-1 and AF-2 respectively. ERα 46 is devoid of 173 amino acids of the ERα 66 protein. It is formed by skipping of exon 1; hence it lacks the AF-1 region (Flouriot et al., 2000). Unlike ERα 66, the ERα 46 isoform does not initiate estradiol dependent cell proliferation. The higher levels of ERα 46 isoform have been correlated with cell cycle arrest in G0/G1 phase that is normally attained during hyper confluent stage of the cells (Penot et al., 2005). ERα 46 acts as potential ligand dependent transcription factor by employing AF-2 domain and antagonize the ERα AF-1 dependent transcription (Flouriot et al., 2000). It also inhibits the estrogenic induction of c-Fos and cyclin D1 promoters that have a role in cell cycle control. ERα 36 is short form of ER α, it lacks both AF1 and AF2 transactivation function domains but retains the DNA binding domain, partial dimerization and ligand binding domains. Without AF1 and AF2, ERα 36 does not show any transcription factor activity. It acts as negative effector of full length ERα and ERβ dependent transactivation (Figtree et al., 2003). This isoform is assumed to show interaction with plasma membrane because of presence of myristoylation sites in the N-terminus of the protein (Wang et al., 2005b). ERα 36 is expressed on plasma membrane and observed to take part in ERα 36 initiated non-genomic signaling such as MAPK/ERK signaling in response to E2.

ERα 80 isoform has been also observed in MCF7:2A cell lines grown in the absence of E2 (Pink et al., 1996). However, the function of this isoform remains to be investigated.

1.3 ER Signaling

1.3.1 Genomic signaling

In classical/genomic dependent signaling, in the absence of estrogen ligand, the estrogen receptors remain inactive in nucleus and cytoplasm of the cell by forming a complex with heat-shock proteins. It is assumed that Hsp90 and Hsp70 associated chaperone machinery prevents the ER from binding to response elements and also retains the capability of ER to bind estrogen ligand with higher affinity. Once the estrogen hormone binds to estrogen receptor, the conformational changes are induced to the receptor. Then ER complex migrates from cytosol of cell to nucleus, it forms a homo dimer and shows high affinity binding to palindromic consensus DNA sequences called estrogen responsive elements (EREs) (AGGTCAnnnTGACCT) (Mason et al., 2010) located within the regulatory region, proximal promoter region and distal enhancer regulatory sites of target genes (Carroll et al., 2006). The DNA/receptor complex then recruits coactivators, corepressors and chromatin-remodeling complexes and regulates the transcriptional activity of the gene (Nilsson et al., 2001).

1.3.2 Non Genomic signaling

Non-genomic regulation is a very rapid process, as it does not involve the estrogen receptors in gene transcription. This mechanism was first observed when ovarectomized rats were administered the E2, an increase in uterine cAMP levels was observed within 15 seconds (Szego and Davis, 1967). It is assumed that the estrogen receptors localized in the plasma membrane and cytoplasm mediate the rapid initiation of intracellular secondary messengers such as Nitric Oxide (NO), Receptor Tyrosine Kinases (RTKs), G protein coupled receptors (GPCRs) and protein kinases such as phosphatidylnositol-3-kinase, serine threonine kinase (AKT) and protein kinases (PKA & PKC, Coleman and Smith, 2001).

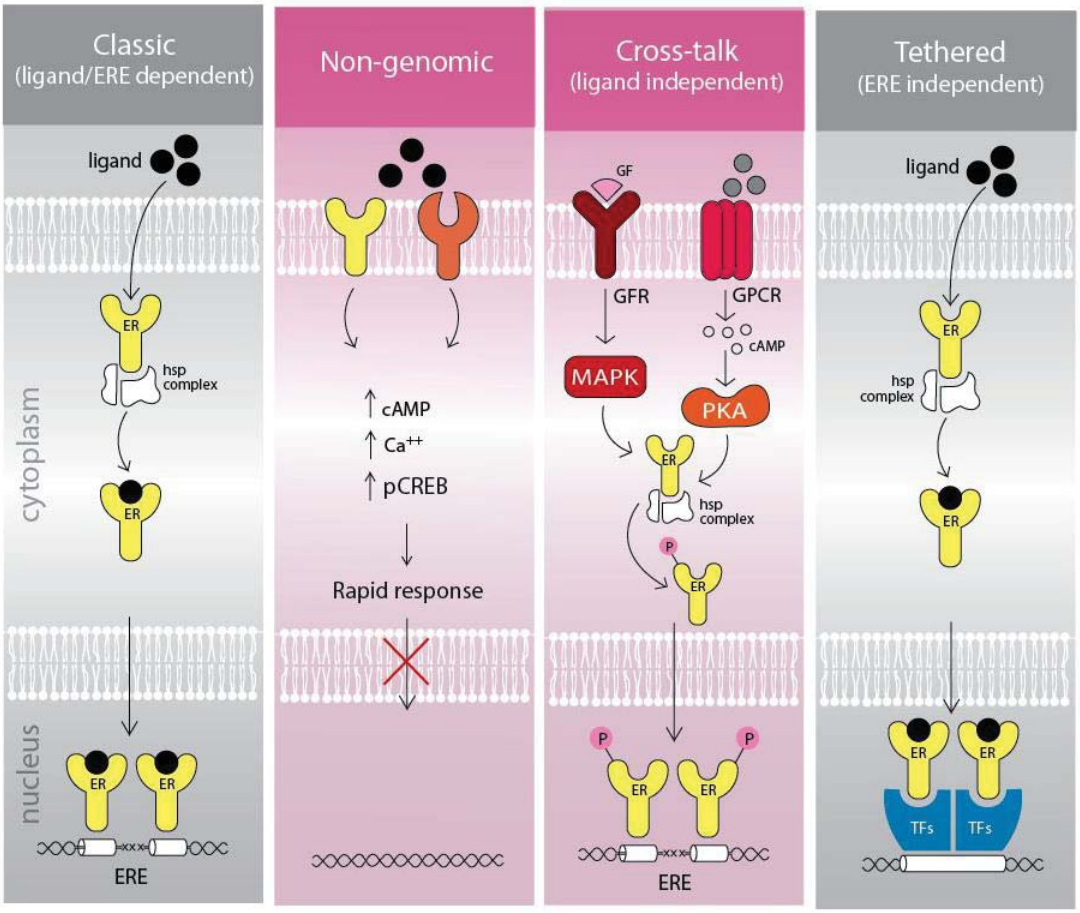


Figure 4: Mechanism of Estrogen signaling. Four pathways involved in Estrogen signaling are presented. (A) In the classic ligand/ERE dependent pathway, latent ER gets activated and translocates to nucleus to bind ERE of target genes. (B) In Non-genomic pathway, ERs increase the levels of intracellular calcium (Ca++) and stimulate cAMP response element binding protein (CREB) and other second messengers to facilitate responses in cells. (C) Ligand independent, in the absence of estrogen ligand ERs initiate the transcription via other messengers like MAPK and PKA. (D) In ERE independent, ER does not bind to ERE, instead they recruit other transcriptional factors (Charitidi et al., 2009, With permission from Elsevier).

1.3.3 Ligand independent signaling

Ligand independent signaling does not involve the estrogen ligand mediated activation. The gene transcription regulation by ER is initiated by growth factors such as Insulin-like growth factor-1 (IGF-1) and Epidermal Growth factor (EGF). These growth factors activate mitogen-activated protein kinase and subsequently MAPK phosphorylates ser118 in AF1 domain of ER (Kato et al., 1995). Because of involvement of AF1 domain, ERα and ERβ show antagonizing activity in this mode of activation.

1.3.4 ERE independent signaling

In ERE independent mechanism, ligand activates ER, but ER does not bind to ERE in DNA. Instead, ERs act as co-regulators rather than transcription factors. They interact with transcription factors such as Activating protein (AP-1) and FOS/JUN complex to initiate the

gene transcription. Hence this mechanism is referred as transcription factor cross-talk (Björnström and Sjöberg, 2005).

1.4 Immune system

The immune system protects the organism by a two-layered defense mechanism. If pathogens such as bacteria and viruses breach the physical barrier, then the innate immune system orchestrates immediate non-specific response. Macrophages and neutrophils provide the first line of defense against different microorganisms. If pathogens evade the first line of defense, the lymphocytes of adaptive immune response provide versatile form of defense and retain the immunological memory to mount the faster response on next encounter of pathogens. The lymphocyte population of adaptive immune system is mostly made up of Thymus derived Lymphocytes (T-lymphocytes), Bone marrow derived Lymphocytes (B-lymphocytes) and Natural-Killer cells (NK cells). T lymphocytes and B lymphocytes are responsible for cell-mediated immunity and humoral mediated immunity that work in close collaboration to provide adaptive immunity (Janeway et al., 2001).

T lymphocytes arise from hematopoietic stem cells and undergo maturation in thymus. These cells are characterized by the presence of T cell antigen receptor (TCR) on their cell surface. Notch signaling in thymus changes the fate of hematopoietic precursors to T-cell by stimulating the expression of T-cell receptor (Zhang et al., 2012). CD4+ T cells commonly termed as T helper and CD8+ T cells as cytotoxic, constitute the majority of T lymphocytes. CD4+ T cells predominantly express CD4 glycoprotein on their cell surface. This cell also assists in maturation of B cells to plasma and memory B cells.

1.4.1 T helper cell phenotypes

The seminal studies by Mossman & Coffman in 1986 (Mosmann et al., 1986) and Bottomly (Killar et al., 1987) revealed the existence of distinct subsets of CD4+ T cells after observing the distinguished pattern of production of cytokines. They introduced the Th1-Th2 dogma that dominated the immunology field for almost 20 years. However, to explain the complexity of T cell immune responses, since 2003, several studies have been documented the new emerging players such as Th17, Treg, Tfh and Th9 cells based on their distinct biological function (Harrington et al., 2005, Doisne et al., 2011, Sakaguchi, 2005).

Th1 cells

Th1 cells produce proinflammatory cytokines TNFα, TNFβ and IFNγ as signature cytokines to initiate the innate and T cell responses. They protect the host against the intracellular pathogens (bacteria & protozoa). The master transcription factor for Th1 cells is Tbx21 (T-bet) that regulates the expression of IFNγ gene. T-bet also facilitates the development of Th1 cells by suppressing GATA3 and IL4 production that are specific to Th2 cells. STAT4 plays critical role in maintaining transcriptional regulation in Th1 cells, IL12 mediates up regulation of IFNγ by recruiting STAT4 and STAT4 in conjunction with STAT1 induces the expression of T-bet that further up regulates the expression of IL12 receptor (IL12Rβ2; Afkarian et al., 2002). CXCR3 is the characteristic chemokine receptor of Th1 cells (Langenkamp et al., 2003). This receptor recognizes CXCL9, CXCL10 and CXCL11 chemokine ligands. The ligands act as T cell chemoattractant that recruit Th1 cells to the site of inflammation (Groom and Luster, 2011).

Th2 cells

Th2 cells are defined based on the production of key effector cytokines IL4, IL5, IL9, IL10 and IL25. Th2 cells mount immune response against extracellular forms of pathogens, such as helminthes & nematodes. IL4 promotes IgG1 and IgE class switching through B cells (Firestein et al., 1989). GATA3 is the key transcription factor of Th2 cells. IL4 activates the STAT6 signaling which up regulates the expression of GATA3. The GATA3 protein also suppresses the Th1 differentiation by inhibiting STAT4 expression (Usui et al., 2003). In STAT6 knockout mice, the Th2 cells failed to develop immune response even more completely than in IL4 deficient mice (Shimoda et al., 1996). STAT6 regulates more than 80% of IL4 target genes in Th2 cells (Elo et al., 2010). Presence of STAT5 observed to affect the Th2 lineage commitment in IL4 and GATA3 independent manner (Kim et al., 1999). Cytokine IL2 initiates the STAT5 binding to different sites in IL4 locus where GATA3 is unable to interact. Recent reports showed that STAT3 promotes synergistic interaction with STAT6 in binding IL4 loci (Stritesky et al., 2011). Otherwise, STAT6 failed to interact with IL4 loci. Th2 cells preferentially express CCR4 (Bonecchi et al., 1998) along with CRTH2 (Chemoattractant receptor homologous molecule expressed on Th2 cells) receptor on their surface.

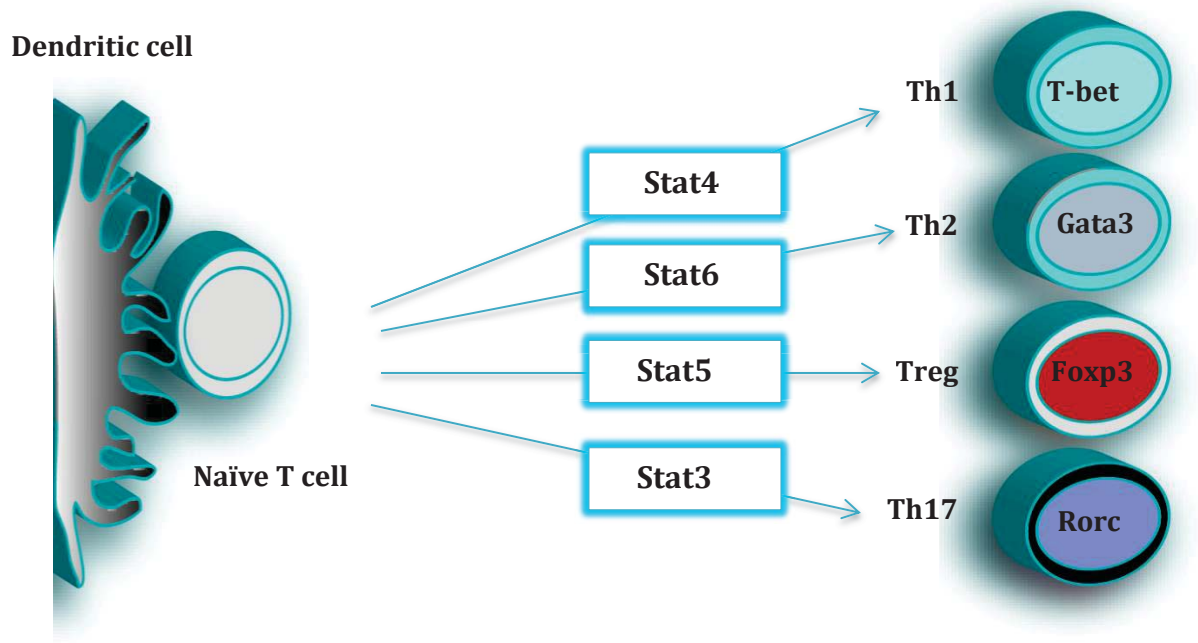


Figure 5: T helper cells lineages and master regulators. Lineage-specifying transcription factors (GATA3, T-bet, Rorγt and Foxp3) and signal transducing regulators.

Treg Cells

Natural regulatory T cells (nTregs) are a special subset of CD4+ T cells that secrete effector cytokines with inhibitory effect. Treg cells are also observed to suppress the proliferation of antigen-stimulated naive T cells in cell-to-cell contact manner (Thornton and Shevach, 1998). nTregs develop from thymus as a distinct lineage with Foxp3 already expressed in the cells. nTreg constitute 5-10% of peripheral CD4 T cells and they express higher levels of interleukin 2 receptor α-chain (CD25), cytotoxic T lymphocyte antigen 4 (CTLA4) and GITR. The other groups of Treg cells, Induced regulatory T cells (iTreg) differentiate from naïve T cells at periphery regions in the presence of TGFβ after T cell receptor (TCR) stimulation. Treg cells are defined by the expression of Foxp3, lineage specific transcription factor that belongs to the Fork-head protein family. The continuous expression of Foxp3 is absolutely necessary for Treg cells to maintain suppressing activity. Wan et al has generated a mouse model with attenuated levels of endogenous Foxp3 in Treg cells and observed the Treg cells conversion into Th2 cells due to elevated levels of Il4 cytokine production from the Treg cells (Wan and Flavell, 2007). In arthritic conditions, Treg cells were observed to lose Foxp3 expression and differentiate into Th17 owing to synovial fibroblast-derived IL-6 cytokine in inflamed joints (Komatsu et al., 2014). IL2 signaling via STAT5 is critical for maintenance of Treg

differentiation. STAT5 was observed to initiate Foxp3 by binding to its promoter and enhancer sites in the gene (Burchill et al., 2007).

Th17 cells

Th17 cells express IL17A, F as signature cytokines. They are also active producers of IL21 and IL22. They protect against extracellular bacteria and fungi. Th17 cells have been associated with induction and propagation of many autoimmune diseases such as Multiple sclerosis, RA, Psoriasis and IBD (Langrish et al., 2004). Retinoic acid-related orphan receptors (ROR) are master regulators in Th17 cell differentiation. RORα and RORγt are indispensable for Th17 development. IL6, IL21 and IL23 direct downstream STAT3 and RORγt to bind with IL17A and IL17F promoters. The transcription factors STAT3 and RORγt act synergistically and studies showed that deletion of either factors impaired the IL17 production (Yang et al., 2008). Mutation of STAT3 which results in defect in Th17 cell development leads to hyper-immunoglobulin E syndrome (HIES, Job's syndrome). Aryl hydrocarbon Receptor (AhR) is ligand dependent transcription factor that promotes the development of Th17 cells by antagonizing the negative regulators of Th17 cells such as STAT1 and STAT5. IRF4/ BATF transcription factor complexes are induced transcriptionally in Th17 cells after TCR stimulation. This complex then initiates the expression of STAT3 and also Th17 target genes. Then the induction of RORγt activates the key Th17 genes (Ciofani et al., 2012).

Th17 cells express high levels of CCR6 (Annunziato et al., 2007). CCL20 acts as ligand and it engages Th17 cells towards the specific part of mucosal lymphoid tissue such as peyers patches (Wang et al., 2009).

Emerging Phenotypes

Recently identified phenotypes of Th cells are Tfh, Th9 and Th22. Tfh (follicular helper T cells) can be observed in edge of B cell zones, follicular areas and germinal regions. They promote development of antigen specific B cell immunity (Vinuesa et al., 2005). They express high levels of CXCR5 and IL21. Bcl6 is the master transcription regulator of Tfh cells.

Th9 cells were initially characterized as Th2 cells but Stockinger (Veldhoen et al., 2008) and Kuchroo et al (Dardalhon et al., 2008) independently reported them as new subset of Th cells. TGFβ is assumed to promote the differentiation of Th2 cells towards Th9 cells (Veldhoen et al., 2008).

Th22 cells are skin homing T cells with role in epithelial innate immune reactions (Soler et al., 2003). They express CCR10 and CCR4 (Duhen et al., 2009).

1.5 Estrogen, ER and T cells

ERα is expressed in thymocytes, bone marrow, T cells, B cells and non-hematopoietic cells. In most immune cells, ERα (*ESR1*) is detected at lower levels and their expression level increases in the presence of estrogen (Inui et al., 2007). CD4+ T cells show higher levels of ERα compared to B cells. However, B cells express more ERβ. In CD8+ cells, both ERα and ERβ are expressed at lower and equivalent levels (Phiel et al., 2005). The functional differences between ERα and ERβ are not yet clear but it appears that estrogen endows its effect through T cells by ERα signaling and B cells by ERβ signaling.

Estrogen significantly modulates both innate and adaptive immune system (Straub, 2007). It exerts dose dependent biphasic effect on T helper cell responses. Low doses of Estrogen initiate Th1 response and higher doses mediate Th2 response (Maret et al., 2003, Bebo et al., 2001).

1.6 Estrogen alpha receptor and autoimmune diseases

ERα & ERβ are expressed in many immunological cells such as T cells, B cells, macrophages and dendritic cells suggesting the effect of estrogen on immune responses (Haruki et al., 1983, Shim et al., 2006). SLE is predominantly observed in women and estrogen is likely to play a deleterious role. In SLE models, estrogen induced cytokine modulation is mediated by estrogen receptor and the endogenous and exogenous levels of estrogen intensified the development of the disease, whereas ERα disruption alleviated the disease (Bynoté et al., 2008).

The development of autoimmune glomerulonephritis was observed in ERα-/- mice indicating that the ERα deficiency may lead to the development of autoimmunity (Shim et al., 2004). Estrogen deficiency increases the differentiation of Th17 cells by up regulating STAT3, RORγt and RORα (Tyagi et al., 2012) and ERα signaling was observed to be critical in T cells, for E2-mediated inhibition of Th1/Th17 cell differentiation and protection from EAE (Lélu et al., 2011).

2. Objective of study

Owing to the role of estrogen and estrogen receptor in human autoimmune diseases and the considerable expression of estrogen receptor in T-helper cells, the overall objective of this study is to explore the role of *ESR1* in the differentiation of Th1, Th2, Th17 and iTreg cells. The first aim is to determine the expression levels of estrogen receptor alpha in vitro cultured different human T helper (Th1, Th2, Treg and Th17) subsets with real-time quantitative PCR. Secondly, specific siRNA is used to down-regulate the expression of *ESR1* in CD4+ T cells and to evaluate the contribution of estrogen receptor alpha in T helper cells polarization. Expression of cytokines and master transcription factors that play major role in determining the respective lineages were evaluated after silencing the expression of *ESR1.*

3. Materials and Methods

3.1 Isolation of CD4+ cells from umbilical cord blood

Umbilical cord blood was used to isolate the CD4+ non-stimulated T cells, in which naïve T cells are dominating. The blood was obtained from healthy neonates born at Turku University Hospital. Blood samples were collected into 50 ml Falcon tubes to which 200 µl of heparin (LEO Pharma, Denmark) was added to prevent coagulation. PBMC isolation was carried out according to Ficoll-paque manufacturer's instructions with little modifications. Blood was mixed with PBS in 1:1 volumes and carefully poured over 15 ml Ficoll (GE Healthcare, Uppsala, Sweden). Then the Ficoll tubes with diluted blood were subjected to density gradient centrifugation at 300 g for 30 mins. Five layers were obtained in the 50 ml tubes. The bottom layer consists of aggregated erythrocytes that had sediment through Ficoll. The second layer contains granulocytes. Above this layer, Ficoll layer was obtained and in the interphase between Ficoll and serum a mononuclear cell layer was formed as indicated in the manufacturer's brochure. The mononuclear cells (include lymphocytes (T cells, B cells, and NK cells), monocytes, and dendritic cells) were recovered and CD4+ T cells were further purified by using positive selection of magnetic Dynabeads (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Dynabeads are super paramagnetic beads (4.5 µm diameter) covalently coated with a monoclonal antibody specific for the CD4 membrane proteins. Dynabeads bind to the CD4+ T cells in mononuclear cells during the 20 min incubation. Then bead-bound cells were separated from mononuclear cells using a magnetic stand. Isolation of CD4+ T cells was carried out by detaching the CD4+ T cells using DeTACHaBead antibodies.

The blood usage from anonymous donors was approved by the Ethics Committee of Hospital District of Southwest, Finland in compliance with the Declaration of Helsinki.

3.2 siRNAs and Transfection

Silencing of target gene was carried out by introducing chemically synthesized short interfering RNAs (siRNAs) into cells. siRNAs are generally 21 bp double stranded RNA molecules designed complementary to target mRNA. siRNA duplex with phosphorylated 5' ends and hydroxylated 3' ends consists of a guide strand and a passenger strand. The endonuclease Argonaute (Ago2), part of RNA induced silencing complex (RISC) binds to guide strand (such as microRNA (miRNA) or short interfering RNA (siRNA)). Then the guide strand directs RISC to endolytic cleavage of complementary target mRNA. siRNAs can be introduced into target cells by liposome transfection, electroporation and viral gene transfer (Ovcharenko etal., 2005). Transfection of ERα and STAT4, 6, 3 siRNAs into CD4+T Cells was carried out by Nucleofector device (Amaxa, Cologne, Germany) using U-14 program (see Appendix 4 for siRNA sequences). Nucleofections were performed in 100 µl Opti-MEM I (Invitrogen, Carlsbad, CA, USA) media and cuvettes were washed with complete 1 ml of RPMI 1640 medium containing 10% heat inactivated FBS (PromoCell), 2 mM L-Glutamine (Sigma) and 50 U/ml Penicillin and 50 µg/ml Streptomycin (Sigma). The transfected cells were rested for 48 hours at 37°C to induce the effect of siRNAs. Then, the cells were added to culture plates and polarizing cytokines and antibodies were added.

3.3 Cell activation and Polarization culture

Activation of CD4+ cells was carried out by plate-bound anti-CD3 (Immunotech, Marseille, France) and soluble anti-CD28 (Immunotech, Marseille, France) in X-VIVO 20-medium (Lonza) supplemented with 2 mM L-Glutamine (Sigma), 100 U/ml Penicillin and 100 µg/ml Streptomycin (Sigma) at 37°C in 5% CO2.

Th17 polarization was initiated with IL6 (20 ng/mL; Roche), IL1β (10 ng/mL) and TGFβ (10 ng/mL) in the presence of neutralizing anti-IFNγ (1 µg/mL) and anti-IL4 (1 µg/mL). Control Th0 cell were cultured in a medium containing only the neutralizing Abs. Th1, Th2, and iTreg differentiation was initiated with IL12 (2.5 ng/mL) and anti-IL4, IL4 (10 ng/mL) and anti-IFNγ, and TGFβ (10 ng/mL), IL2 (10 ng/ml), respectively. All cytokines and neutralizing Abs were from R&D Systems unless otherwise stated.

Table 1: Following antibodies and cytokines were added to polarize the respective conditions.

	Th0	Th1	Th2	Th17	Treg
Anti CD28	x	x	x	x	x
Anti-IFNγ	x	_	x	x	x
Anti-IL12	x	_	x	x	x
Anti-IL4	x	x	_	x	x
IL12	_	x	_	_	_
IL4	_	_	x	_	_
IL6	_	_	_	x	_
TGFβ	_	_	_	x	x
IL2	_	_	_	_	x
IL1β	_	_	_	x	_
IL23	_	_	_	x	_

X: added; _: not added

3.4 RNA isolation and cDNA Synthesis

Total RNA from polarized CD4+ T cells was isolated by using RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA concentration was measured using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, US). cDNA synthesis was carried out using Superscript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA). The reaction for 20 µl was prepared with 4 µl of 5X VILO reaction mix (includes random primers, MgCl2, and dNTPs), 2 µl of 10X Superscript Enzyme mix, 150 ng of total RNA and water to make up to 20 µl. The conditions used for reaction were 42°C for 60 min and 85°C for 5min. cDNA samples were diluted in 1:10 volumes for quantitative amplification.

3.5 Real-time Quantitative Polymerase chain reaction

Real-time PCR is a variant of PCR with ability to quantify the DNA molecules in real time. qPCR instruments determine the fluorescent signal during the course of amplification cycles of reaction. qPCR employs two chemistries for measuring the fluorescent signal (1.Taqman, 2. SYBR Green). In this study, Taqman probes were used for quantification of cDNA. Taqman fluoregenic probes- sequence-specific oligonucleotides that contain reporter (e.g. FAM or VIC dye label on the 5' end) and quencher (e.g. minor groove binder (MGB) and non-fluorescent quencher (NFQ) on the 3' end). In the second strategy, SYBR green dye (intercalating dye) in reaction mix preferentially binds to newly forming double-stranded DNA and increasing fluorescence is measured throughout the cycles. The parameter measured in qPCR is Ct

(threshold cycle) value, which is intersection between amplification curve and threshold line. The Ct values are inversely proportional to the target gene expressed in the sample (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_5279.pdf). The obtained Ct value is normalized with endogenous control genes like EF1α, HPRT, Actin-B and GAPDH.

Relative quantification of gene expression was done by using ABI 7900HT Fast Real-Time PCR System (Applied Biosystems Inc, Foster City, CA). Primers were ordered from Oligomer (Helsinki, Finland) and probes were obtained from Universal Probe Library (Roche Applied Science) with FAM (reporter) and TAMRA (quencher). 10 µL reaction volume of master mix was prepared for each well with 2 µl of cDNA, 2 µl of primer pair, 1 µl of probe (see Appendix 5 for primers and probe information) and 5 µl of PROBE FAST ABI Prism 2X qPCR Master Mix (Kapa biosystems, Woburn, MA). Samples were added to 96-well PCR plate and plates were spun at 300 g for 30 sec. The following PCR conditions were used: 95°C for 10 min and 95°C for 15 sec, 60°C for 1 min for 40 cycles. The average cycle threshold (Ct) value for each sample was normalized against HPRT to obtain ΔCt and gene expression quantification by comparative Ct value was calculated using $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001). The standard curves were plotted for each gene to verify the amplification efficiency.

3.6 Western Blotting

CD4+ T cells were lysed by adding Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 5% Glycerol, 1% SDS) containing proteinase and phosphatase inhibitors (Roche) on ice for 30 mins. Sonication of samples (Bioruptor UCD-200; Diagenode) was carried out for 5 mins with every 30 seconds ON-OFF cycles. The samples were centrifuged for maximum speed at 4°C for 30 mins and supernatant was collected. For equal loading, the protein quantification was carried out by detergent compatible protein assay kit (Bio-Rad, Hercules, CA) (Appendix 1). Measurement was done in a photometer in triplicates at a wavelength of 750 nm filter. Before loading into 12% SDS-PAGE gel, the 6X loading dye (330 mM Tris-HCl, pH 6.8, 330 mM SDS, 6% β-Mercaptoethanol, 170 µM bromophenol blue, 30% Glycerol) was added to 30-50 µg of protein and boiled for 5 mins. The PAGE was run initially at 70 volts till the protein reached the resolving gel as the lower voltage during the first minutes of the run increases band sharpness. Then the voltage was increased to 120 volts. Proteins were transferred from gel to PVDF membrane (Bio-Rad, Hercules, CA) by instrument Bio-Rad trans-blot for 30 mins using the built-in program. The membrane was treated with ponceau to ensure the transfer of protein onto PVDF membrane. Then washing was

performed thrice and blocking of the membrane was done for 1 hour by using 5 % BSA in 1X TBST (0.1%). Primary antibodies (Appendix 2: list of primary antibodies used in this study) were 1:1000 diluted with 5 % BSA in 1X TBST (0.1%) and incubated with membrane at 4°C for overnight. After washing for three times with TBST buffer, membrane was incubated with HRP conjugated secondary antibody at room temperature for 1 hour. The protein was detected by enhanced chemiluminescence using the Pierce ECL Western blotting substrate (Thermo Scientific, Rockford, IL) and Fuji X-ray film (Fuji Medical 100 NIF), which was developed using a Curix 60 X-ray film processor (AGFA).

3.7 Flow cytometry

The 24-hour cultured T-helper subset cells were washed twice with PBS and collected into FACS tube. 100 µL of fixation buffer containing 4% paraformaldehyde (eBioscience, San Diego, CA) was added to the cells and incubated at 4°C for 30 mins. Cells were washed twice with 500 µL of permeabilization buffer (eBioscience, San Diego, CA) containing 0.1% saponin and centrifuged at 450g for 3mins. The supernatant was discarded and 100 µl of permeabilization buffer containing staining antibodies were added (see Annex. 3 for list of antibodies). The tubes were kept at 4°C for 45 mins and washed with 500ul of permeabilization buffer. Tubes were spun at 450g for 2 mins and 350 µl of FACS buffer was added in the end. The stained cells were detected by flow cytometer FACSCalibur with CellQuest software (Becton Dickinson, San Jose, CA).

3.8 Flow cytometry data analysis

30,000 cells/sample were collected for data analysis. Isotype controls and unstained control were used. FACS data was analyzed by FlowJo software (Treestar, San Carlos, CA). Initially, the gating of live cells was performed and the plots were exported into .emf format.

4. Results

4.1 TCR activation induces expression of ERα 66kDa isoform in T cells

TCR mediated activation is an important stimulus for Th cell differentiation. Naïve precursor helper cells differentiate into different Th subset cells only after TCR stimulation in the presence of antigens, co-stimulatory molecules and cytokines. TCR stimulation induces the activation of different transcription factors. To determine the effect of TCR stimulation on estrogen receptor expression under in vitro conditions, I activated naïve T cells with anti-CD3 (stimulating TCR) and anti-CD28 (co-stimulatory molecule). TCR activation alone without

simultaneous co-stimulatory receptor signaling leads to anergy, a state that prevents the naïve T cells from proliferating and differentiating into effector cells (Schwartz, 2003).

After performing Western blotting, I observed that un-activated naïve precursor helper T cells mostly expressed 46 and 36 kDa of ERα and showed low expression of the 66 kDa isoform. However, TCR stimulation up-regulated the level of 66 kDa ERα, suggesting the role of ERα 66 protein in T-cell differentiation. I also observed 46 and 36 kDa isoform bands for ERα in naïve and other subset type of T cells (Figure 6). In naïve Th cells ERα 46 was expressed more than ERα 36. The antibody used in Western blot is specific for the carboxy terminus of the ERα 66kDa isoform but also detects carboxy terminus of ERα 46kDa isoform. However, it is impossible to detect the 46kDa of ERα specifically at mRNA and protein levels because of its identical protein sequence with ERα 66kDa isoform (Irsik et al., 2013).

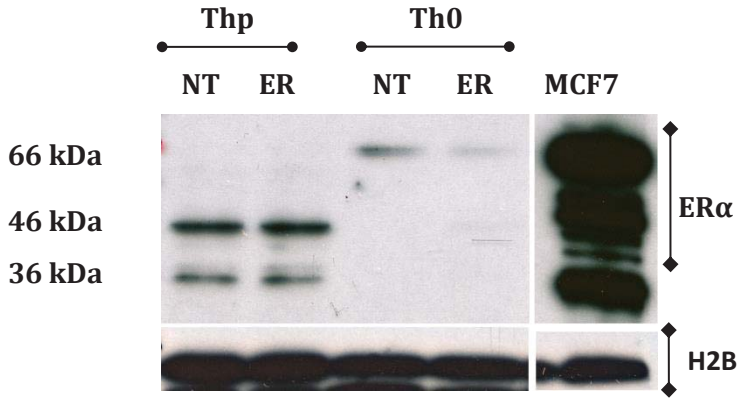


Figure 6: Western blot analysis representation of ERα 66 kDa, 46 kDa and 36 kDa isoforms expression. siRNA nucleofected but without TCR activated Thp cells were collected at 0 hour. The other subsets of cells were cultured under Th0 conditions for 72hours after activation. MCF7 (human breast cancer cell line) cells were added as positive control to verify the size of ERα bands. NT: Non-targeting (siRNA control), ER: Estrogen receptor siRNA.

4.2. Expression of ERα in Th subsets

To determine the expression levels of ERα in Th subset cells, the naïve CD4+ cells were polarized into Th0 (with anti-CD3 and anti-CD28 activation, without polarizing cytokines), Th1, Th2, Treg and Th17 cells for 24 and 72 hours. To confirm whether cells could polarize toward the specific lineages under the culturing conditions, the mRNA levels for transcription factors of GATA3 (Th2), Foxp3 (Treg) and cytokine IFNγ (Th1), IL17 (Th17) were determined

(Figure 7 A) by real-time quantitative PCR (TaqMan®). IFNγ, GATA3 and IL17 were expressed highly in only Th1, Th2 and Th17 polarizing cells respectively. IFNγ and GATA3 were observed in higher levels already during 24 hours, whereas Foxp3 and IL17 expression was confirmed at 72 hours. mRNA quantifications for all Th subset cells (Figure 7 A) and Western blot detections for Th1 and Th2 (Figure 7 B) master regulators indicate that the Th cells differentiated to the right lineages under the conditions used in this study.

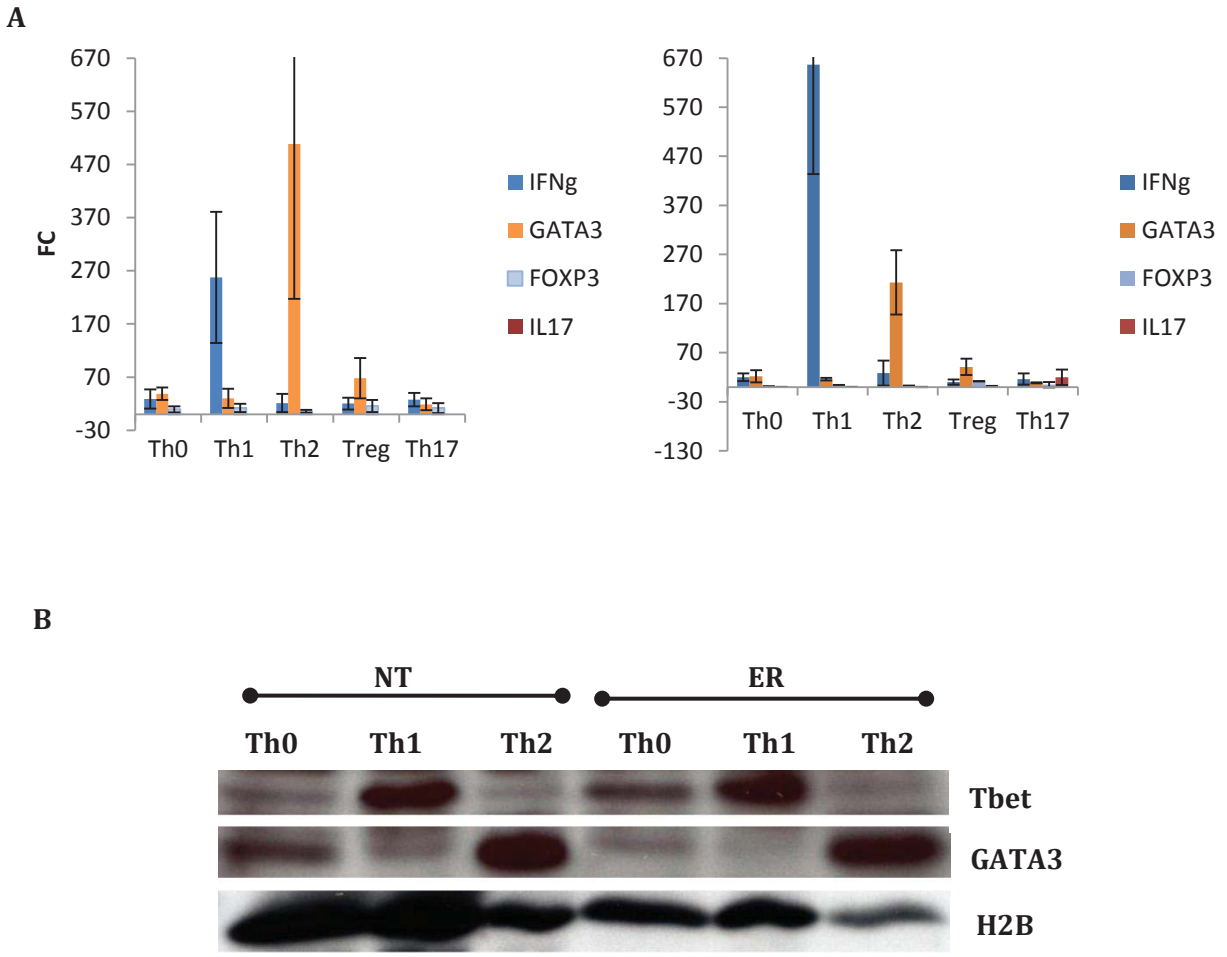
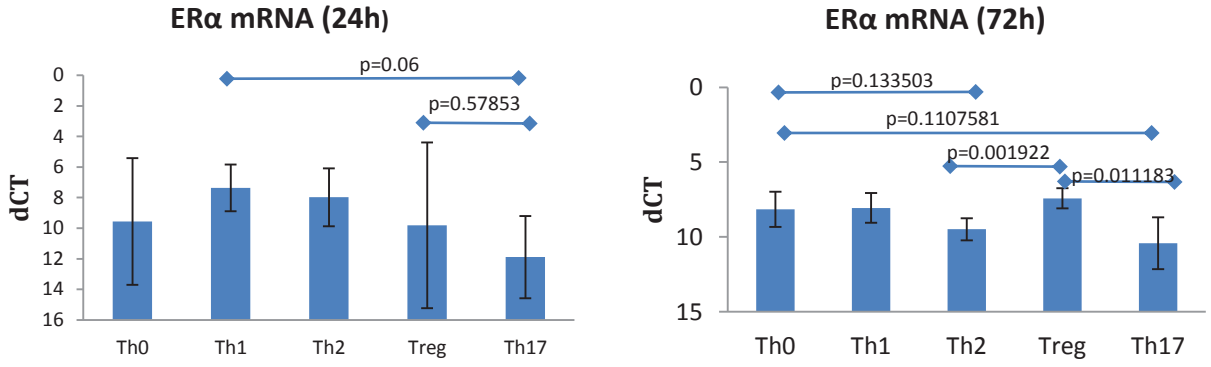


Figure 7: (A) T cells were cultured into Th1, Th2, Treg and Th17 for 24 hours (left) and 72 hours (right) and mRNA for master regulators and cytokines of Th subsets were quantified. (B) Protein levels of T-bet and GATA3 verified for 24 hour culture in polarizing Th0, Th1 and Th2 cells

To determine how ERα and ERβ are expressed in the *in vitro* cultured Th subsets, I performed real-time quantitative PCR. TaqMan RT-PCR results showed that ERα mRNA was expressed highly in Th1, Treg cells and lowly in Th2, Th17 cells during 72 hours (Figure 8 A).

Interestingly, in another independent experiment, in Th2 and Th17 cells higher mRNA levels of ERβ were observed in comparison to ERα (Figure 8 B). ERα and ERβ have been observed to have opposing effects in cell proliferation and apoptosis (Thomas and Gustafsson, 2011). Lindberg et al reported that ERβ inhibits ERα-mediated gene transcription in the presence of ERα. In liver and bone tissues it is reported that ERβ reduces 85% of ERα-regulated transcription of genes (Lindberg et al., 2003) and in the absence of ERα, ERβ partially compensates the function of ERα.

A



B

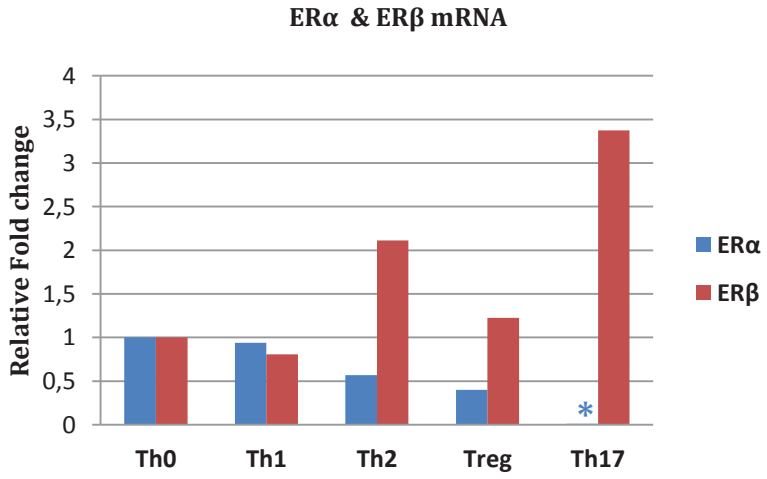


Figure 8: (A) Expression of ERα was detected in Th0, Th1, Th2, Treg and Th17 for 24 and 72 hours using TaqMan. Error bars showing SEM and representative of three independent experiments **(B)** Th0, Th1, Th2, Treg and Th17 were cultured for 72 hours and mRNA levels for ERα and ERβ were quantified using TaqMan RT-PCR. *indicates that RT-PCR signal is not detectable.

4.3 Expression of ERα is regulated by STATs

STATs (signal transducers and activators of transcription) are transcription factors that play crucial role in the differentiation and expansion of T helper cells. Extracellular binding of cytokines activates STAT proteins, and then they mediate the induction of the master transcription factors and further regulate the expression of cytokine inducible genes. Each subset of T helper cells is generally characterized based on the STAT protein expression (O'Shea and Paul, 2010). To study whether expression of ERα in Th subsets is regulated by STAT proteins, I silenced the STAT4, STAT6 and STAT3 in Th1, Th2 and Th17 conditions respectively, then detected expression of ERα at mRNA level. As aforementioned, I observed the reduction of ERα in control Th2 and control Th17 polarizing cells, importantly, down-regulation of STAT6 in Th2 and STAT3 in Th17 cells resulted in enhanced expression of ERα at mRNA level (Figure 9).

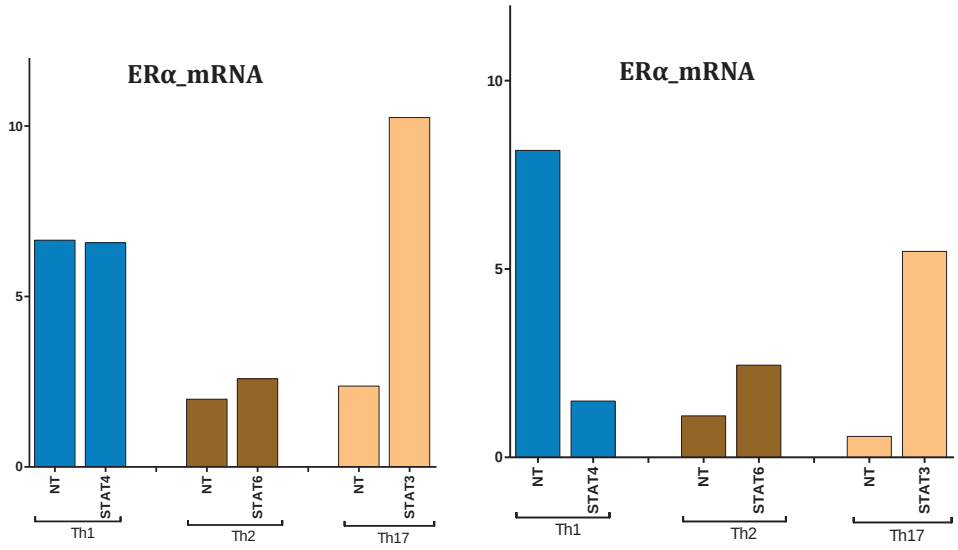


Figure 9: ERα mRNA levels were detected by Taqman RT-PCR in Non-target (siRNA control) and STAT siRNA nucleofected cells. STAT4, STAT6 or STAT3 was silenced in Th1, Th2 or Th17 lineage accordingly and cells were cultured for 24 (left) and 72 hours (right).

These results suggest that STAT6 and STAT3 signaling negatively and STAT4 positively regulate the ERα expression in Th2, Th17 and Th1 cells, respectively. Interestingly, coincidently elevated levels of ERβ were also observed in Th2 and Th17 cells. The regulation of STAT6 on ERα is not yet known, but protein phosphatase 2A (PP2A) was observed to affect

both STAT6 (Munz et al., 2011) and ER signaling (Lu et al., 2003). A previous study has reported that STAT3 is activated by IL-6 down-regulates ERα in cancer cells (Wang et al., 2005), indicating that STAT3 down-regulates the expression of estrogen receptor alpha in several cell types.

4.4 ERα knockdown influences expressions of master regulators of Th1, Th2 and Treg cells

To determine the role of ERα in human Th cell differentiation, the ERα gene was silenced in polarizing Th subset cells using siRNA transfection. The knockdown effect of siRNA was verified by using the MCF7 cell line in which ERα is highly expressed and therefore can serve as positive control for estrogen receptor isoforms (Figure 10). Again, 66kDa, 46kDa and 36kDa isoforms were detected in MCF7 cells and the ERα siRNA was observed to be specifically and efficiently targeting the ERα 66 and 46 isoforms (Figure 10).

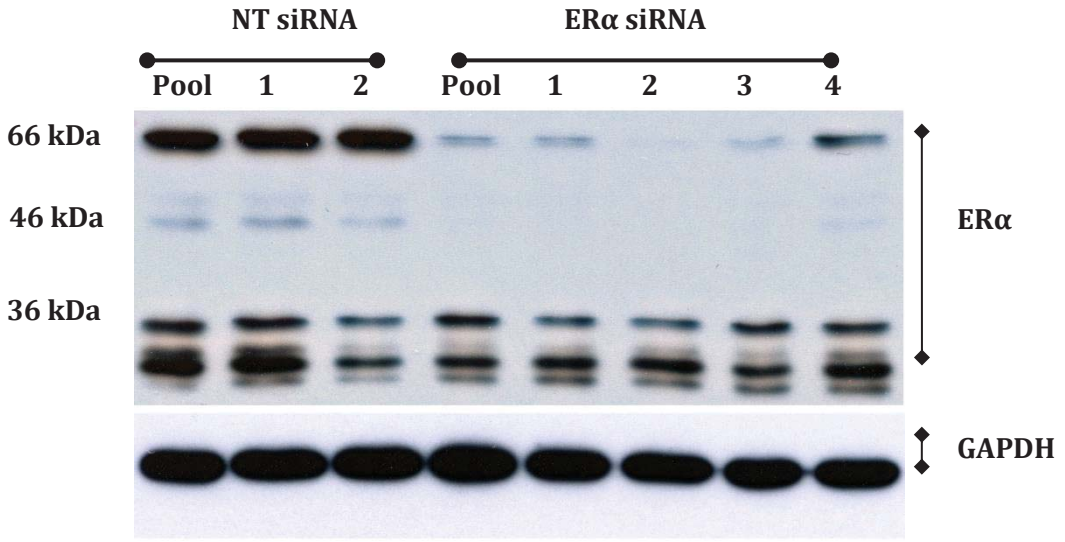


Figure 10: MCF7 cells were nucleofected with pooled non-targeting siRNA, two different individual non-targeting siRNAs (siRNA controls), combined pool of ERα siRNAs and 4 different individual ERα siRNAs. The MCF7 cells were nucleofected using E-14 program according to manufacturer's protocol (Amaxa Biosystems) and cultured for 24 hours.

The same siRNA was used to knockdown ERα in T cells and the influence of ERα silencing on Th1, Th2, Th17 and Treg cells was investigated by TaqMan, Western blotting and Flow cytometry. The knockdown effect of siRNA was observed to be transient in Th0 cells (Figure 11 A). The silencing effect of siRNA was observed clearly in Th1 and Treg cells as they express

high levels of ERα and the silencing of ERα in Th2 and Th17 cells was not observed because ERα was expressed at very low level (Figure 11 B).

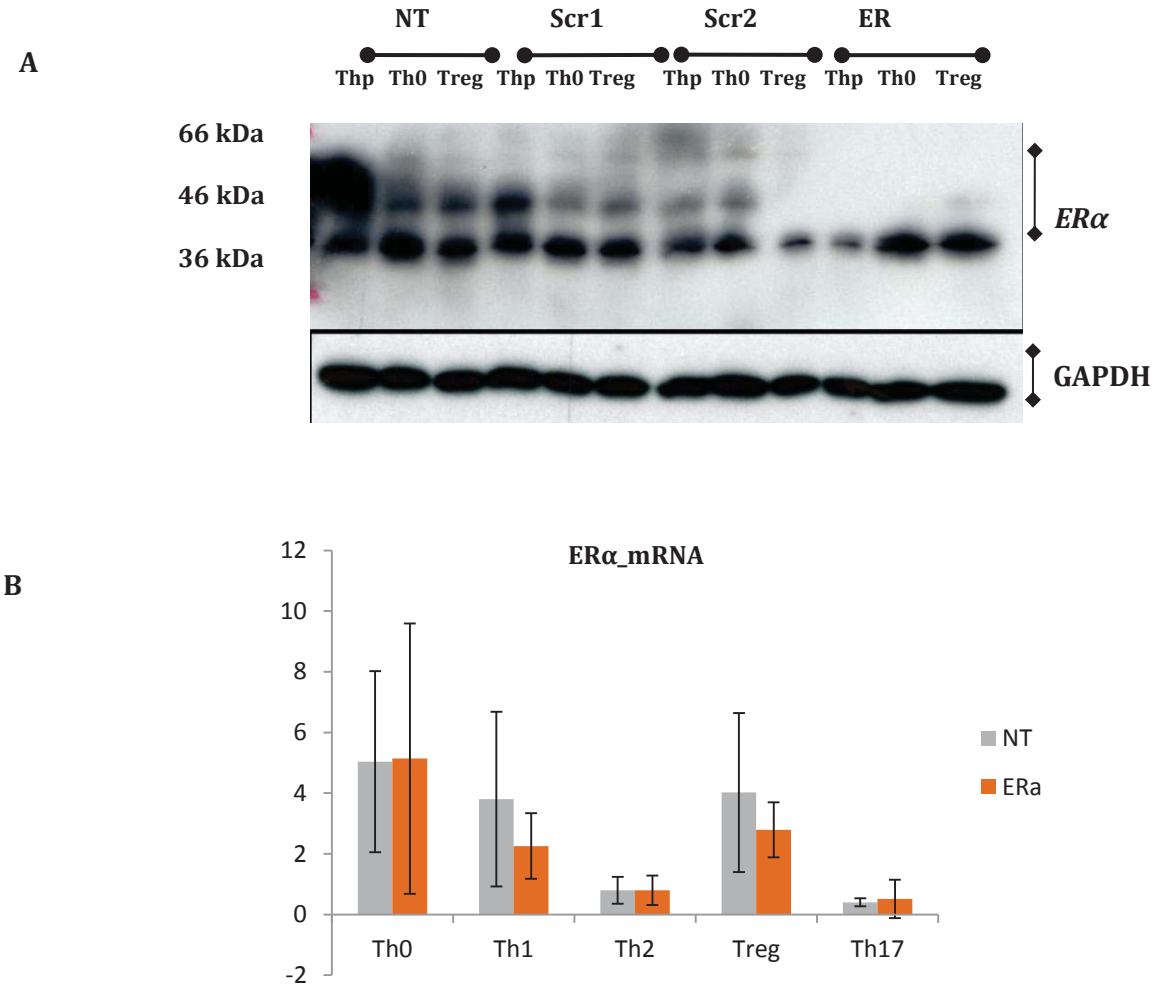
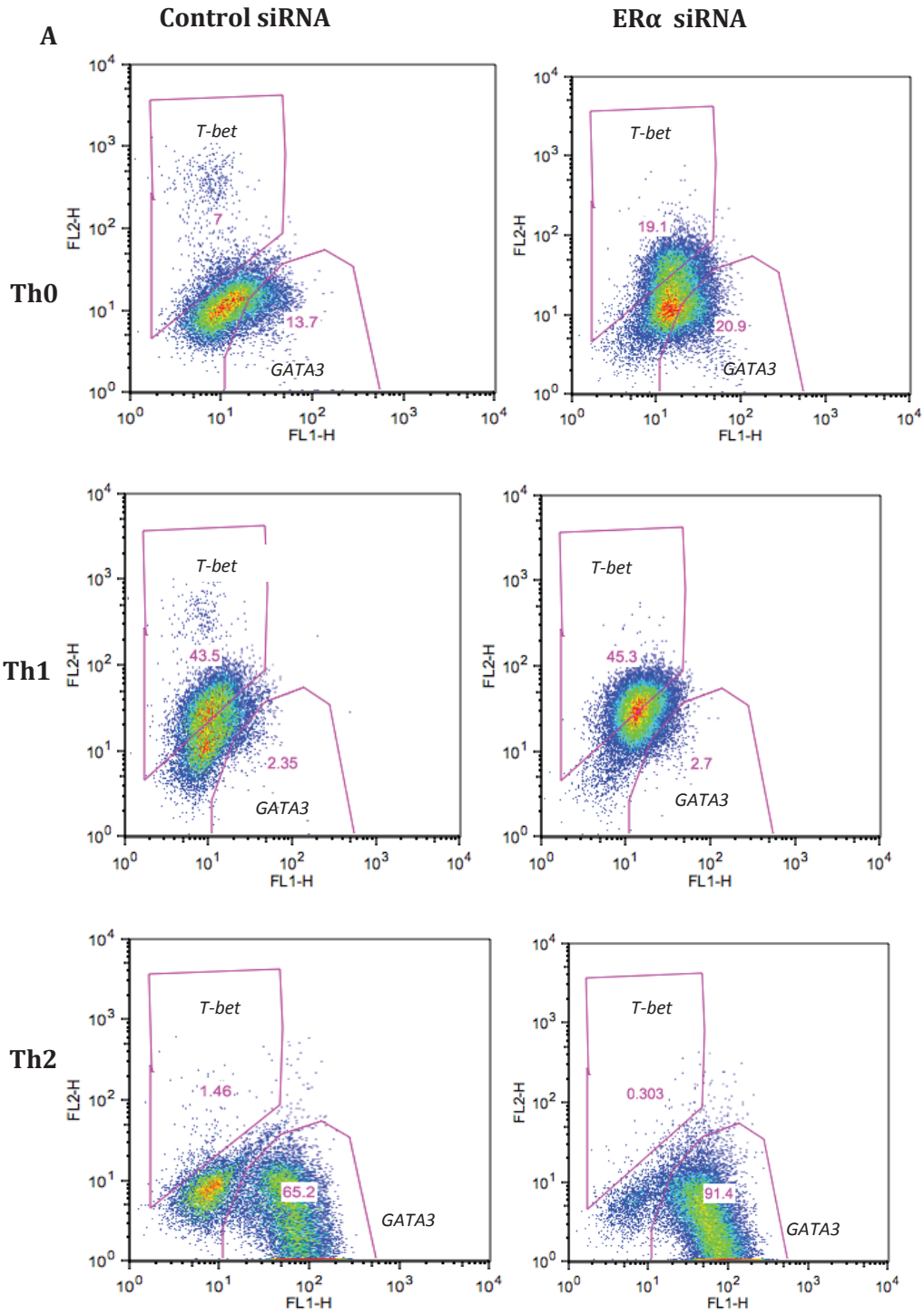


Figure 11: (A) Western blot representative for Treg cells that were nucleofected with non-targeting siRNAs (NT, Scr1 & Scr2) and ERα siRNA and cultured for 24 hours (B) Silencing of ERα was verified by measuring the mRNA levels of ERα after culturing the cells for 72 hours.

Intracellular staining of transcription factors T-bet and GATA3 in ERα knockdown T cells showed slightly increased levels of T-bet in Th1, increased GATA3. In Th2 cells, ERα knockdown increased GATA3 at protein level during 48 hours (Figure 12 A) and at mRNA level IL4 expression also increased during 24 hours (Figure 12 B). It can be assumed that ERα silencing increased Th2 cells polarization.


A
Control siRNA
ERα siRNA
Th0
Th1
Th2
T-bet
GATA3
FL2-H
FL1-H
7
13.7
19.1
20.9
43.5
2.35
45.3
2.7
1.46
65.2
0.303
91.4

B

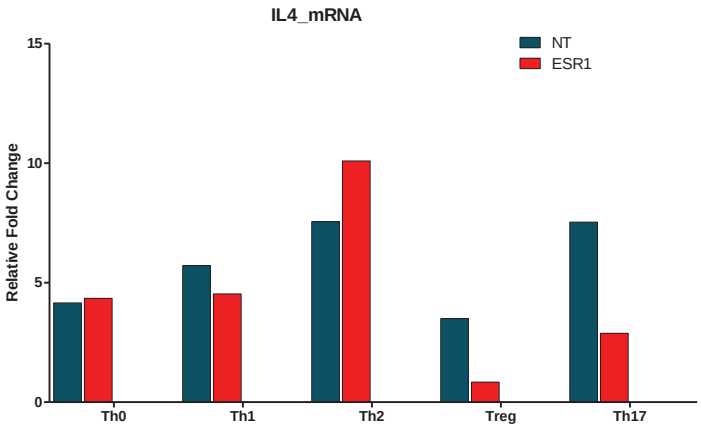
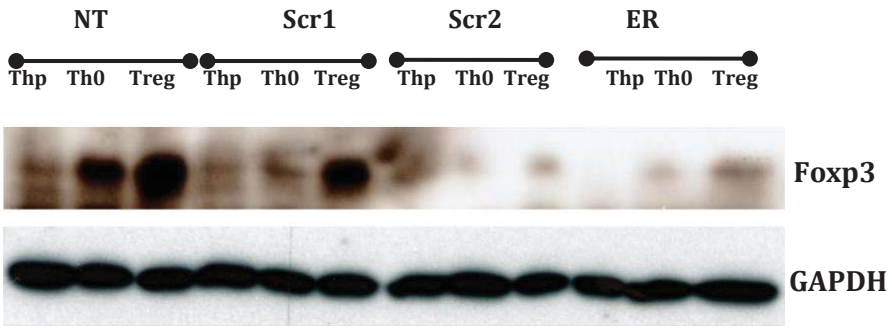


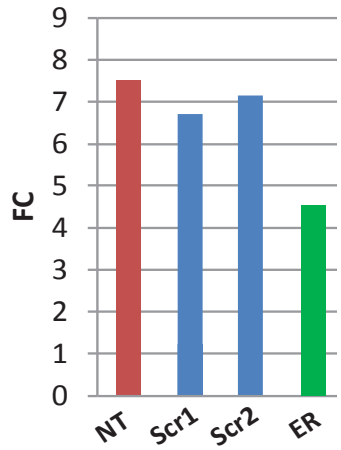
Figure 12: (A) T-bet and GATA3 protein were analysed in siRNA nucleofected and cultured Th0, Th1 and Th2 cells. The proteins were detected by using intracellular flow cytometry detections. **B)** mRNA levels of IL4 measured in polarizing Th0, Th1, Th2 , Treg and Th17 cells using Taqman RT-PCR.

In Treg cells, Western blotting performed for 24 hour cultured cells showed Foxp3 was highly expressed in differentiating Treg cells and I observed reduced levels of Foxp3 (Figure 13 A) compared to control siRNA transfected Treg cells. To further validate the influence of ERα for expression of Foxp3, TaqMan RT-PCR (Figure 13 B) and intracellular staining of Foxp3 (Figure 13 C) were performed. The results obtained from different detection methods were consistent, and indicated that down-regulation of ERα reduces expression of Foxp3.

A



B



C

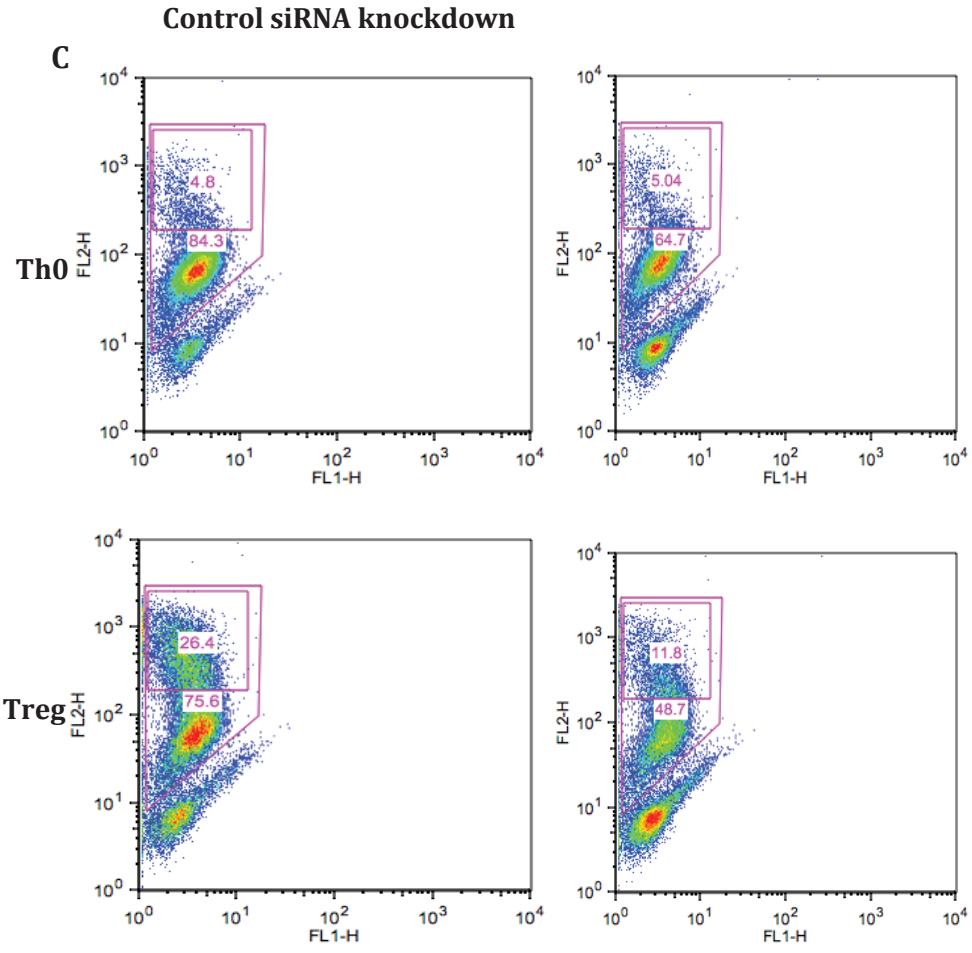


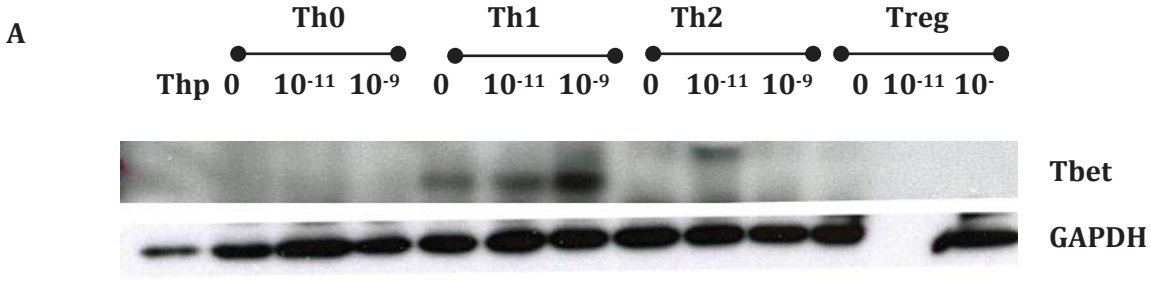
Figure 13:(A) Treg cells were nucleofected with Non-targeting siRNAs (NT, Scr1 & Scr2) and Estrogen siRNA, cultured for 24 hours (B) RT-PCR was performed for Non-targeting siRNAs (NT, Scr1 & Scr2) and ERα siRNA nucleofected Treg cells to determine the levels of Foxp3 mRNA. (C) Foxp3 levels were determined by flow cytometry in siRNA nucleofected and cultured Th0 and Treg cells. Above gated box in Foxp3 (Foxp3High) indicates nTreg cells.

4.5 Addition of ERα ligand, Estradiol increased T-bet and reduced Foxp3 levels

Estrogen has been implicated as protective factor in autoimmune diseases. Polanczyk et al reported that estrogen administration protected the mice from developing EAE and also observed the elevated levels of Foxp3 in Treg cells (Polanczyk et al., 2004). To study the effect of estrogen on Th subset cells, naïve T cells were activated and cultured with polarizing cytokines for 3 days without (control) and with 10-11 and 10-9 M of 17β estradiol.

As the master regulator of Th1 differentiation, high expression of T-bet was detected only in Th1 cells. Addition of Estradiol (E2) further increased T-bet's expression in Th1 cells (Figure 14 A). Consistently, estradiol addition also increased the IFNγ mRNA levels in Th1 cells (Figure 14 B).

In Treg cells, as shown by intracellular staining, expression of Foxp3 was induced by iTreg culture condition and addition of E2 reduced Foxp3 expression in Treg cells (Figure 14 C) in dose-dependent manner.



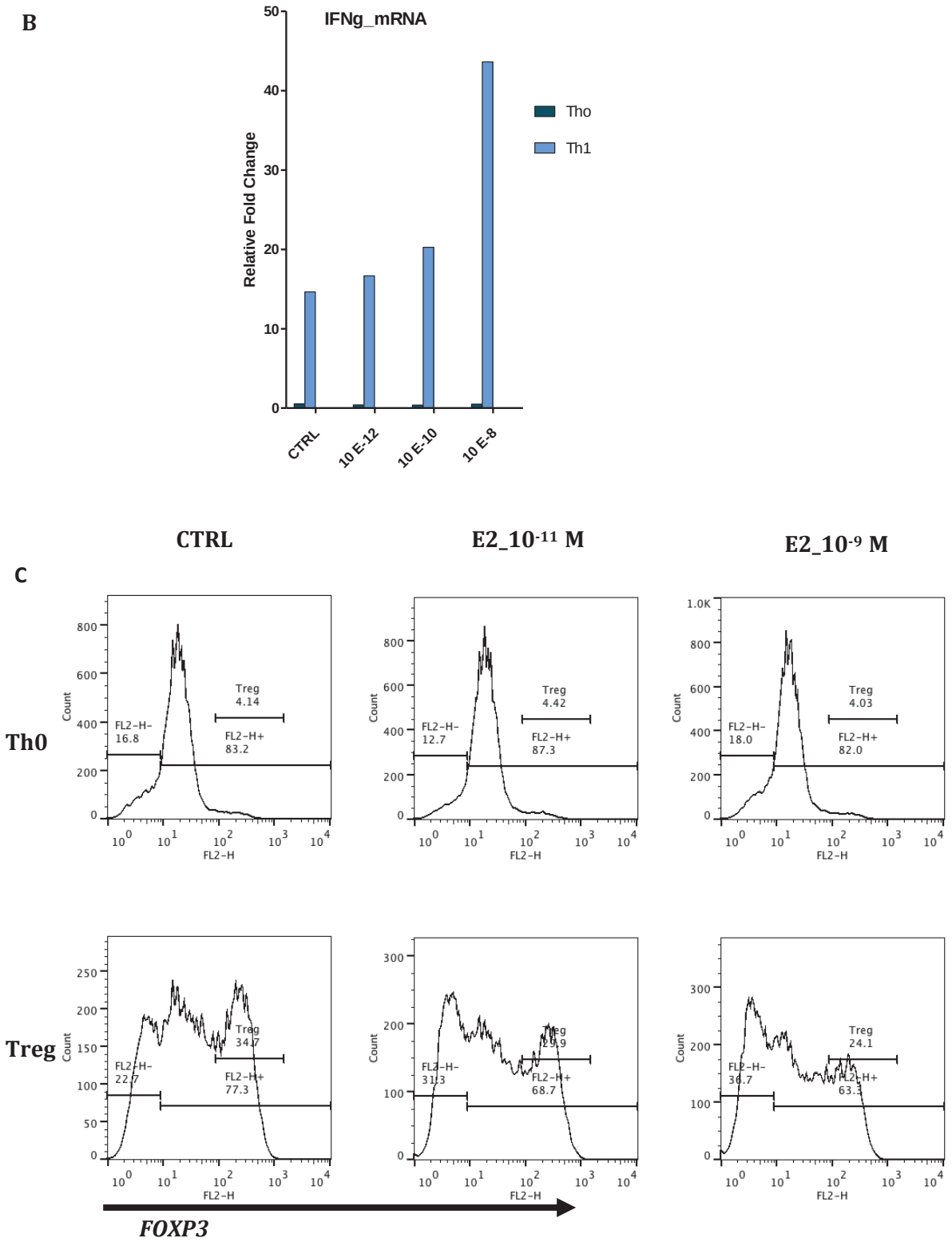


Figure 14: A). Representative Western blot detection of T-bet in estradiol (E2) treated cells. Th0, Th1, Th2 and Treg cells were cultured with 0, 10-11 and 10-9 M of estradiol for 72 hours. GAPDH was used as protein loading control. (B) IFNγ mRNA levels increased in estradiol treated Th1 cells in dose dependent manner (C) Reduced Foxp3 expression in estradiol treated Treg cells in dose-dependent way. Intracellular detection of Foxp3 through FACS staining in Treg cells cultured with 0, 10-11 and 10-9 M of estradiol for 48 hours.

5. Discussion

Differentiation of naïve T helper cells into functionally distinct subsets is of critical importance to human health. The differentiation process is tightly controlled by a complex intracellular signaling network. In this study, I demonstrated the involvement of estrogen receptor in transcriptional regulation in T cells and its effect on the differentiation of T helper cells. After the activation of naïve T cells, I observed the changes of ERα isoforms, specifically prominent was the up-regulation of ERα 66. The full activation of T cell includes CD28 co-stimulation. It has been reported that CD28 co-stimulation in T cells promotes alternative splicing events in activated cells compared to naïve cells (Butte et al., 2012). This might lead to the expression of ERα 66 isoform in activated T helper cells.

The master transcription factors T-bet, GATA3 and Foxp3 were observed to cross-regulate each other and affect the clinical outcome in terms of autoimmunity and infection (Díaz et al., 2010). Therefore, it is important to study the effect of ERα depletion on master transcriptional regulators and cytokines in T helper cells.

In Th1 cells, I observed up-regulation of T-bet in both ERα depleted and estradiol treated cells. Increased IFNγ was also observed in estradiol treated Th1 cells. Moreover, a similar finding was also reported in mice by Karpuzoglu et al. According to that study, the mechanism behind up-regulation of IFNγ levels is not apparent but it is assumed that STAT4β is up-regulated by IL12 in estradiol treated mice (Karpuzoglu et al., 2009) and the rapid activation of STAT4β in nongenomic or genomic manner might be the result of transcriptional cross-talk between STAT4-estrogen or in between estrogen receptors-protein kinase signaling pathways.

In Th2 cells, ERα is lowly expressed and ERβ is expressed in high levels. ERα silencing led to increased GATA3 expression. The up-regulation of GATA3 in ERα silenced Th2 cells is highly surprising as GATA3 and ERα were reported to be highly correlated in terms of expression in other cells such as breast cancer cells (Eeckhoute et al., 2007)

In Treg cells, Foxp3 levels were reduced after ERα silencing and estradiol addition. In ERα -/- mice, the loss of estrogen receptor reduced TGFβ (Hasson et al., 2014). TGFβ is required for iTreg and Th17 cell differentiation in dose-dependent manner. At higher concentrations, TGFβ suppresses IL23R expression and supports Foxp3+ Treg cells. At lower concentrations, TGFβ up-regulates IL23R expression and promotes Th17 cell differentiation (Zhou et al., 2008).

Hence, it can be assumed that loss of ER might be affecting TGFβ in Treg cells and decreasing the levels of Foxp3.

Most of the studies related to protective effect of estrogens for autoimmunity were performed in EAE, a mouse disease model for multiple sclerosis (Bodhankar et al., 2012, Wisdom et al., 2013). Estrogen receptor β signaling and microglia involvement was reported independently by the groups of Ake Gustafsson (Wu et al., 2013) and Rhonda Vokshul (Wisdom et al., 2013). However, these studies are mostly related to murine models. Our study demonstrates estrogen response and cellular mechanisms in human immune cells as I have used human cord blood as a model to study. To develop therapeutic strategies combating human autoimmune diseases, understanding the molecular mechanisms how estrogen through its receptor influences the differentiation of Th subsets is particularly valuable.

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8. Appendix

1. Protein quantification

Protein quantification was performed using Biorad DC (detergent compatible) protein assay kit. For measurement, 1 µl of lysed protein sample was taken and added with 25 µl of reagent A and reagent S. 200 µl of reagent B was pipetted to the above reagents in 96-well plate and incubated for 10 mins at room temperature. Finally, at 750nm the protein concentration was measured with Multiskan ascent plate reader.

2. List of Antibodies used for Western blotting

Antibody	Catalog No.	Company	Dilution
T-bet	SC-21749	Santacruz	1:5000
GATA3	SC-268X	Santacruz	1:5000
Foxp3	14-4776-82	eBioscience	1:5000
ERα	8644	Cell signaling	1:5000
Gapdh	5G4Mab6C5	Hytest	1:10,000
H2B	SC10808	Santacruz	1:5000
Anti-rabbit	18-8816-33	eBioscience	1:10000
Anti-mouse	SC-2005	Santacruz	1:10000
Anti-rat	SC-2956	Santacruz	1:10000

3. List of Antibodies used for FACS

Antibody	Catalog No.	Company	Dilution
T-bet-PE	SC 21749	Santacruz	1:100
GATA3-Alexa Fluor® 488	560163	Santacruz	1:100
Foxp3-PE	12-4776-42	eBioscience	1:100

4. siRNA sequence

siRNA	sense strand	anti-sense strand
Scr_siRNA_1	GCGCGCUUUGUAGGAUUCG dT][dT]	CGCGAAACAUCCUAACG[dT][dT]
Scr_siRNA_2	AUUCUCCGAACGUGUCACGU[dT][dT]	UAAGAGGCUGCACAGUGCA[dT][dT]
***ESR1*_siRNA_1**	GAUCAAACGCUCUAAGAAG[dT][dT]	CUUCUUAGAGCGUUUGAUC[dT][dT]
***ESR1*_siRNA_2**	GAAUGUCGGUGGCUAGGA[dT][dT]	UCUCUSGCCAGGCACAUUC[dT][dT]
***ESR1*_siRNA_3**	GAUGAAAGGUGGGAUACGA[dT][dT]	UCGUAUCCCACCUUUCAUC[dT][dT]
***ESR1*_siRNA_4**	GCCAGCAGGUGCCCUACUA[dT][dT]	UAGUAGGGCACCUGCUGGC[dT][dT]
STAT4	GGUACAACGUGUCAACCAA[dT][dT] GGCAACGAUUCUUCUUCAA[dT][dT]	UUGGUUGACACGUUGUACC[dT][dT] UUGAAGAAGAAUCGUUGCC[dT][dT]
STAT6	AAGCAGGAAGAACUCAAGUUU[dT][dT] CAGUUCCGCCACUUGCCAAU [dT][dT] GAAUCAGUCAACGUGUUGUCA[dT][dT]	UUCGUCCUUCUUGAGUCAAA[dT][dT] GUCAAGGCGUGACGGUUA[dT][dT] CUUAGUCAGUUGCACAACAGA[dT][dT]
STAT3	CGUUAUAUAGGAACCGUAA[dT][dT] GGAGAAGCAUCGUGAGUGA[dT][dT] CCACUUGGUGUGUUUCAUAA[dT][dT] UCAGGUUGCUGGUCAAAUU[dT][dT]	UUACGGUUCCUAUAUAACG[dT][dT] UCACUCACGAUGCUUCUCC[dT][dT] UUAUGAAACACCAAAGUGG[dT][dT] AAUUUGACCAGCAACCUGA[dT][dT]

5. Primers used in the study

Primer	Forward /Reverse/Company	Probe library /Catalog No.
IFNG	Applied Biosystems	Hs00174143
GATA3	Applied Biosystems	Hs00231122
FOXP3	TCTTGAGTCCCGTGCAGAC GAGACACGGGGTATTTTTGG	5
Il17	Applied Biosystems	Hs00174383
ESR1	TTACTGACCAACCTGGCAGA ATCATGGAGGGTCAAATCCA	24

ESR2	ATGATGGGGCTGATGTGG TTCTACGCATTTCCCCTCA	50
HPRT	Applied Biosystems	4326321E-0601007