PhD Program in Complex Systems for Life Sciences

The effects of estrogens and Interferon-β on T helper 17 cells in Multiple Sclerosis: analysis of -omics data as a tool for identifying molecular targets

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1. Table of abbreviations

Abbreviation	Full Name
5hmC	5-Hydroxymethylcytosine
5mC	5-Methylcytosine
AF	Activation Function Domain
APC	Antigen-Presenting Cell
ARC	Activator-Recruited Co-Factor
ARR	Active Regulatory Region
BAD	Bcl2 Associated Agonist Of Cell Death
BCL2A1	Bcl2 Related Protein A1
BET	Bromodomain And Extra-Terminal Motif
BMDM	Bone Marrow-Derived Macrophages
Breg	Regulatory B Cell
CARM1	Coactivator-Associated Arginine Methyltransferase 1
CBP/p300	Creb-Binding Protein/P300
CCL2	Chemokine C-C Motif Ligand 2
CD	Cluster Of Differentiation
CFLAR	Casp8 And Fadd Like Apoptosis Regulator
ChIP-qPCR	Chromatin Immunoprecipitation Followed By Quantitative Polymerase Chain Reaction
ChIP-Seq	Chromatin Immunoprecipitation Followed By Sequencing
CI	Confidence Interval
CINC	Cytokine-Induced Neutrophil Chemoatractant
CIS	Clinically Isolated Syndrome
CNS	Central Nervous System
CNS0	Conserved Non-Coding Sequence 0
CNS1	Conserved Non-Coding Sequence 1
CNS2	Conserved Non-Coding Sequence 2
CSF2RB	Colony Stimulating Factor 2 Receptor Beta Common Subunit
CSR	Cell-Type Specific Regulatory Regions
CTLA-4	Cytotoxic T Lymphocyte-Associated Protein 4
CTSL	Cathepsin L
CTSS	Cathepsin S
CXCL10	C-X-C Motif Chemokine Ligand 10

DBD	DNA Binding Domain
DC	Dendritic Cell
DDX60	Dexd/H-Box Helicase 60
DE	Differentially Expressed
DICE	Database Of Immune Cell Expression, Expression Quantitative Trait Loci, And Epigenomics
DMDs	Disease Modifying Drugs
DMTs	Disease-Modifying Therapies
DNA	Deoxyribonucleic acid
DNMT3A	Dna Methyltransferase 3a
DRIP	Vitamin D Receptor Interacting Protein
E1	Estrone
E2	17β-Estradiol
E3	Estriol
EAE	Experimental Autoimmune Encephalomyelitis
EAN	European Academy Of Neurology
EBV	Epstein–Barr Virus
ECTRIMS	European Committee For Treatment And Research In Multiple Sclerosis
EIF2AK2	Eukaryotic Translation Initiation Factor 2 Alpha Kinase 2
EIF2AK3	Eukaryotic Translation Initiation Factor 2 Alpha Kinase 3
eQTL	Expression Quantitative Trait Loci
ER	Estrogen Receptor
ERE	Estrogen Response Element
ERα	Estrogen Receptor Alpha
ERα36	36 KDa Erα Variant
ERα46	Af-1 Domain-Truncated 46 KDa Variant Of ERα
ERα66	Full-Length 66 KDa ERα
ERβ	Estrogen Receptor Beta
ESR1	Estrogen Receptor 1 human gene
ESR2	Estrogen Receptor 2 human gene
FASLG	Fas Ligand
FBS	Fetal Bovine Serum
FDR	False Discovery Rate
FDR	False Discovery Rate
FIMO	Find Individual Motif Occurrences Software
FN1	Fibronectin 1

FOXP3	Forkhead Box P3
FPKM	Fragments Per Kilobase Of Exons Per Million Fragments Mapped
GC	Germinal Center
GCN5	Histone Acetyltransferase Gcn5
GO	Gene Ontology
GREAT	Genomic Regions Enrichment Of Annotations Tool
H3K27ac	Histone 3 Acetylation Of Lysine 27
H3K27ac	Histone H3 Acetylated Lysine 27
H3K27me3	Histone 3 Trimethylation Of Lysine 27
H3K4me3	Histone 3 Trimethylation Of Lysine 4
HD	Healthy Donors
HERC5	Hect And Rld Domain Containing E3 Ubiquitin Protein Ligase 5
HERC6	Hect And Rld Domain Containing E3 Ubiquitin Protein Ligase Family Member 6
HLA	Human Leukocyte Antigen
hPSCs	Hematopoietic Pluripotent Stem Cells
HSF4	Heat Shock Transcription Factor 4
IAP	Inhibitors Of Apoptosis Protein
IFI27	Interferon Alpha Inducible Protein 27
IFI44L	Interferon Induced Protein 44 Like
IFI6	Interferon Alpha Inducible Protein 6
IFIT1	Interferon Induced Protein With Tetratricopeptide Repeats 1
IFIT2	Interferon Induced Protein With Tetratricopeptide Repeats 2
IFIT3	Interferon Induced Protein With Tetratricopeptide Repeats 3
IKZF2	Ikaros Family Zinc Finger 2
IKZF4	Ikaros Family Zinc Finger 4
IL	Interleukin
INF-β	Interferon-Beta
iNOS	Inducible Nitric Oxide Synthase
IRF1	Interferon Regulatory Factor 1
IRF8	Interferon Regulatory Factor 8
ISG	Interferon-Stimulated Gene
ISG15	Isg15 Ubiquitin Like Modifier
JIA	Juvenile Idiopathic Arthritis
JUN	Jun Proto-Oncogene, Ap-1 Transcription Factor Subunit
KAT5/TIP60	Histone Acetyltransferase Kat5/Tip60
LBD	Ligand Binding Domain

LGALS3BP	Galectin 3 Binding Protein
Limma	Linear Models for Microarray Analysis
lincRNA	Long Intergenic Non-Coding Rna
mAbs	Monoclonal Antibodies
MAF	Maf Bzip Transcription Factor
MD	Mean Difference
MMP	Matrix Metalloprotease
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
MX1	Mx Dynamin Like GTPase 1
MxA	Myxovirus-Resistance Protein A
Nabs	Neutralizing Anti-Interferon-Beta Antibodies
NCoR	Nuclear Receptor Co-Repressor
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NFKB1	Nuclear Factor Kappa B Subunit 1
NGS	Next-Generation Sequencing
NK	Natural Killer
NOTCH1	Notch Homolog 1, Translocation-Associated
Nrf2	Nuclear Factor Erythroid-Derived-2-Like 2
NURD	Nucleosome Remodeling Deacetylase
OAS3	2'-5'-Oligoadenylate Synthetase 3
OASL	2'-5'-Oligoadenylate Synthetase Like
p160/SRC	P160/Steroid Receptor Coactivator
PBMCs	Peripheral Blood Mononuclear Cells
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death-Ligand 1
PIK3R1	Phosphoinositide-3-Kinase Regulatory Subunit 1
PPMS	Primary-Progressive Multiple Sclerosis
PRDM1	Pr/Set Domain 1
PRIMS	Pregnancy In Multiple Sclerosis study
PWMs	Positional Weight Matrices
qPCR	Quantitative Polymerase Chain Reaction
REA	Repressor Of Estrogen Receptor Activity
RELA	Rela Proto-Oncogene, NF-Kb Subunit
RNA	Ribonucleic acid
RORC	Rar-Related Orphan Receptor C

ROSE	Rank Ordering Of Super Enhancers
RR	Relative Risk
RRMS	Relapsing-Remitting Multiple Sclerosis
RSAD2	Radical S-Adenosyl Methionine Domain Containing 2
SIGLEC1	Sialic Acid Binding Ig Like Lectin 1
SMRT	Silencing Mediator For Retinoid Or Thyroid-Hormone Receptors
SNP	Single Nucleotide Polymorphism
SOX2	Sry-Box Transcription Factor 2
SPMS	Secondary Progressive Multiple Sclerosis
SRA	Steroid Receptor RNA Activator
SWI/SNF	SWItch/Sucrose Non-Fermentable
T-bet	T-Box Transcription Factor Tbx21
TET	Ten-Eleven Translocation
TF	Transcription Factor
TFH	Follicular Helper T Cell
TGF-β	Transforming Growth Factor Beta
Th	T Helper Cell
TLL	T-Lymphoblastic Leukemia
TNC	Tenascin C
TNF	Tumor Necrosis Factor
TNFRSF10B	Tnf Receptor Superfamily Member 10b
TPM	Transcripts Per Million
TRAF1	Tnf Receptor Associated Factor 1
TRAIL	Tnf-Related Apoptosis-Inducing Ligand
TRAP	Coactivator Complex And The Thyroid Hormone Receptor-Associated Proteins
Treg	Regulatory T Cell
TSS	Transcription Start Site
USP18	Ubiquitin Specific Peptidase 18
VCAM1	Vascular Cell Adhesion Molecule 1
Weka	Wakaito Environment For Knowledge Analysis
WT	Wild-Type
XAF1	Xiap Associated Factor 1

2. Abstract

Neuroinflammation in Multiple Sclerosis (MS) is mediated by the infiltration of myelin reactive T cells into the central nervous system (CNS). In particular, T helper (Th) 17 cells promote neurodegeneration while regulatory T cells (Treg) are protective. Transcriptomic and epigenomic data allow to observe global changes in response to a hormone or a drug in immune system cells. Moreover, data integration is a powerful tool to explore complex patterns of regulation.

In this PhD project we analyzed genome-wide dynamics behind two phenomena related to MS: estrogen and interferon-β induced transcriptional activity in Th17 cells.

The first part aimed to elucidate the molecular mechanisms behind the anti-inflammatory role of estrogens in MS patients, originating from the correlation between high levels of circulating estrogens during pregnancy and the reduction in relapse rates. We used Chromatin Immunoprecipitation followed by sequencing (ChIP-Seq) data of Histone H3 acetylated lysine 27 (H3K27ac) to identify a set of specific genomic regulatory regions in Th17 and Treg cells. We compared the transcriptome of these T helper subtypes to identify key transcription factors of their specification. We used an enrichment analysis of the estrogen response elements (ERE) within selected regulatory regions to identify genomic targets of estrogen receptor alpha (ERα). Best candidate regions were validated with ChIP followed by quantitative PCR (ChIP-qPCR) in *in-vitro* polarized Th17 cells treated with 17β-estradiol at pregnancy levels. These data indicated that ERα is involved in chromatin remodeling at specific regulatory regions in Th17 cells and their dysfunctional activation may reflect disease progression.

The second part aimed to identify a gene signature of interferon-β (INF-β), the most widely used first-line drug for the treatment of MS. IFN-β reduces the annual rate of relapse in patients with MS, but the classification of non-responders and the identification of therapy biomarkers are still under discussion. We exploited and integrated data sets of IFN-β transcriptome modulation in large cohorts of MS patients. We obtained a transcriptional signature of IFN-β therapy in diverse state of MS progression. Then, we used this signature to highlight the molecular targets of IFN-β in CCR6+ myelin reactive Th17 cells. These results can be useful to explain the mechanisms related to Th17 cells that support the success or failure of IFN-β therapy.

To conclude, omics data integration, common thread of this PhD thesis, constitute an approach to understand the pathogenesis, course and progression of MS that expands the analysis perspective of the mechanisms related to treatment and to particular life situations including pregnancy.

3. Introduction

3.1. Multiple Sclerosis

3.1.1. Etiopathogenesis of Multiple Sclerosis

MS is a chronic inflammatory demyelinating disease of the CNS that lead to axonal damage and is characterized by the infiltration of T cells, B cells, macrophages, and natural killer (NK) cells [1]. The experimental evidence based on Experimental Autoimmune Encephalomyelitis (EAE), the murine model of MS, and samples from MS patients, give us the current definition of the immunological process in the pathogenesis of MS. First, autoreactive T cells and B cells are activated in peripheral lymph nodes and differentiate into effector cells. Among the effector CD4+ T cells, Th1 and especially Th17 cells have important roles in the pathogenesis of this disease. MS patients have shown an increased number of Th17 in the peripheral blood [2], and in cerebrospinal fluid and perivascular space in the CNS [3–6]. Activated T and B cells cross the blood–brain barrier, that is disrupted in the early stages of MS, and arrive in the CNS, where antigen-presenting cells (APC) re-activate them. In the CNS, activated immune system react against myelin components and sustain the inflammation recruiting other autoreactive cells from peripheral blood by producing cytokines and chemokines. Activated B cells mature to antibody-producing plasma cells that induce, maintain, and reactivate CD4+ T cells and produce proinflammatory cytokines. The overall process increases inflammation and cause demyelination and axonal damage. In the later stages of the disease, the inflammatory response is sustained by microglial activation and lead to chronic neurodegeneration [7].

To date, the causes that influence the development and course of MS are still not clear. MS is a multifactorial disease of unknown etiology. Its onset, course and progression depend on both genetic and environmental factors. MS is not inheritable, although first-degree relatives of patients show susceptibility for MS [8,9]. Human leukocyte antigens (HLA) in the class II region, especially the HLA-DRB1*1501 and DQB1*0602 alleles, have been shown to be significantly associated with MS and are currently recognized as predisposing genetic factors [10], whereas the HLA class I region HLA-A*02:01 is recognized as protective [11]. Among environmental factors, the Epstein–Barr virus (EBV) infection [12,13], smoking habitude [14] and vitamin D deficiency [15] exert epigenetic changes and have been linked to the risk of developing MS disease. The more recent evidence has highlighted also intestinal microbiota [16] and oral contraceptive therapy [17] as risk factors for MS. Epidemiology shown a sex difference in the prevalence and progression of MS disease. The relapsing form of MS is more frequent in young

women [18] while in men show more incidence at an older age and a more severe progressive course [19].

3.1.2. MS course and treatment

Relapsing-remitting MS (RRMS) is the most common course of MS since 85%–90% of patients are initially diagnosed with RRMS [20]. RRMS is characterized by the alternation of relapse and remission phases. After RRMS, most patients transition to secondary progressive MS (SPMS) characterized by a progressive accumulation of disability. A primary-progressive MS (PPMS) form is instead the initial form of disease in ~ 10%–15% of patients [20].

MS has no curative treatment available. MS therapeutic strategy is to reduce the risk of relapses avoiding accumulation of disability. Indeed, the early intervention with disease modifying drugs (DMDs) has been shown to reduce long-term disability [21]. MS therapy is a result of balancing considerations on efficacy, side effects and potential damage [20]. The European Committee for treatment and research in multiple sclerosis (ECTRIMS) and the European Academy of Neurology (EAN) provided recommendations for the treatment of MS patients [20]. Currently, 11 disease-modifying therapies (DMTs) have been approved by the European Agency for Medicine [20].

Beta interferons and glatiramer acetate mechanisms of action have been not fully elucidated. However, they inhibit antigen presentation; they induce a shift from proinflammatory phenotype of T cells to a regulatory phenotype of T cells that suppress the inflammatory response; they reduce the entry of T cells into the CNS. Given to a resemblance in the peptide composition of Glatiramer acetate to myelin basic protein, this drug seems to act as a decoy, diverting the autoimmune response against myelin. Dimethyl Fumarate instead, act on the activation of the nuclear factor erythroid-derived-2-like 2 (Nrf2) in the antioxidant response pathway promoting neuroprotection. Other DMDs act blocking the entry of lymphocytes into the CNS (Natalizumab, Laquinimod) or preventing lymphocyte egression from secondary lymphoid tissues (Fingolimod). Another class of DMDs is composed of monoclonal antibodies against cluster of differentiation (CD) 25 (Daclizumab) that selectively inhibits activated T cells, or against CD20 (Ocrelizumab) that targets B lymphocytes, or CD52 that cause the depletion of mature lymphocytes and monocytes (Alemtuzumab). Finally, other DMDs act as antineoplastic (Mitoxantrone) and immunosuppressors inhibiting the de novo pyrimidine synthesis (Teriflunomide) or acting as purine analog that targets lymphocytes and selectively suppresses the immune system (Cladribrine) [22].

Moreover, guidelines also define the starting, the switching, and the interruption of DMTs [22]. It is suggested to continue a DMTs in stable patients, while consider switching DMTs in patients when relapses or side effects appear. Moreover, it is indicated the stopping of DMTs before conception and during pregnancy, with

exception for Interferon-β and Glatiramer acetate that recently, have been indicated as safe to continue during pregnancy and breastfeeding [21].

3.2. Estrogens and MS

3.2.1. Pregnancy protects women with RRMS from relapses

Pregnancy is protective for MS patients. A pattern of remissions at the end of pregnancy and exacerbations during postpartum has been obtained by both retrospective [23] and later prospective studies [24–27]. In 1998, The Pregnancy in Multiple Sclerosis (PRIMS) study prospectively assessed 254 MS patients during pregnancy and reported a 70% reduction in the annualized relapse rate of the third trimester of pregnancy compared with the annualized relapse rate of the year before pregnancy [24,25]. Later, a meta-analysis [26] that included 1221 women with MS confirmed a significant decrease in the relapse rate during pregnancy. Moreover, a larger multicenter retrospective study supported these results [27]. According to the evidence it became clear that the relapse rate decreases during late pregnancy as hormonal secretions increase [24]. Interestingly, the immune response in MS patients is regulated by estradiol that is capable to modulate the expression and release of inflammatory and anti-inflammatory cytokines in CD4+ T helper cells, orchestrating a regulatory immune response [28]. These data suggest a potential application of estrogens in MS therapy, although mechanisms that underlie this process are under investigation and few clinical trials have been completed so far [29–32].

3.2.2. Estrogens and MS pregnancy

Estrogens are sex steroid hormones. They are present in both men and women, but they circulate at significantly higher levels in women during fertility age.
The level of circulating estrogens varies during all stages of a woman's life, starting from childhood until menopause [33]. Endogenous estrogens include estrone (E1), 17β-estradiol (E2), and estriol (E3). E2 is the predominant form of premenopausal period, while E3 is mainly produced during pregnancy, together with high levels of E2 [33,34]. Estrogens primarily promote the development of female secondary sexual characteristics and regulate the menstrual cycle. In addition to sexual development, estradiol influences the functionality of various organs and tissues, including the skin, muscles, adipose tissue, the brain, the cardiovascular system, and bones, and it actively protects against osteoporosis and various cardiovascular diseases [35].
Immune system behavior is affected by the levels of circulating estrogens, especially during pregnancy, when it adapts to establish fetal tolerance [36].
In physiological pregnancy, maternal Treg cells expand in both the peripheral blood and in the placenta in which they suppress the aggressive allogeneic

response directed against the fetus [37,38]. A lack of Treg cells leads to pregnancy failure due to an immunological rejection of the fetus [39,40]. In autoimmune diseases, including MS, Treg cells suppress the autoimmune response. The protective effect of estrogens, observed during pregnancy in MS, is believed to partially result from estrogen-mediated anti-inflammatory cytokine production and Treg cell expansion [37,40].

3.2.3. Estrogen Receptors in the immune system

Estrogens have direct and indirect way of acting that depends on the involvement of their receptors, called estrogen receptors (ERs) [41]. ERs are nuclear steroid receptors that are able to dimerize upon activation and translocate to the nucleus, where they regulate gene expression. Activated ERs can bind directly to specific DNA sequences called estrogen response elements (EREs) and act as transcription factors (TFs) by regulating a broad range of estrogen-responsive genes. Alternatively, ERs can indirectly bind DNA through protein–protein interactions with other transcription factors [42,43]. However, also in hormonal deprived environment, ERs have been shown to bind extensively to the genome of luminal breast cancer cells and regulate the expression of hundreds of genes with developmental functions [44]. ERs exist in two main forms, ER alpha (ERα) and ER beta (ERβ), which are encoded by the human genes Estrogen Receptor 1 (ESR1) and Estrogen Receptor 2 (ESR2). ERα and ERβ share high homology, particularly in the DNA binding domain [45]. The general structure of ERα consists of an N-terminal activation function (AF) -1 domain, which is followed by a DNA binding domain (DBD), a dimerization domain, and the ligand binding/AF-2 domain (LBD). The AF domains are responsible for the recruitment of coregulators; cofactor recruitment by AF-1 is ligand-independent, whereas cofactor recruitment by AF-2 is ligand-dependent [46]. Three main different isoforms of ERα, derived from alternative splicing events, have been described: the full-length 66 kDa ERα (ERα66), the AF-1 domain-truncated 46 kDa variant of ERα (ERα46), and a 36 kDa ERα variant (ERα36) that lacks both AF-1 and AF-2 domains [47–49]. Similarly, ERβ is transcribed from at least two additional upstream promoters and undergoes alternative splicing, leading to at least five protein isoforms (ERβ1–5) [45].
The ovary, uterus, and breasts express ERs in abundance and, therefore, represent the main target tissues of estrogens. However, estrogens affect many other tissues, including the immune system, in which ER signaling contributes to the regulation of the immune response. ER expression in peripheral blood mononuclear cells (PBMCs) has been explored by using different techniques: quantitative PCR (qPCR), flow cytometry, and Western blotting have indicated that ERs are differentially expressed in PBMC subsets [50,51]. Gene expression analysis by qPCR has shown that ERα and ERβ are endogenously expressed in Th lymphocytes [50], and their expression levels in B lymphocytes seem to be higher than those expressed in CD4+ T cells and CD8+ T cells; this is especially the case for ERβ.

Comparisons between CD4+ T cells and CD8+ T cells suggest that CD4+ T lymphocytes express higher levels of ERα. The immunostaining approach has been used to confirm and better characterize the expression of a specific receptor in the same cell type. This approach has shown that CD4+ and CD8+ T lymphocytes, B lymphocytes, and NK cells contain intracellular ERα and ERβ, and data suggest that ERβ is expressed at a lower level with respect to ERα [51]. Interestingly, the short isoform ERα46 is the most represented isoform in T cells compared with ERα66 [51]. The ERα46 protein is also predominantly expressed by human macrophages in addition to the full-length ERα66 [52]. ERα46 is formed by skipping exon 1, which encodes the AF-1 domain that is responsible for ligand-independent transactivation. ERα46 and ERα66 share a ligand-binding site and a DNA binding site, but they differ in the AF-1 domain. As a result of this difference, the mechanisms of coregulator recruitment differ between cells with high levels of the short isoform and target tissues in which the long isoform predominates and is constitutionally expressed at very high levels. Specific tissue mechanisms depend on expression, the heterodimerization of receptor isoforms, competition for DNA binding sites, or a combination of these processes [47]. Moreover, ERα66 and ERα46 have similar estrogen binding affinity, but they bind differentially to some estrogen receptor agonists and antagonists. In particular, a classical estrogen receptor antagonist, ICI 182,780 (Fulvestrant), was found to have a higher affinity for ERα66 than ERα46 [53]. In the age of NGS, gene expression databases are a popularly used tool to explore cell-type-specific gene expression levels [54]. Interestingly, gene expression of ERα and ERβ is higher in B and T lymphocyte subtypes and NK cells in their non-activated state compared with in vitro activated lymphocytes and circulating monocytes (Figure 1).

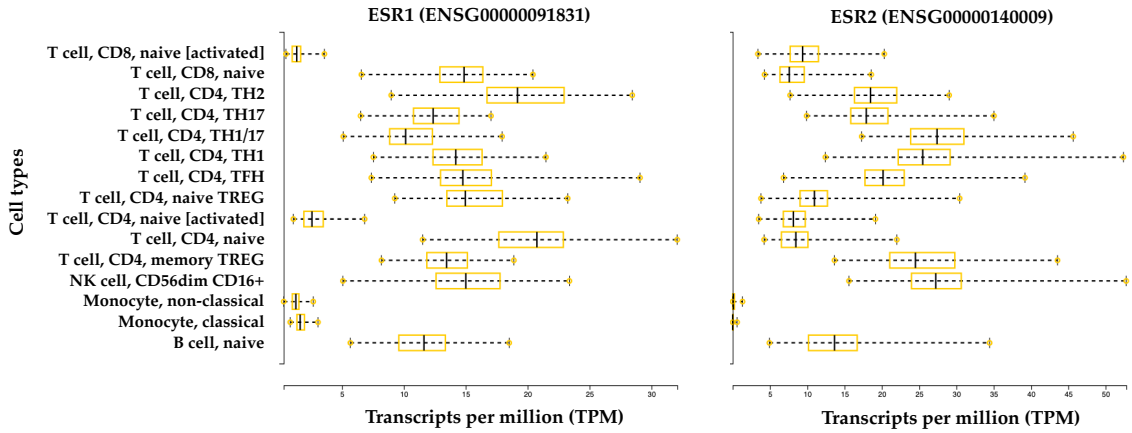

Figure 1. ERα and ERβ expression in the immune system. The bar plots represent gene expression data of the human genes ESR1 and ESR2, which encode for ERα and ERβ, respectively. Data were retrieved from the Database of Immune Cell expression, expression quantitative trait loci (eQTL), and epigenomics (DICE) [54]. RNA-Seq data are normalized between samples and expressed in transcripts per million (TPM). Data were generated from 13 immune cell types from 91 healthy subjects. The cell types include: three innate immune cell types (CD14high CD16− classical monocytes, CD14− CD16+ non-classical monocytes, and CD56dim CD16+ NK cells); four adaptive immune cell types that have not encountered their cognate antigen in the periphery (naive B cells, naive CD4+ T cells, naive CD8+ T cells, and naive Treg cells); six differentiated T cell subsets (Th1, Th1/17, Th17, Th2, follicular helper T cells (TFH), and memory Treg cells); and two ex vivo activated cell types (naive CD4+ and CD8+ T cells).

Along with gene expression, a fundamental aspect of ER function in the cell is the recruitment of coregulating proteins that are necessary for mediating the transcriptional activity of ERs. The resulting complexes contribute to epigenetic modifications and chromatin remodeling that transform the response to hormones or pharmacological ligands involved in regulatory activity [55]. Epigenetic modifications are hereditary modifications that do not alter the DNA sequence but regulate gene expression. At the DNA level, the most frequent epigenetic modification is the methylation of cytosine in CpG islands. Usually, hypomethylated CpG islands are associated with active genes, while CpG hypermethylation tends to silence gene expression. At the chromatin level, on the other hand, histone acetylation and methylation model chromatin and form active regulatory regions as enhancers and promoters or repressed heterochromatic regions (e.g., histone H3 lysine 27 acetylation in active regulatory regions increases the accessibility of chromatin to TFs). DNA methylation and demethylation often contribute to the inheritable organization of chromatin, while histone modifications are able to confer cellular identity but remain sufficiently malleable to regulate response to stimuli. Once activated, ERs recruit chromatin remodeling complexes in a timed and sequential manner. These mechanisms have been described in detail in the model MCF-7 breast cancer cell line [55,56]. The described ER*α*-associated transcriptional coactivator complexes include histone arginine methyltransferases (e.g. p160/SRC, CARM1), histone acetyltransferases (e.g. CBP/p300, KAT5/TIP60, GCN5), RNA-processing factors (e.g. SRA), and polymerase II mediator complexes (e.g. TRAP/DRIP/ARC). Conversely,

corepressors include chromatin remodeling complexes (e.g. SWI/SNF, NURD) and basal corepressors with histone deacetylase activity (e.g. NCoR, SMRT). The Next-generation sequencing (NGS) technologies have broadened the understanding of these processes by showing estrogen binding to ERs in distal regulatory regions to modulate the expression of several hundreds of target genes [57,58]. In recent years, the recruitment of coregulators has been shown to lead to the remodeling of chromatin's 3D organization [59]. This 3D rearrangement results in the formation of functional chromatin loops between ERα binding sites at the enhancers and promoters of target genes that are activated [60–62]. The formation of loops mediated by ERα is also involved in the mechanisms of gene repression. Estrogen-mediated DNA looping represses diverse chromosomal regions through DNA methylation and repressive chromatin modifications that inhibit gene expression [63]. Furthermore, ERα activity is influenced by the tissue-specific presence of coactivators and transcriptional corepressors and their differential interaction with ERα in the presence of estrogens or anti-estrogens [64,65].

3.2.4. Effects of estrogens on the immune system of MS patients

Increasing evidence highlight the action of estrogens on the immune system. These aspects have been described in both physiological (e.g., pregnancy) and pathological conditions of the immune system (e.g., autoimmunity and the tumor microenvironment) [66,67]. The role of ERs in the regulation of innate immune system cells has been described in recent reviews [68–70], which have suggested estrogens' potential contribution to sex differences in the innate immune response by affecting both progenitor and mature cells. Moreover, changes in circulating estrogen levels can affect progenitor and mature cells of both the innate and adaptive immune systems. ERα is present in most cells from the early stages of hematopoietic development to lymphocyte development in the thymus [66,68,71]. In the early stages, E2 enhances the expansion of hematopoietic pluripotent stem cells (hPSCs) [71] the differentiation of monocytes to macrophages [52], thymus trophism, and the maturation of double positive cells (CD4+ CD8+) [72,73] through ERα-dependent pathways.

3.2.4.1. Effects of estrogens on the innate immune system

The role of ERs in the regulation of the development and functions of innate immune cells has been discussed in details [18,66,67]. Main findings are reported in a recent review by our group [33]. Estrogens affect the innate immune system by regulating the number of cells and their specific biological functions: in neutrophils, they regulate chemotaxis, infiltration, and the induction of cytokine-induced neutrophil chemo attractants (e.g., CINC-1, CINC-2β, CINC-3) and cytokines (e.g., TNF-α, IL-6, IL-1β); in macrophages, they regulate chemotaxis, phagocytic activity, and the production of cytokines (e.g., IL-6, TNF-α); in NK cells,

they decrease cytotoxicity; in dendritic cells (DCs), they promote differentiation and regulate chemokine (e.g., IL-8 and CCL2) and cytokine (e.g., IL-6, IL-10) expression [33]. In the context of MS, ERα activation delays the onset of EAE, while ERβ activation sustains later neuroprotection. Indeed, both ERα and ERβ signaling reduce demyelination, axonal loss, and neuronal pathology in EAE, but only ERβ activation induces the recovery of motor performance [74]. The anti-inflammatory action of ERα is connected to the modulation of microglia, which survey the CNS for infections and have functions that are similar to macrophages in the periphery [75]. ERα regulates the inflammatory pathway in microglia, likely by reducing the time of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcriptional activity and thus regulating inflammatory signaling [76,77]. The later neuroprotection mediated by ERβ activation is connected to the observed effects on macrophages in the CNS. ERβ activation induces CD11c+ DCs and macrophages to express less inducible NOS (iNOS) and T-box transcription factor TBX21 (T-bet) and more IL-10, and these effects favor immunotolerance in EAE mice. Furthermore, ERβ activation induces the maturation of oligodendrocytes and enhances remyelination [78]. The innate and adaptive immune systems are closely connected, and it has become evident that estrogens can regulate the interactions among immune cell types. Indeed, ERs sustain neuroprotection in EAE by regulating the interactions between innate immune cells and both T [79] and B cells [80].

3.2.4.2. Effects of estrogens on the adaptive immune system

Estrogens act on the adaptive immune system by modulating the production of cytokines and interleukins (IL) and influencing the differentiation of lymphocytes and the inflammatory environment [33]. Estrogens have profound effects on B cell differentiation, activity, and survival [81–83]. Estrogens increase the numbers of plasma cells and autoantibody-producing cells [84]. Estrogens promote IL-10 secretion in regulatory B cells (Breg), a specific subset of B cells that can negatively regulate T cell immune responses, thereby controlling the follicular T cell response in germinal centers [85]. Together with Treg cells, the frequency of Breg cells increases during pregnancy [86]. B cells contribute to the pathogenesis of MS by producing anti-myelin antibodies, acting as antigen-presenting cells, and producing cytokines [87,88]. Interestingly, recent evidence showed that B cells are required for E2-mediated protection against EAE. The effects of E2 on Breg cells are mediated through ERα and the programmed cell death protein 1 (PD-1) pathway. Treatment with E2 upregulates programmed death-ligand 1 (PD-L1) in B cells and increases the percentage of Breg cells that produce IL-10. These results suggest that the anti-inflammatory effects of estrogens are also mediated by Breg cells, which suppress neuroinflammation during EAE and reduce the number of proinflammatory cells that infiltrate the CNS [89–91].

E2 modulates cytokine secretion by CD4+ T cells from healthy subjects and self-reactive CD4+ T cell clones isolated from MS patients. Low concentrations of E2 (i.e., levels during the pre-ovulatory phase of the menstrual cycle) induce IFN-γ production in T cells in mice [92,93], humans [94], and MS Th clones [95]. IFN-γ is the principal cytokine secreted by activated T cells as well as other cell types, such as NK, B, and APCs, in order to promote cell-mediated immunity. IFN-γ stimulation by estrogens is mediated by ERα regulation of the IFN-γ gene [92], the Th1-specific transcription factor T-bet [96], or both. On the other hand, high doses of E2 (i.e., levels during pregnancy) in these immune cells induce the expression of the transforming growth factor beta (TGF-β) and anti-inflammatory IL-10 [95,97]. Although E2 is able to stimulate both IFN-γ and IL-10 at the same time, the results of these two events do not seem to conflict. An increase in the concentration of estradiol favors immunotolerance by significantly decreasing the IFN-γ/IL-10 ratio [98]. Moreover, in human CD4+ T cells, the production and secretion of Tumor Necrosis Factor-α (TNF-α) were seen to increase at low E2 concentrations and be inhibited at high E2 concentrations [95]. Estrogens have a less marked effect on IL-4 production in CD4+ T cells [95,97,98]. IL-4 antagonizes the effects of IFN-γ and thus inhibits T cell-mediated immunity. During the menstrual cycle, a positive correlation exists between estrogen levels and IL-4 [84]. The hormone progesterone induces IL-4 production in Th cells [99] but does not affect IL-12, IFN-γ, IL-10, and TNF-α [98]. During pregnancy, the modulation of IL-4 is attributed to progesterone, and the immune-tolerance environment can be realized and maintained by the combined action of progesterone and estrogen, which affect the synthesis of various anti-inflammatory cytokines [100].

Estrogens at pregnancy levels enhance the expression of the transcription factor forkhead box P3 (FOXP3), which is specific for Treg, in mice [101]. We recently demonstrated that FOXP3 expression is promoted in human PBMCs upon stimulation with pregnancy levels of estradiol from Th17 cells undergoing polarization in vitro [102]. Moreover, estradiol potentiates the suppressive function of Treg cells by promoting their proliferation [103]. Estrogens also regulate immune checkpoints. Immune checkpoints involve protein-protein interactions that modulate the signaling pathways responsible for immunological tolerance. PD-1 and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) are immune checkpoint proteins, and their expression is regulated by ERα-mediated signaling [104,105].

The anti-inflammatory effect of estrogens also involves Th17 cells. Th17 cells, which are characterized by the production of the proinflammatory cytokine IL-17, have been associated with the pathogenesis and outcome of several autoimmune diseases, including MS [2,106]. The importance of estrogens in the modulation of the adaptive immune system during MS is supported by data from the EAE murine model of MS. In mice with EAE, pregnancy limits cell infiltration and reduces CNS demyelination. Induced immunization during pregnancy leads to a reduction in the incidence of EAE and a decrease in clinical severity, while immunization during the postpartum period increases the severity of the disease [107]. In addition, the

effects of pregnancy are evident even when the pregnancy occurs after the onset of EAE [108]. The protective effect is mediated by a reduction in TNF-α- [109] and IL-17-secreting cells and an increase in IL-10-secreting cells. E2 promotes immune tolerance by enhancing the Treg cell compartment and FOXP3 expression [101]. E2 treatment in mice strengthens the expression of PD-1 in Treg cells in a dose-dependent manner and correlates with the efficiency of EAE protection. E2 at pregnancy levels, but not at lower concentrations, increases the frequency of Treg cells and drastically reduces the production of IL-17 in the peripheral blood of immunized EAE mice. Treatment with E2 does not protect against EAE in mice with PD-1 deficiency [110]. Moreover, Esr1 -/- immunized mice are not protected against EAE in the presence of E2. The splenocytes of Esr1 -/- mice produce more TNF-α, IFN-γ, and IL-6, even in the presence of E2. In contrast, in wild-type (WT) mice and Esr2 -/- mice, E2 treatment produces clinical signs of EAE suppression and eliminates inflammatory lesions in the CNS [111]. These results show that the reduction in EAE severity involves the genomic action of E2 via ERα [74] and that the anti-inflammatory effect is mediated by ERα but not ERβ [74,111]. Moreover, experiments using ERα-deficient mice have demonstrated that T lymphocytes (but not macrophages or dendritic cells) require ERα for the E2-mediated inhibition of Th1/Th17 cell differentiation and protection from EAE [112]. The results of these studies emphasize the role of Th17 and Treg cells in ERα-mediated E2 modulation in EAE.

3.2.5. Estrogens modulate the T Helper Epigenome in MS

The genomic regulatory landscape of cells controls gene expression and defines cell identity. The phenotypes of Th cells are determined by their cytokine secretion, gene expression, and surface molecules, which guide their action in the adaptive immune system. Th cells react to environmental stimuli by repolarizing to different cell subtypes in a phenomenon defined as plasticity [113]. Epigenetic reprogramming is a series of events that underlie plasticity, and this process determines the difference between a pro-inflammatory and an anti-inflammatory environment [114]. In this context, chromatin states described by epigenetic modifications play a role in the regulation of the immune response. Histone modifications regulate transitory responses to stimuli. Histone modifications are able to maintain a stable cellular state while remaining sufficiently malleable to allow the plasticity in Th cells. In fact, the histone modifications that determine the accessibility of chromatin to TFs change in response to different contexts and stimuli [115]. Pioneering studies on this subject described changes in histone modification at the promoter of lineage-determining TFs in T cells as a molecular mechanism that occurs during cell plasticity [116]. Epigenome dynamics in T cells have been described and discussed, starting from their development in the thymus to their peripheral plasticity [117]. The balance between Th17 and Treg is widely considered to reflect inflammation in MS and is strongly connected to disease

outcomes [118]. Th17 and Treg have a high degree of plasticity, which allows for their functional adaptation to the phases of the immune response. However, Th17-Treg plasticity could also be a critical factor in MS [119]. The integration of epigenomics and transcriptomics data have been used to unravel the intricate gene regulatory circuits underlying these processes in Treg [102,120] and Th17 cells [102,121,122].

Some epigenetic regulation mechanisms and targets have been associated with EAE and the Th17–Treg axis. In encephalitogenic T cells of EAE mice, signaling through CD44 causes increased methylation of Ifng/Il17a and demethylation of Il4/Foxp3 [123]. Since CD44 expression is chronically elevated in MS demyelinating lesions, this mechanism has been proposed to sustain inflammation at the sites of CNS lesions [123]. Conversely, the CD27 and CD70 costimulatory pathway results in the epigenetic silencing of the IL17a gene, thus inhibiting Th17 differentiation [124]. In particular, FOXP3, given its role as a key transcription factor in Treg cells, has long been studied in the context of epigenetic regulation and autoimmunity. The demethylation of the conserved non-coding sequence 0 (CNS0) in the FOXP3 locus helps to stabilize the identity of Treg cells [125]. In addition to CNS0, at least two other known CNSs are responsible for FOXP3 regulation (i.e., CNS1 and CNS2) [126]. Recent studies on CNS1—a FOXP3 intronic enhancer that is essential for the development of peripheral Treg cells—have reported that the adaptation of the immune system during pregnancy enabled maternal–fetal tolerance [125]. Moreover, the deletion of CNS2—a FOXP3 enhancer—led to reduced stability and the loss of FOXP3 expression in proliferating Treg cells [125,127,128]. However, FOXP3 alone does not control all aspects of Treg biology and is not the initiating factor in Treg development. DNA demethylation of Treg signature genes is required for the stable maintenance of the Treg phenotype and function [129,130]. The establishment of the Treg-specific epigenome starts before FOXP3 expression. Indeed, FOXP3 exploits a pre-existing enhancer landscape and a TF network of Treg cells [131–133]. Ten-eleven translocation (TET) proteins regulate DNA methylation and gene expression by converting 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC). Treg cells in mice with specific Tet2/Tet3 deficiency begin to express IL-17. This phenotypic shift occurs not only at the level of known CNSs but also in new regions identified as FOXP3's upstream enhancer, which could contribute to stable FOXP3 expression [134]. DNA methyltransferase 3A (DNMT3A), responsible for "de novo" methylation, prevents methylation of the FOXP3 locus [135], thus supporting Treg cell identity at sites of inflammation by keeping CNS2 in a demethylated state and allowing for the maintenance of its suppressive function. Interestingly, the epigenetic reprogramming of peripheral Treg cells is possible to achieve in vitro through the demethylation of the RAR-related orphan receptor C (RORC) locus and the development of Th17-like cells [136]. The role of estrogens and ER in the complexity of epigenetic regulation mechanisms in T cells has been poorly studied, but some evidence has emerged from recent studies. As previously described,

estrogens promote the activation of ERα and its transcriptional activity through interactions with ERE. ERα binding at the RORC and FOXP3 regulatory regions has been recently demonstrated. In both in vitro experiments and pregnant MS patients, E2 at pregnancy levels inhibited Th17 polarization, thereby reducing RORC expression and enhancing FOXP3 transcription as a result of ERα binding to their promoters and enhancers [102]. The molecular mechanisms of this process remain elusive. However, the suppressive action of ERα in Th17 cells could be mediated by the recruitment of the repressor of estrogen receptor activity (REA). The ERα/REA complex recruits histone deacetylases to the RORC promoter to suppress its expression [137]. In the orchestration of chromatin architecture, ERα may mediate epigenetic modifications at chromatin hubs in CD4+ T cells to affect their differentiation and plasticity. In this respect, ERα may act as a cooperative TF in the T cell epigenome dynamic. Understanding the steps that lead to this mechanism may open doors to new therapeutic approaches that exploit this property of T cells. Moreover, estrogens have been combined with DMDs, including IFN-β, in clinical trials with clinical safety and promising results [32]. Molecular investigations will elucidate mechanisms behind hormonal therapy in combination with DMDs.

3.2.6. Estrogens as a potential therapy for MS patients.

The protective effects of estrogens have been explored in a first clinical trial in 2002, using estriol, the form of estrogens that is mainly produced during pregnancy [29]. Estriol was administered in 10 female MS patients decreasing the volume of MRI lesions in all patients. This beneficial effect was lost 3 months after treatment was stopped [29]. Estriol was well tolerated, no serious side effects were observed; there were neither significant alterations in any laboratory measures including sexual hormone levels [29]. In parallel, estriol treatment showed reduced IFN-γ levels and the production of TNF-α, an increase of two anti-inflammatory interleukins. IL-5 production increased in CD4+ T cells and IL-10 production increased in macrophages, leading to an immune tolerant environment [29].

These promising results leaded to a larger phase 2 trial where estriol have been used in combination with DMDs. This trial enrolled 164 MS patients and estriol was used as an add-on therapy compared with glatiramer acetate alone [30]. Estriol in combination with glatiramer acetate reduced the annualized relapse rate (0.25 relapses per year versus 0.37 relapses per year in the control group). Estriol concentration in sera were inversely correlated with the number of relapses and the number of active lesions on brain Magnetic Resonance Imaging (MRI) [30]. No differences were observed in the number of cerebral lesions (enhancing or T2 lesions), however post-hoc volumetric study, showed less cortical grey matter atrophy in the estriol group than in the control group [30]. Moreover, safety analysis indicated safety of the hormonal treatment [30]. In 2009, a double-blind placebo-controlled phase 3 study enrolled 300 pregnant MS women with the aim

to prevent postpartum MS relapses by treatment with Nomegestrol Acetate (LUTENYL® 10 mg/day) combined with transdermal estradiol (DERMESTRIL SEPTEM® 75 μg, once a week) for a period of 24 weeks after delivery. The results did not show clear beneficial effects [31].

More recently, a new phase 2 clinical trial was conducted to evaluate the effects of estradiol in combination with DMDs [32]. In this trial MS patients received high-dose ethinylestradiol and desogestrel in addition to IFN-β. The group treated with estradiol showed a significant decrease in new gadolinium-enhancing lesions compared with IFN-β group over a 96-week period and clinical safety was assessed [32]. These results enhance the role of estrogens and make the prospect of a hormonal combination with DMDs in the therapeutic field a more concrete step.

3.3. Interferon-β and MS

3.3.1. Interferon-β protects RRMS patients from relapses

IFN-β therapy was the first proposed and approved DMD for the MS treatment and, to date, it still remains one of the most used first line DMD for the treatment of MS [138,139].

IFN-β therapy is most effective in early RRMS with little benefit on progression in the later stages of MS [140]. Because of the benefits of early intervention and their safety profile, beta interferons are offered to patients since they manifest a clinical isolated syndrome and an abnormal MRI with lesions suggestive of MS but who do not fulfil criteria for MS diagnosis [20,141–143].

The therapeutic efficacy of IFN-β in RRMS has been confirmed by several clinical trials. Interferon resulted in a lower annualized relapse rate at 48 weeks' follow-up (RR=1.15, 95% CI: 1.08–1.23) [144] and at 104weeks' follow-up (n=960; RR=1.73, 95% CI: 1.35–2.21) [138,145,146]. Interferon reduced disability worsening confirmed at 3 months over 48weeks' follow-up (RR = 0.61, 95% CI: 0.39–0.93; n = 1012) [144] and on disability worsening confirmed at 6 months over 2 years' follow-up (RR=0.71, 95% CI: 0.51–0.98; k=2; n=1069) [146,147]. Interferon reduced new or newly enlarging T2 lesions (MD = −7.30, 95% CI: −8.85 to −5.75) [144]. Lower annualized relapse rate and fewer new or newly enlarging T2 lesions have been observed also at 2 and 4 years' follow-up [148,149].

Regarding SPMS, a significant reduction in disability worsening has been confirmed at 3months (RR = 0.78, 95% CI: 0.66–0.92) [150] and a smaller effect on disability has been confirmed at 6 months (RR = 0.92, 95% CI: 0.80–1.06) [140,150,151].

3.3.2. Interferon-β and Interferon-β stimulated genes

Most of MS patients have low levels of serum interferon (IFN) [152]. Moreover, recent high-throughput genetic screening in MS patients highlighted the

occurrence of many genetic risk variants in the proximity of immunologically relevant genes interferon-stimulated genes (ISG), suggesting a genetic dysfunction of the interferon pathway in MS [153]. IFN-β mechanisms of action in MS are partially known. Its beneficial effects are imputed to the combined effect of different mechanisms including the down-regulation of class II major histocompatibility complex molecules on antigen presenting cells (dendritic cells and B lymphocytes) [154], the induction of IL-10 in T cells [154–156], the inhibition of T cell migration as a result of blockade of metalloproteases and adhesion molecules [154,157,158], the induction of Th17 cells and B cells apoptosis [2,159]. Unfortunately, not all patients respond to IFN-β therapy and some MS patients experience disease progression and the needing of another more effective therapy [160,161]. The early identification of IFN-β responders and non-responders MS patients would help to select patients needing a rapid transition to another therapy and ultimately to avoid the accumulation of permanent disability over time. Indeed, a clear and shared clinical definition of IFN-β responders is not established yet and the use of blood biomarkers is still debated [162,163]. The prediction of IFN-β response remains one of the big challenges to manage the therapy of MS patients and studies indicate to enlarge cohorts or focus on the mechanisms related to IFN-β in a specific cell type connected with MS [160–163].

3.4. Public data re-use and integration as a tool for the identification of molecular targets

In past decades, hypothesis-based approaches have predominated in the search for the etiology of MS, although with limited success and poor understanding of the key molecules and processes involved in MS progression. The NGS techniques in research have had relatively rapid development and diffusion, revolutionizing the way we approach basic genomic research for a personalized precision medical diagnosis that allows the selection of molecular drugs target for individual treatment. The implementation of integrative approaches of large amounts of data, produced by NGS techniques, for the understanding of complex disorders has led to the increase of new hypotheses to explain diseases and therapeutic mechanisms [164]. The analysis and integration of large amounts of data determine the identification of networks significantly preserved deregulated in a given disorder (hypothesis) and can be further validated through traditional procedures. In this consists the systems biology, where several significant molecules of numerous studies are integrated and analyzed simultaneously (epigenetics, transcriptomics, proteomics) [165]. This work implements different systems biology approaches to produce visions of Th17 molecular mechanisms involved in two different processes related to MS pathology, applying statistical filters, different bioinformatics pipelines and visualization tools. In the first example we retrieved and reused publicly available NGS data. In particular we retrieved the epigenetic and

transcriptomic profile of different T helper lymphocyte subtypes from healthy donors and developed an integration pipeline aimed to identify specific targets for the epigenetic action of estrogens in the Th17 / Treg balance. In the second example we performed an integrative analysis of studies on the gene expression profile from PBMC of a large number of MS patients treated with interferon-beta and identified a set of interferon-modulated genes that identify treatment in MS. We then speculated on the action of the drug on the pathological Th17 transcriptome, exploiting a second public data set of autoreactive T cell clones in MS.

4. Results

In the first part of the results we will show that ERα is involved in chromatin remodeling at specific regulatory regions in Th17 cells. These regions have been selected by data integration analysis and validated by ChIP-qPCR in in-vitro polarized Th17 cells treated with 17β-estradiol at pregnancy levels. In the second part of the results we will show how the transcriptional signature of IFN-β therapy in MS was obtained and how we used this signature to highlight the molecular targets of IFN-β in myelin reactive Th17 cells.

4.1. Estrogens inhibit Th17 polarization by chromatin remodeling at FOXP3, RORC, MAF and SATB1 loci

Genomic regulatory regions are integrative hubs for cellular pathways activated upon environmental stimuli. These are the site on the genome in which TFs are recruited to form the transcriptional complexes that regulate gene expression. Each cell activates a specific pattern of genomic regulatory regions to exert its biological functions. In order to identify genomic targets of estrogens signaling in Th17 and Treg cells, we designed a computational approach composed of four consecutive NGS data integration steps: (i) SEs prediction in CD4+ T cell subtypes, (ii) chromatin states analysis for identification of active regulatory regions, (iii) overlap between these regions and SEs detected in Th17 and Treg cells, (iv) reconstruction of a core TFs regulatory network of Th17 and Treg cells and identification of putative ERα targets (Figure 2) [102].

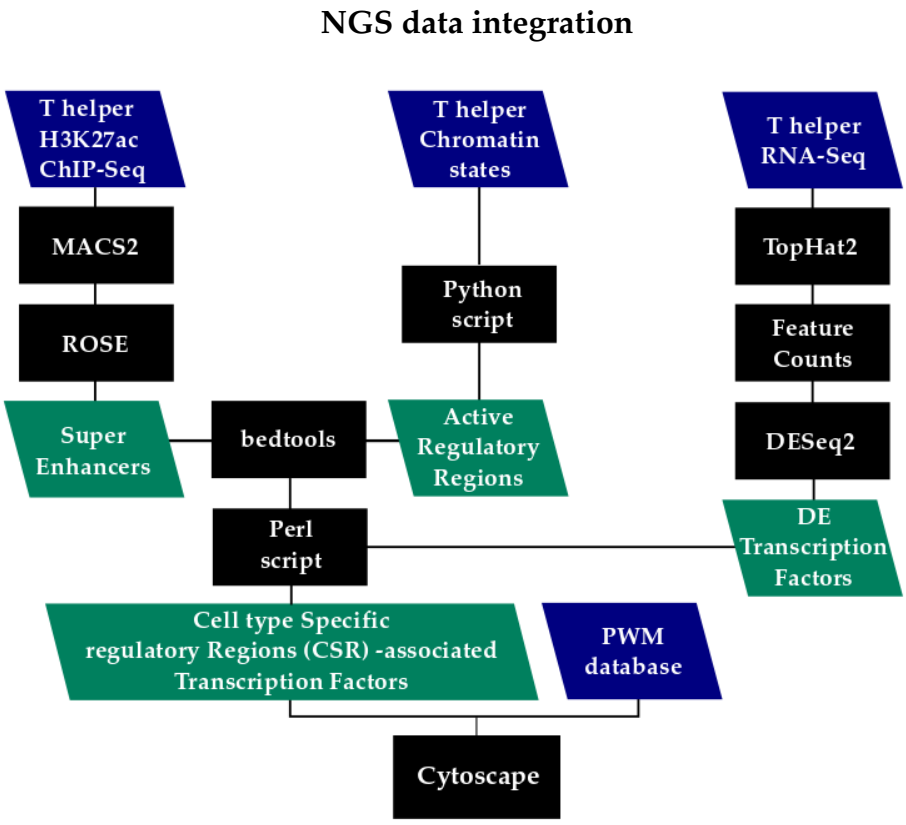

Figure 2. Workflow representation of our approach for data integration.

4.1.1. *Super Enhancers and chromatin states define Active Regulatory Regions of Th17 and Treg cells*

We predicted SEs using public H3K27ac ChIP-Seq data of human CD4+CD25–CD45RA+ cells (Naive T), CD4+CD25– T cells (Th), CD4+CD25–IL17+ T cells (Th17), and CD4+CD25+CD45RA+ T cells (Treg) from the Roadmap Epigenomics Project (30), identifying 658, 676, 999, and 851 SEs in Naive T, Th, Th17, and Treg cells, respectively (Figure 3, Table S1A).

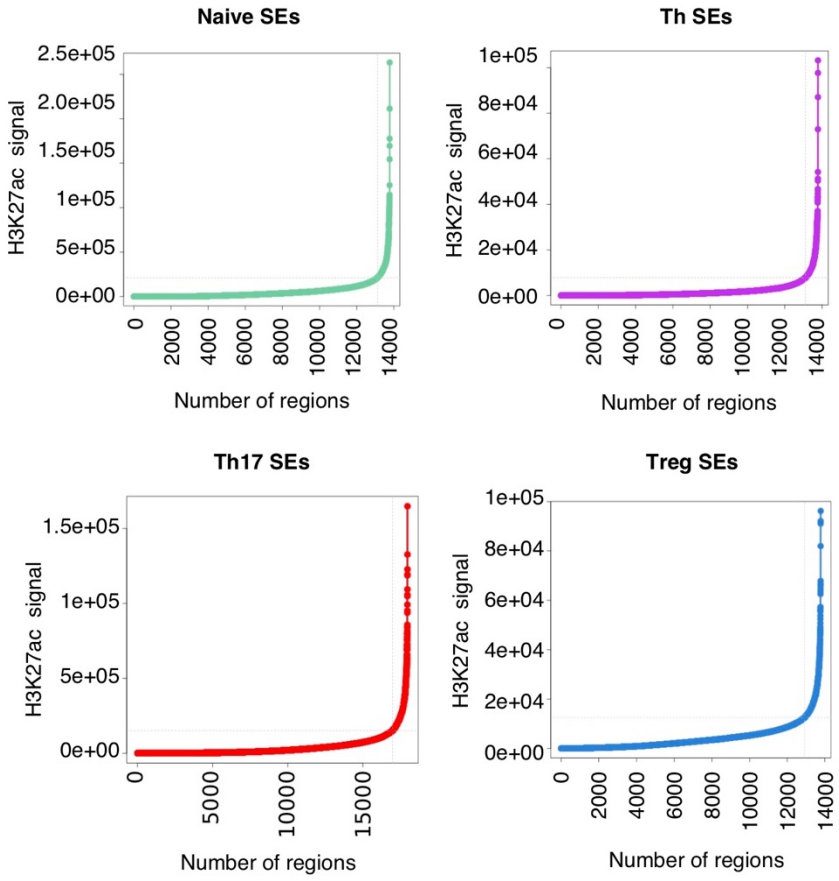

Figure 3. Prediction of SEs in Th17, Treg, Naive T, and Th cells by Rank Ordering of Super Enhancers (ROSE) algorithm. Line plot reports the cumulative number of enhancers identified in Th17 and Treg cells as function of the number of H3K27ac ChIP-Seq reads over the input dataset. Vertical lines represent the threshold over which H3K27ac signal intensity defines SEs.

Interestingly, Gene Ontology (GO) analysis of genes mapped in proximity of SEs showed an association with "immune response" and "regulation of immune system" processes (Table S1B). We evaluated the enrichment of Single Nucleotide Polymorphisms (SNP) associated to a set of 41 diseases, within Th17 and Treg SEs. SNPs associated with autoimmune diseases, overlapped more often with Th17 and Treg SEs than with a random set of regions of the same length. This enrichment is stronger for autoimmune-disease-associated SNPs in respect to the control group of other-disease-associated SNPs, meaning that these chromatin hubs affect immune system behavior and can be related to disease development (Figure 4).

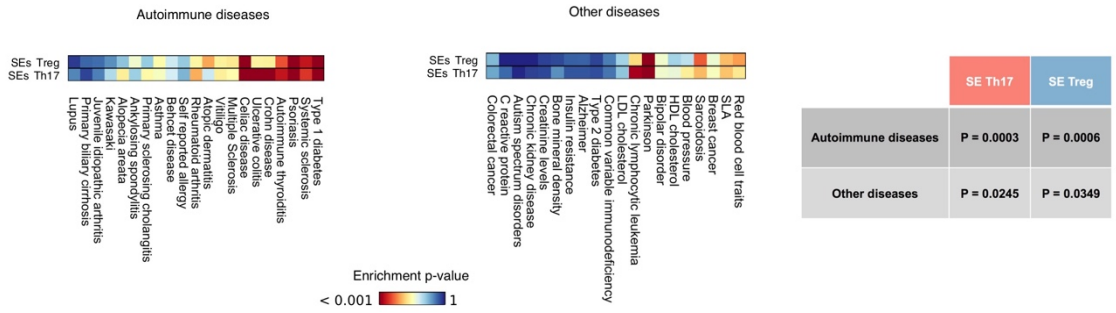

Figure 4. Results of SNPs analysis performed on predicted SEs in Th17 and Treg cells. Heatmap shows results of the single-trait-associated SNPs enrichment analysis. Table shows results of the enrichment analysis considering the two groups of diseases as a single trait.

To identify Active Regulatory Regions (ARRs) with a more precise spatial resolution we analyzed chromatin states data predicted by ChromHMM [166] in the aforementioned CD4+ T cell subtypes. This model consists of 25-chromatin states model based on imputed data for 12 epigenetic marks defined for 127 cell types and provides a 200 bp human genome segmentation with the corresponding predicted functional annotation. Using this data, we selected a subset of 65,581 genomic regions characterized by high enrichment of histone marks that define active promoters and enhancers. To distinguish these regions according to their level of regulatory activity among CD4+ T cells, we compared their epigenetic state and found 4,610 (7.03%), 7,508 (11.45%), 4,720 (7.20%), and 5,608 (8.55%) ARRs exclusive to naive T, Th, Th17, and Treg cells, respectively (Table S1C).
Then, to further isolate ARRs characterized by the highest predicted regulatory activity, we overlapped ARRs with predicted SEs in these cell subtypes. The 2.27, 2.73, 14.60, and 8.10% of naive-, Th-, Th17-, and Treg-ARRs, respectively, overlapped with SE regions (Table S1D). As expected, SE-overlapped ARRs (SE-ARRs) showed significantly higher levels of H3K27ac compared with ARRs (Figure 5).

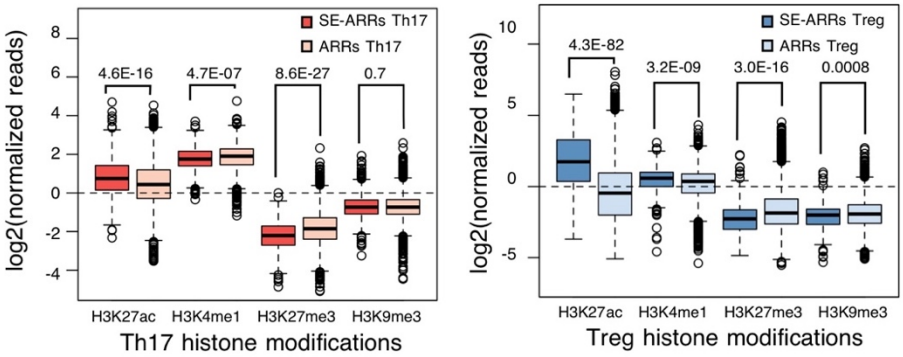

Figure 5. Box plot shows the log2 normalized H3K27ac, H3K4me1, H3K27me3, and H3K9me3 ChIP-Seq signal measured in Th17- and Treg- SE-ARRs in comparison with ARRs. The P-values are calculated by Wilcoxon Rank-sum test.

Moreover, the comparison of Th17 and Treg SE-ARRs underlines that H3K27ac in SE-ARRs has a cell-type specific enrichment (Figure 6).

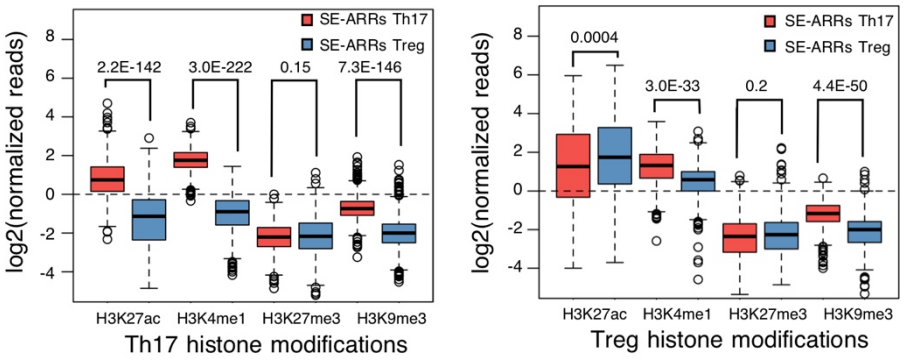

Figure 6. Box plot shows the log2 normalized H3K27ac, H3K4me1, H3K27me3, and H3K9me3 ChIP-Seq signal measured in Th17- and Treg- SE-ARRs. The P-values are calculated by Wilcoxon Rank-sum test.

GO analysis for genes mapped in proximity of Th17 SE-ARRs showed an association with immune system and inflammatory processes, whereas Treg SE-ARRs are associated with chromatin remodeling and metabolism (Tables S1E-F).

4.1.2. Reconstruction of Cell Type-Specific Regulatory Networks Identifies ERα-Regulated Genomic Regulatory Regions in Th17 and Treg Cells

In order to obtain an overview of gene expression profiles associated to SE-ARRs in Th17 and Treg cells, we re-analyzed raw data from a paired-end tag poly (A+) RNA-Seq datasets performed on purified CD4+ T cells, including Th17 and Treg cells, from five human healthy donors (35). We found 1,291 significantly Differentially Expressed (DE) genes between Th17 and Treg cells (DESeq2 adjusted p-value < 0.001), 147 of which associated to SE-ARRs mapped within a distance of 100 kbp (Table S2A). Comparison of the expression specificity among CD4+ T cells highlighted that upregulated genes in Treg cells were more specific of this CD4+

subtype, while upregulated genes in Th17 cells were similarly expressed in Th1 and Th2 subtypes (Figure 7).

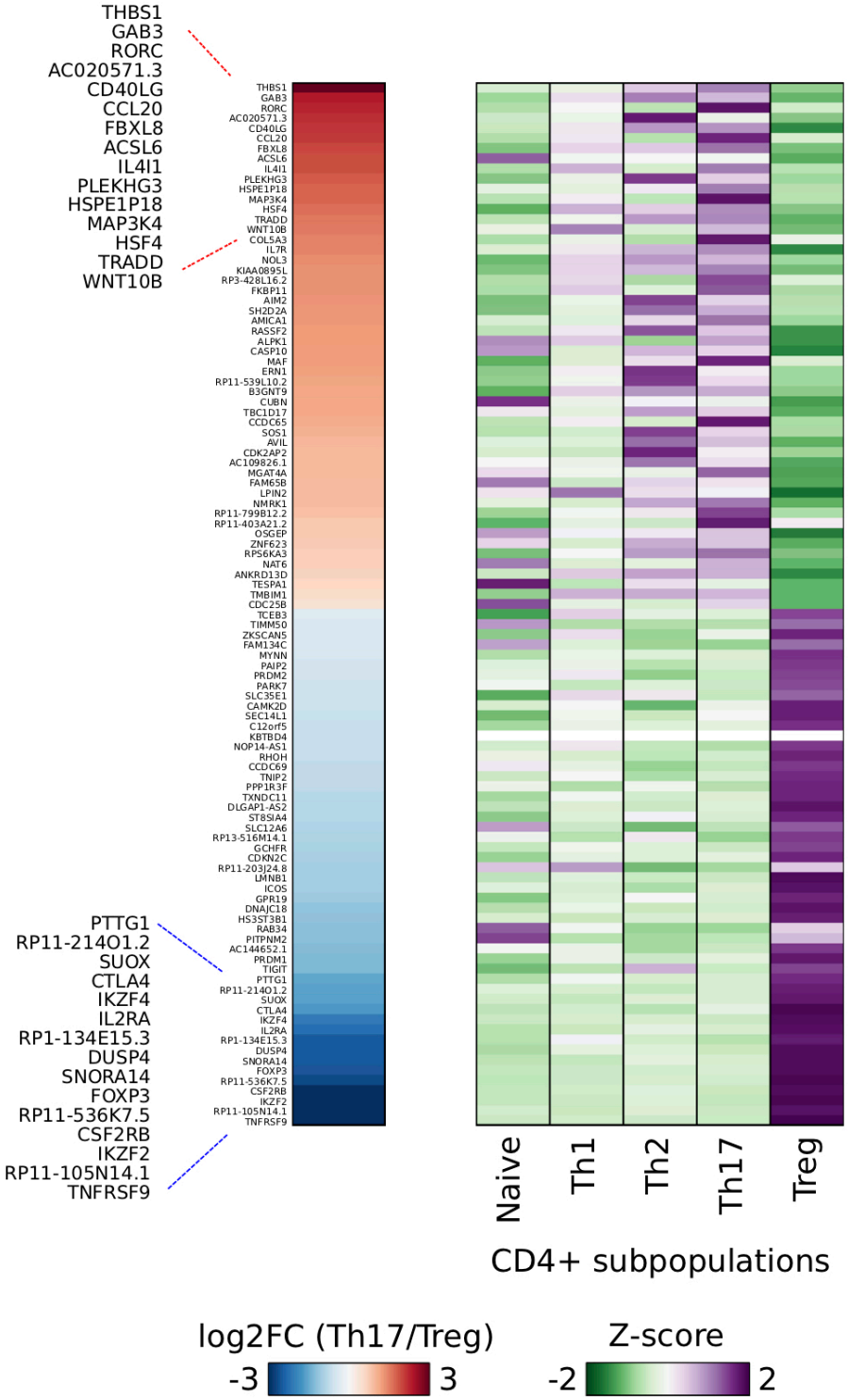

Figure 7. The first heatmap (left) represents SE-ARRs associated DE genes between Th17 and Treg cells. Genes are sorted by decreasing Th17/Treg log2FC. The second Heat map (right) represents the computed Z-score between CD4+ subpopulation.

Interestingly, among most upregulated genes in Th17 cells (log2FC Th17/Treg cells > 1.5 and DESeq2 FDR adjusted P-value < 0.001), the top DE TF-coding genes associated with Th17 SE-ARRs were the RAR Related Orphan Receptor C (RORC), the Heat Shock Transcription Factor 4 (HSF4) and the MAF BZIP Transcription Factor (MAF) (Table S2A). Among most upregulated genes in Treg cells (log2FC Th17/Treg cells < -1.5 and DESeq2 FDR adjusted P-value < 0.001), the top DE TF-coding genes associated with Treg SE-ARRs were the IKAROS Family Zinc Finger 2 (IKZF2), the Forkhead Box Protein 3 (FOXP3), the IKAROS Family Zinc Finger 4 (IKZF4) and the PR/SET Domain 1 (PRDM1) also known as BLIMP-1 (Table S2A).
To identify putative regulatory interactions between SE-ARRs associated TFs, we explored the sequence of SE-ARRs for the binding motif of a list of human TFs. Results of this analysis were used to reconstruct a core TF regulatory network in which the indegree of nodes, representing TF-coding genes, is given by the number of significant TF binding motifs enriched at gene-associated SE- ARRs. Conversely, the outdegree of nodes is the sum of predicted TF bindings to another gene-associated SE-ARRs (Figure 7).

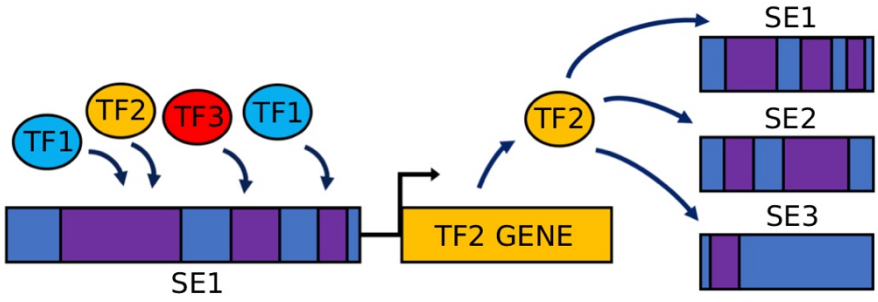

Figure 7. Methodology for network reconstruction.

We extracted information on key candidate TFs involved in Th17 or Treg lineage determination by computing the differential gene expression between Th17/Naive and Treg/Naive CD4+cells. We identified 4 and 10 SE-ARR-associated DE TFs (FDR adjusted P-value < 10^{-7}) in Th17/Naive and Treg/Naive comparison respectively (Tables S2B–E). We used these TFs to create subnetworks of the total regulatory networks (Figure 8). We enriched these subnetworks with activation and inhibition regulators inferred by a correlation analysis of gene expression (Figure 8 and Table S2F). Our network reconstruction highlighted RORC and MAF as nodes with highest indegree in the Th17 network, and FOXP3, IKZF2, IKZF4 and SATB1 as nodes with highest indegree in the Treg network, highlighting the importance of these regions as regulatory hubs in the definition of the phenotype of Th17 and Treg cells (Figure 8). We called the regions associated to these TFs Cell-type Specific Regulatory regions (CSR). Finally, since our main interest was to identify targets for genomic pathway of estrogens, we computed the enrichment of EREs within Th17 and Treg CSRs (Figure 8). Collectively this analysis revealed the major

candidates of ERα-mediated regulation in Th17 and Treg cell specification (Figure 8).

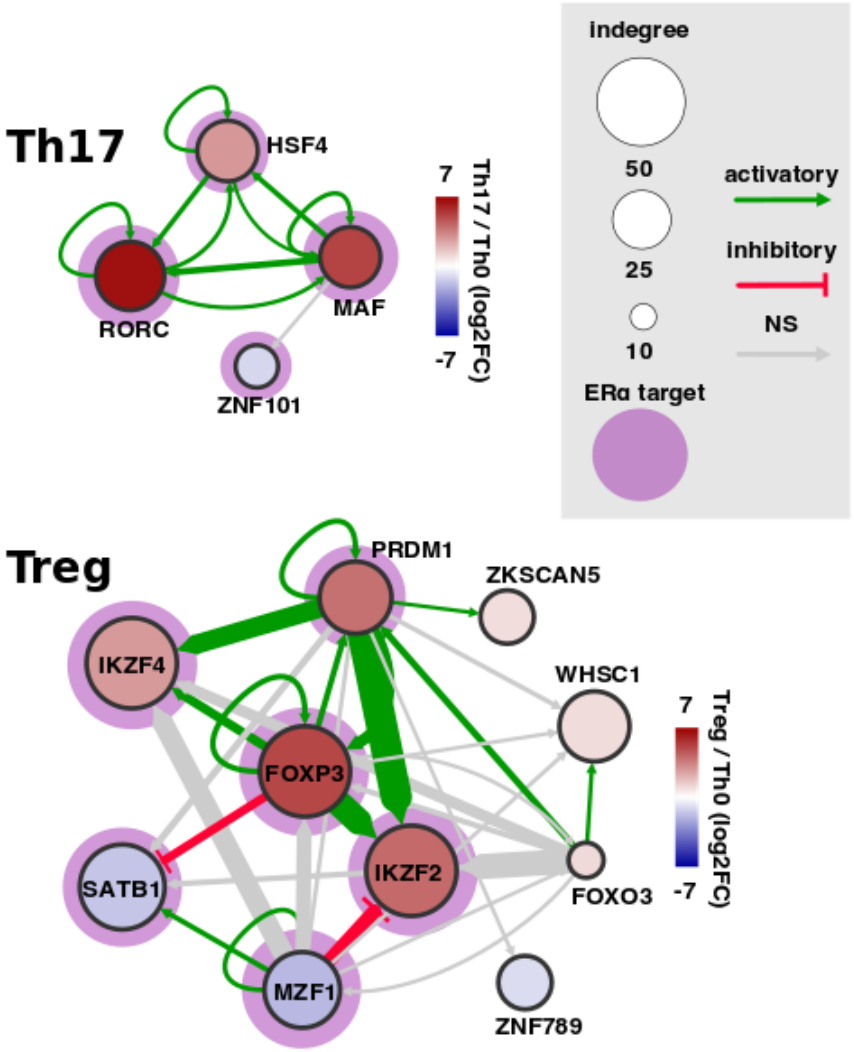

Figure 8. Th17 and Treg core regulatory networks. Node size is scaled to indegree values. Node color represents log2 fold change expression of Th17/Naive CD4+ cells and Treg/Naive CD4+ cells, respectively. Edge thickness is scaled to the sum of predicted TF binding sites at target-associated CSRs. Edge color represents positive (green) or negative (red) regulation inferred by Pearson correlation analysis between regulator and target gene expression (Table S2F). Positive and negative correlations are used to represent activatory and inhibitory network edges, respectively. Since PWMs are not available for all TFs, some interactions could not be predicted. Networks show also predicted ERα binding at CSRs associated TFs in Th17 and Treg cells (pink circles).

4.1.3. ERα network validation

We purified PBMCs from female donor blood (25-48 years of age) and subjected the cells to Th17 polarizing conditions for three days. At day zero, the polarizing cytokines were added, in conjunction with antibodies necessary for the activation of T lymphocytes. Concurrently, in one of the two plates, for each biological

replicate, E2 was added in quantities equal to those observed during the third trimester of pregnancy. We designed specific primers for CSRs interested by ERE occurrence, including both promoters (0-2Kb upstream TSS) and active enhancers. Then, we immunoprecipitated the chromatin fragments bound by ERα to validate the network edges using ChIP-PCR. We used polyclonal antibody against human ERα protein and evaluated the binding proportion between treated and untreated conditions. Moreover, we evaluated epigenetic marks and gene expression associated with E2-regulated genomic regulatory regions : for enhancers, we used histone 3 acetylation of lysine 27 (H3K27ac); for promoters, we used the histone 3 trimethylation of lysine 4 (H3K4me3), the active histone mark and the histone 3 trimethylation of lysine 27 (H3K27me3), the repressive histone mark. We showed the ratio between H3K4me3 and H3K27me3 marks because both marks can be present on the same promoter to indicate different states of activation. Moreover, we evaluated the effect of E2 on gene expression by qPCR

4.1.3.1. RORC

We observed that E2 at pregnancy level reduces promoter (0-2Kbp upstream TSS) activation of RORC gene. This effect can be mediated by the effect of ERα binding in the same region that is increased, accordingly. The epigenetic state of the genomic regulatory region associated with RORC is reflected in a reduction in RORC expression (Figure 9).

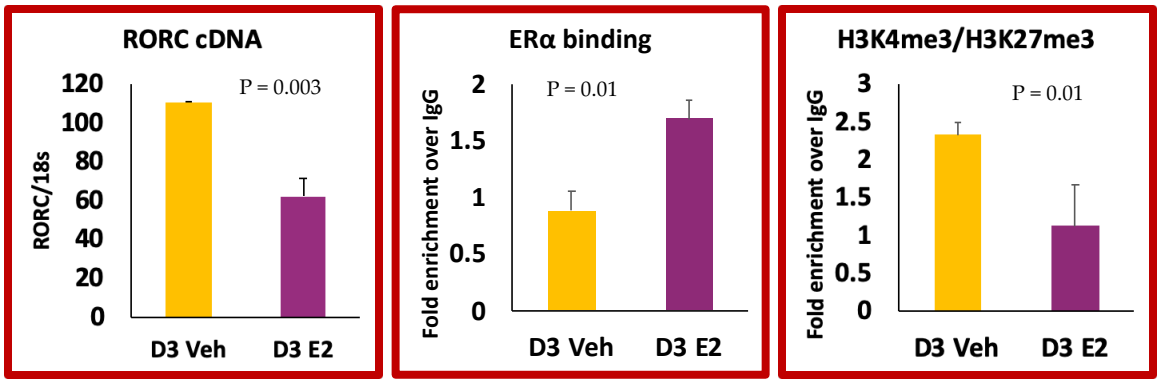

Figure 9. RORC relative gene expression measured by qPCR at 3 days of Th17 polarization. Relative gene expression is normalized over 18s gene expression and compared between E2 treated condition (E2) and Vehicle (Veh). ERα binding at the promoter is measured by ChIP-qPCR and normalized over IgG H3K4me3 (activator histone mark) and H3K27me3 (inhibitor histone mark) enrichment is measured by ChIP-qPCR, normalized over IgG. H3K4me3/H3K27me enrichment is represented. (p-value t-test, n = 4)

4.1.3.2. MAF

We observed that E2 at pregnancy level reduces promoter (0-2Kbp upstream TSS) (Figure 10) and enhancer activation (Figure 11) of MAF gene. ERα binding is enriched in both regions suggesting a role of ERα in the chromatin remodeling of

these regions. The epigenetic state of the genomic regulatory regions associated with MAF is reflected in a reduction in MAF expression (Figure 10).

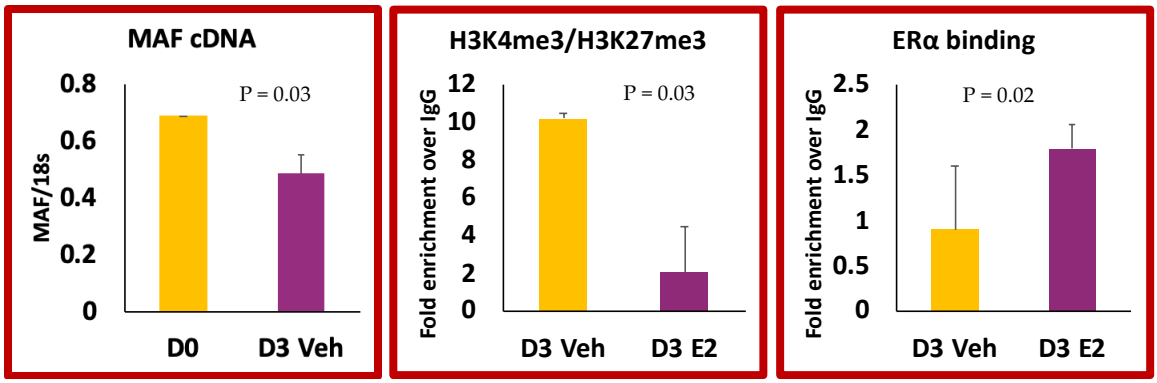

Figure 10. MAF relative gene expression measured by qPCR at 3 days of Th17 polarization. Relative gene expression is normalized over 18s gene expression and compared between E2 treated condition (E2) and Vehicle (Veh). ERα binding at the promoter is measured by ChIP-qPCR and normalized over IgG H3K4me3 (activator histone mark) and H3K27me3 (inhibitor histone mark) enrichment is measured by ChIP-qPCR, normalized over IgG. H3K4me3/H3K27me enrichment is represented. (p-value t-test, n = 4)

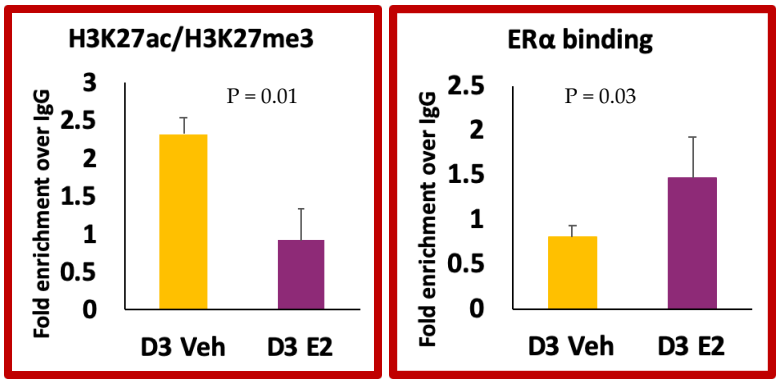

Figure 11. ERα binding at MAF enhancer is measured by ChIP-qPCR and normalized over IgG H3K27ac (enhancer activator histone mark) and H3K27me3 (inhibitor histone mark) enrichment is measured by ChIP-qPCR, normalized over IgG. H327ac/H3K27me enrichment is represented. (p-value t-test, n = 4)

4.1.3.3. FOXP3

We observed that E2 at pregnancy level induce promoter (0-2Kbp upstream TSS) activation of FOXP3 gene. This effect can be mediated by the effect of ERα binding in the same region that is increased, accordingly. The epigenetic state of the genomic regulatory region associated with FOXP3 is reflected in increased FOXP3 expression (Figure 12).

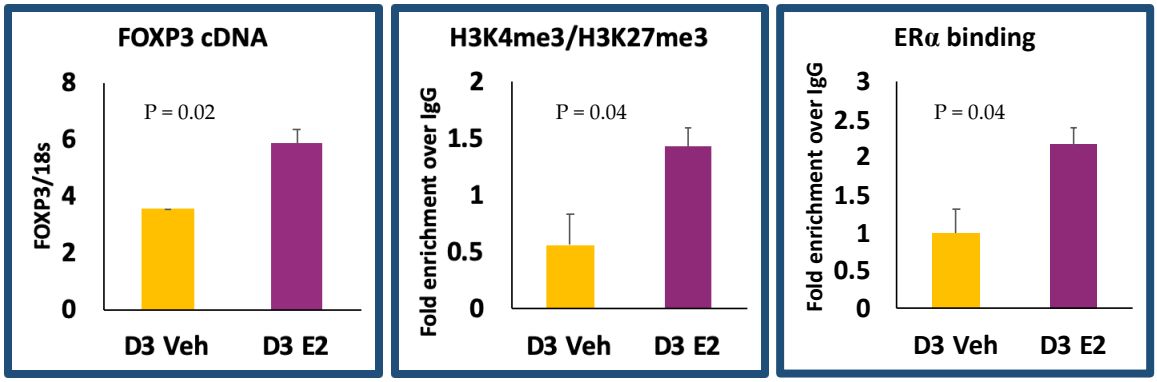

Figure 12. FOXP3 relative gene expression measured by qPCR at 3 days of Th17 polarization. Relative gene expression is normalized over 18s gene expression and compared between E2 treated condition (E2) and Vehicle (Veh). ERα binding at the promoter is measured by ChIP-qPCR and normalized over IgG H3K4me3 (activator histone mark) and H3K27me3 (inhibitor histone mark) enrichment is measured by ChIP-qPCR, normalized over IgG. H3K4me3/H3K27me enrichment is represented. (p-value t-test, n = 4)

4.1.3.4. SATB1

We observed that E2 at pregnancy level reduce promoter (0-2Kbp upstream TSS) (Figure 13) and enhancer activation (Figure 14) of SATB1 gene. ERα binding is enriched in both regions suggesting a role of ERα in the chromatin remodeling of these regions. The epigenetic state of the genomic regulatory regions associated with SATB1 is reflected in a reduction in SATB1 expression (Figure 13).

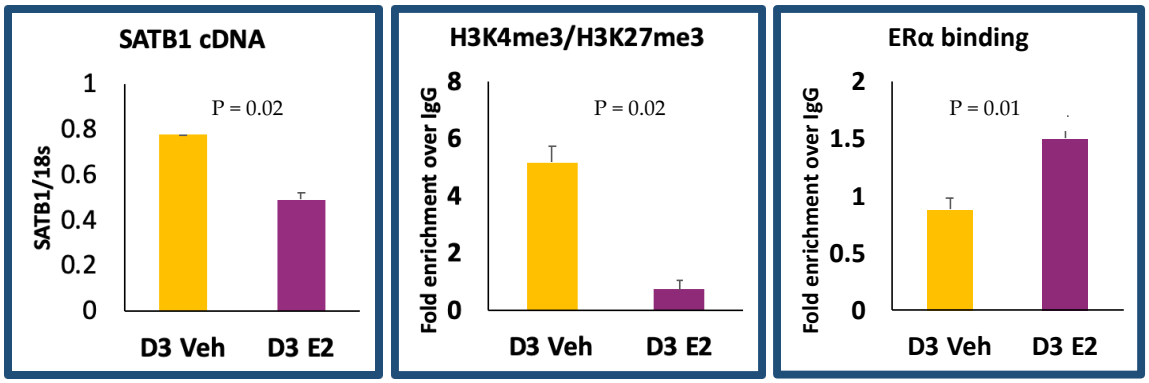

Figure 13. SATB1 relative gene expression measured by qPCR at 3 days of Th17 polarization. Relative gene expression is normalized over 18s gene expression and compared between E2 treated condition (E2) and Vehicle (Veh). ERα binding at the promoter is measured by ChIP-qPCR and normalized over IgG H3K4me3 (activator histone mark) and H3K27me3 (inhibitor histone mark) enrichment is measured by ChIP-qPCR, normalized over IgG. H3K4me3/H3K27me enrichment is represented. (p-value t-test, n = 4)

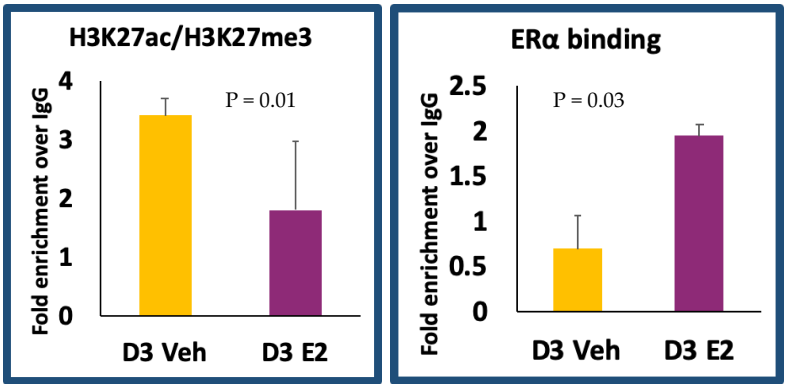

Figure 14. ERα binding at SATB1 enhancer is measured by ChIP-qPCR and normalized over IgG H3K27ac (enhancer activator histone mark) and H3K27me3 (inhibitor histone mark) enrichment is measured by ChIP-qPCR, normalized over IgG. H327ac/H3K27me enrichment is represented. (p-value t-test, n = 4)

All together these results show the epigenetic and transcriptional effect of E2 on Th17 polarization. Increased ERα binding at CSRs suggests a wider epigenetic rearrangement of regulatory landscape of T helper cells favoring the T regulatory phenotype and against Th17 phenotype. These results are supported by cytofluorimetry measurements performed by our group that show an expansion in Treg subpopulation during Th17 polarization upon E2 stimulation at pregnancy level [102]. Moreover, the epigenetic status of the CSRs associated with FOXP3 and RORC has been evaluated in MS patients during pregnancy and postpartum showing that RORC is inhibited during pregnancy and reactivated during postpartum and conversely that FOXP3 is activated during pregnancy and inhibited during postpartum [102].

4.2. Integrated transcriptional analysis highlights Interferon-beta regulated genes in pathogenic Th17 cell clones

Previous studies from the group of Prof. Clerico indicated that Th17 myelin basic protein specific cells increased in active MS [2]. IFNAR1 expression, IFN-beta-induced STAT1 activation, and apoptosis were significantly greater in Th17 rather than Th1 cells after IFN-β treatment [2]. Many ISGs have been identified during years [167–169], although IFN-β signature has been described in peripheral blood without showing enough power to predict the response of MS patients to this drug. In the second part of my PhD, we investigated the IFN-β modulation of CD4+ T cells, focusing on the potential role of ISGs in Th17 cells in MS patients. We confirmed previously IFN-β targets and defined a signature of ISGs related to the IFN-β therapy in MS patients.

4.2.1. Integrative transcriptional analysis of IFN-β treatment in MS depicts an ISGs expression signature related to the disease

To identify a signature of ISGs related to the IFN-β treatment in MS, we collected and integrated public gene expression dataset from three genome-wide studies of IFN-β treated MS patients (GSE16214, GSE41850, and GSE73608) (Table 1).

GEO accession ID	Experiment	DE analysis method	MS course	Sampling time	Number of samples	Reference
GSE16214	Microarray - GPL570	GEO2R	CIS-RRMS	3 months	MS patients (n = 94 treated, n = 82 untreated)	Ottoboni_2012 - PMID: 23019656 [28]
GSE41850	Microarray - GPL16209	GEO2R	CIS-RRMS-SPMS	0 - 1year - 2years	MS patients (n = 106 treated, n = 89 untreated),	Nickles_2013 - PMID: 23748426 [29]
GSE73608	Microarray – GPL571	GEO2R	SPMS	2 years	SPMS patients (n = 20 treated, n = 30 untreated)	Gurevich_2015 - PMID: 26589141 [30]

Table 1. Table illustrate the composition of the cohorts included in the studies considered for the analysis of ISG in MS patients.

The GSE41850 data set consists of whole blood gene expression data of a cohort of 195 MS patients treated with IFN-β. Patients of this cohort were in different stages of the disease (CIS-RRMS-SPMS) [169]. Instead, the GSE16214 dataset reports gene expression data of a cohort of 176 RRMS patients treated with IFN-β for 3 months [168]. Then, in order to consider IFN-β transcriptional modulation in the progressive form of MS, we included the dataset GSE73608 consisting of 50 SPMS patients who have been on therapy for 2 years [170].
For all the datasets, we compared the transcriptome of IFN-β treated and untreated patients. We obtained 92 ISGs for the MS group, 104 ISGs for the RRMS group, and 36 ISGs for the SPMS group (adj. p-value < 0.05; -0.5 < log2FC > 0.5) (Table S3A-C). 23 genes were detected as ISGs in all the comparisons, 24 genes were detected as ISGs in the MS and RRMS but not in the SPMS group, 6 genes were detected as ISGs in the RRMS and SPMS groups but not in the MS group. Finally, 6, 45, and 51

genes were detected specifically in the SPMS, MS, and RRMS groups, respectively (Figure 15, Table S3D).

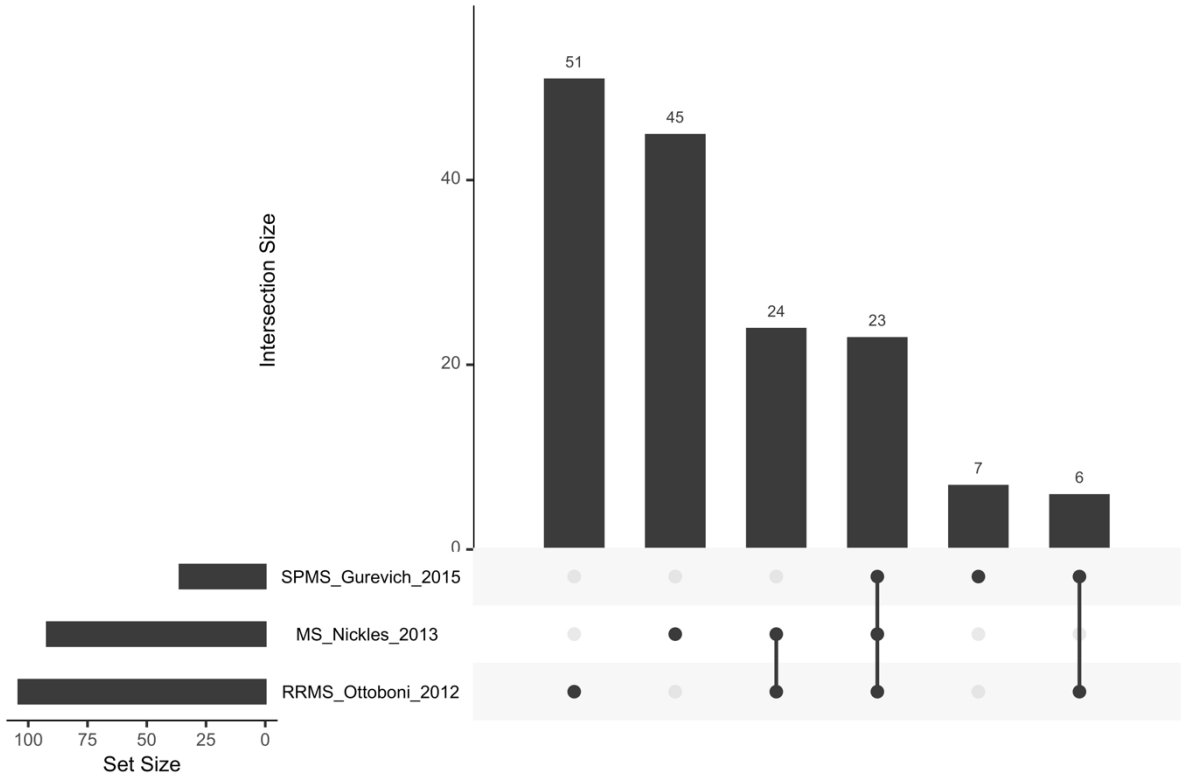

Figure 15. The upset plot shows the overlap between the lists of ISGs for each dataset considered. Datasets are ordered by increasing number of ISGs detected in each dataset (set size) and by the number of genes present in each intersection (intersection size).

Then, we considered the union of 138 ISGs identified in these studies to evaluate the general transcriptional activity of IFN-β in PBMCs from MS patients (Table S3E). As expected, gene set enrichment analysis on these 138 ISGs resulted in a significant enrichment (FDR adjusted p-value < 0.05) in terms related to Type I IFN signaling like "Interferon alpha/beta signaling_Homo sapiens_R-HSA-909733" (adj. p-value = 5.56E-29) and "type I interferon signaling pathway" (GO:0060337) (adj. p-value = 3.16E-27) and in immune system like "Immune System_Homo sapiens_R-HSA-168256" (adj. p-value = 1.01E-14) and "Immune_system" (Jensen Tissues) (adj. p-value = 1.99E-07) (Supplementary Table 1G). Furthermore, several pathways related to the IFN response were enriched including "Antiviral mechanism by IFN-stimulated genes_Homo sapiens_R-HSA-1169410" (adj. p-value = 1.21E-06), "ISG15 antiviral mechanism_Homo sapiens_R-HSA-1169408" (adj. p-value = 1.45E-06) beside more general immune-response related terms like "Cytokine Signaling in Immune system_Homo sapiens_R-HSA-1280215" (adj. p-value = 7.93E-17).
The enrichment analysis was also performed to predict candidate transcriptional regulators of the 138 ISGs. The enrichment with the gene sets from ChIP-Seq experiments resulted in the enrichment of the Interferon regulatory factor 1 (IRF1)

and Interferon regulatory factor 8 (IRF8), although data refers to ChIP-seq experiments performed in monocytes, bone marrow-derived macrophages (BMDM) and Germinal Center (GC)-B cells. Moreover, the RELA Proto-Oncogene, NF-KB Subunit (RELA) and the SRY-Box Transcription Factor 2 (SOX2) resulted as transcriptional regulators although according to experiments performed in fibrosarcoma and the human colorectal cancer cell line SW620, respectively (Table S3F).

Then, we considered the 23 ISGs (Figure 15) resulted to be significantly modulated in all the cohorts (Figure 1). Gene set enrichment analysis of these genes resulted in a significant enrichment (FDR adjusted p-value < 0.05) for different terms related to Interferon alpha/beta signaling and antiviral response including, "Interferon alpha/beta signaling_Homo sapiens_R-HSA-909733" (adj. p-value = 4.08E-24), "Antiviral mechanism by IFN-stimulated genes_Homo sapiens_R-HSA-1169410" (adj. p-value = 3.47E-10), "ISG15 antiviral mechanism_Homo sapiens_R-HSA-1169408" (adj.p-value = 4.16E-10) including Hepatitis C (adj. p-value = 5.48E-06), Influenza A (adj. p-value = 1.97E-04) and Epstein-Barr-virus (adj. p-value = 7.75E-03) (Supplementary Table 1H). The prediction of candidate transcriptional regulators of these ISGs highlighted a candidate direct regulation of IRF1 (ChIP-Seq_MONOCYTES_Human, adj. p-value = 1.28E-03) and IRF8 (ChIP-ChIP_GC-B_Human, adj. p-value = 2.53E-02) (Table S3G).

Among the 23 genes HERC5, IFI44L, IFIT1, MX1, RSAD2, SIGLEC1 [167,168], EIF2AK2, HERC6, IFI6, IFIT3, LGALS3BP, OASL [168] and IFI27, USP18 [167] have been indicated as ISGs in previous studies of transcriptional response to IFN-β in MS patients. Moreover, some of our genes have not been detected by these studies but their use as blood biomarkers for IFN-β therapy in MS is currently under investigation (CXCL10, IFIT2, ISG15, OAS3, XAF1) [162,171,172]. Then, we tried to identify the minimal set of genes in classifying IFN-β treated patients and untreated ones. We used data mining approach on GSE16214 and GSE41850 datasets. First of all, we compared 9 different machine learning algorithms for the evaluation of genes as classifier attributes. The set of 23 genes was able to classify with the same accuracy of the two respective lists of DEGs in the two datasets (Table S4A) and the random forest classifier resulted the best performer compared to the other 8 algorithms in achieving this task (adj. p-value <0.05, Table S4B). We trained the random forest algorithm with a 10-fold cross validation, to avoid overfitting, and tried to evaluate the performance of the model trained on one data set, in the other. We observed that the model based on the expression of our 23 genes trained in the GSE16214 dataset classified with 85.23% of accuracy within the same dataset in 10-fold cross-validation and with 70.43% of accuracy using the GSE41850 dataset as test set (Table S4C). Being the patients of GSE16214 all females, sampled at ~ three months of treatment we decided to filter the GSE41850 dataset removing males and dividing patients sampled at the end of the 1st year of treatment (1st year follow-up) and at the end of the 2nd year of treatment (2nd year follow-up) to evaluate the performance of the model in a sex-matched cohort over

time. We observed that the model correctly classified patients with 70.61% of accuracy in 1st year follow-up patients and with 71.38% of accuracy in 2nd year follow-up patients (Table S4C). Moreover, we ranked the importance of each one of the 23 genes in the classification task, reduced the number of attributes one by one, re-trained and re-tested the performance of the random forest classifier obtaining the best performance with only the first two genes in the ranking that are USP18 and IFI27 (Table S4D). The model trained in GSE16214 with 10-fold cross validation using USP18 and IFI27 as classifiers showed more accuracy than the set of 23 genes, since it performed with 80.00% (1st year follow-up) and 81.13% (2nd year follow-up) of accuracy over time (Table S4D).

4.2.2. *IFN-β signature highlights XAF-1 and LGALS3BP as molecular targets in pathogenic Th17 cells in MS*

We exploited a dataset of the transcriptional profile analysis of myelin-responsive and not responsive single T cell clones generated from MS patients and healthy subjects (GSE66763) [173]. We used Recount2 to retrieve RNA-Seq data and performed a cross-study analysis of ISGs in Th17 myelin-reactive cells [174]. In this study single-cell clones of CCR6+ T cells myelin tetramer-positive (+) and negative (-) cells from HD or MS patients were produced.
As a first step we computed the differential expression between MS- vs. HD-, HD+ vs. HD-, MS+ vs. MS- and MS+ vs. HD+ obtaining 114, 153, 402 and 716 DEGs respectively (Table S5A-D). The overlapping of DEG lists is reported in Table S5E and represented in Figure 16.

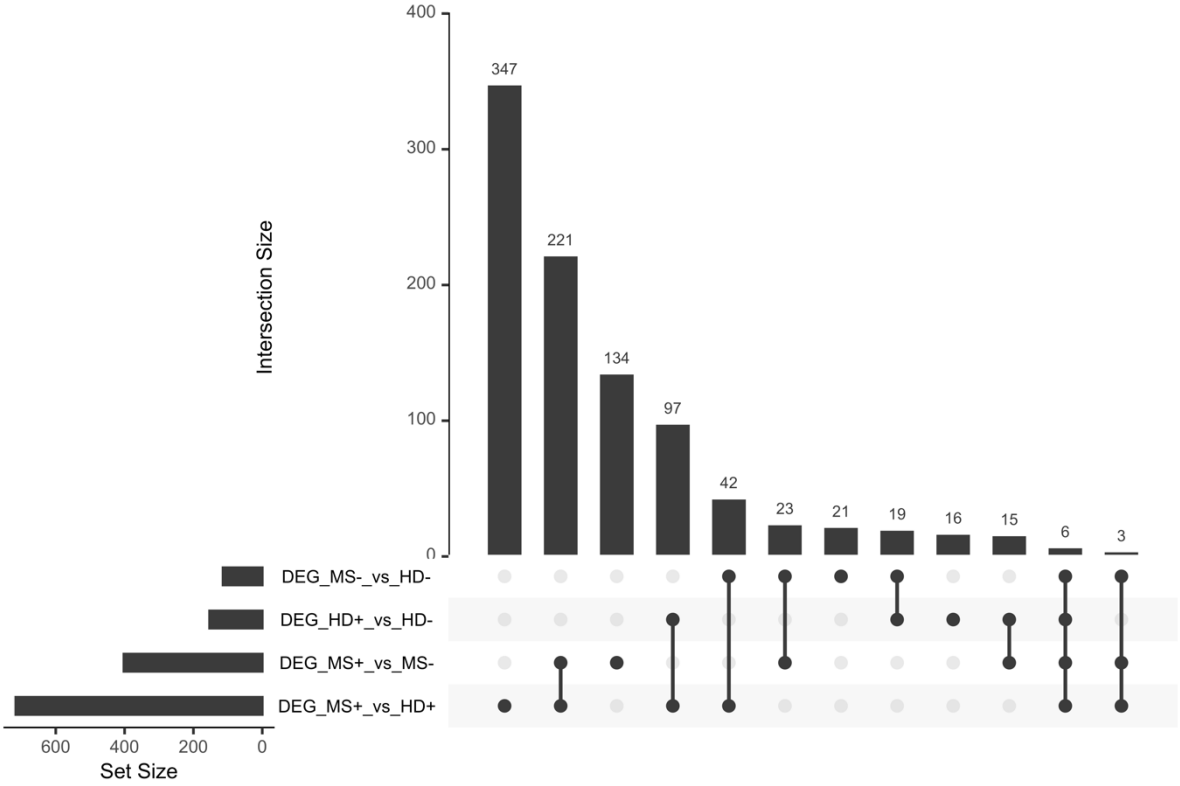

Figure 16. The upset plot shows the overlap between the lists of ISGs for each dataset considered. Datasets are ordered by increasing number of ISGs detected in each dataset (set size) and by the number of genes present in each intersection (intersection size).

We unified DEG lists in a list of 943 genes (Table S5F) and computed z-score normalization among samples (Fig. 17).

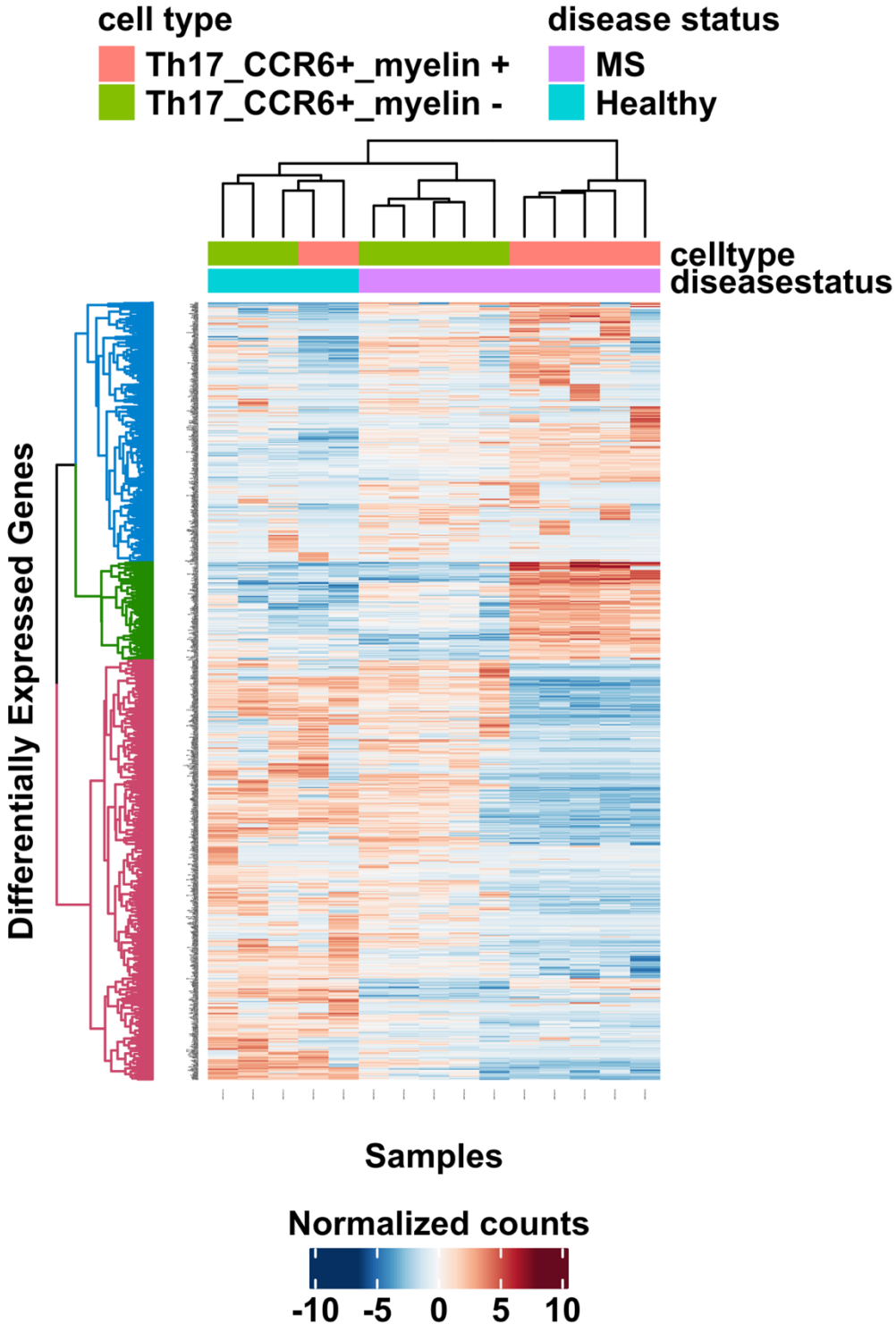

Figure 17. The heatmap shows gene expression variability of DEGs in Th17 cells from MS patients and healthy subjects.

Unsupervised hierarchical clustering based on Euclidean distance between values of gene expression among samples highlighted three clusters of genes: cluster 1 (red), cluster 2 (blue) and cluster 3 (green). Cluster 1 is composed of genes that are progressively downregulated in pathogenic Th17 cells (Table S5F, Fig. 17). gene set enrichment analysis on this cluster of genes resulted in no significant enrichment (FDR adjusted p-value < 0.05) for any GO terms (Table S5G). Cluster 2 is composed of genes that are progressively upregulated in pathogenic Th17 cells (Table S5F, Fig. 3). Gene set enrichment analysis on this cluster resulted in a significant enrichment (FDR adjusted p-value < 0.05) in a term related to the apoptotic process like "Regulation of BAD phosphorylation_Homo sapiens_h_badPathway" (adj. p-value = 2.50E-05) and a term related to cytokine regulation like "Cytokine-cytokine receptor interaction" (adj. p-value = 3.89E-02). The enrichment analysis predicted Notch homolog 1, translocation-associated (NOTCH1) as a candidate in the direct regulation of this cluster of genes, although according to a ChIP-Seq experiment performed in T-lymphoblastic leukemia (TLL) cells (adj. p-value = 5.52E-03) (Table S5H). Finally, cluster 3 is characterized by the presence of gene expressed at higher levels in MS+ Th17 cells compared to the other groups (Table S5F, Fig. 17). Gene set enrichment analysis on this cluster of genes resulted in a significant enrichment (FDR adjusted p-value < 0.05) in different terms related to Th17 cell differentiation and inflammatory response like "Th17 differentiation" (adj. p-value = 1.55E-06), "JAK-STAT signaling pathway" (adj. p-value = 3.66E-08) and "NF-kappaB_complex" (adj. p-value = 1.05E-17) (Table S5I). Moreover, this enrichment analysis highlighted some specific terms related to Th17 cell expansion under IL-2, IL-27 and IL-23 signaling like "IL2-mediated signaling events_Homo sapiens_a2a1883c-6193-11e5-8ac5-06603eb7f303"(adj. p-value = 2.33E-05), "interleukin-27_complex" (adj. p-value = 3.74E-04), "IL23-mediated signaling events_Homo sapiens_b71d0ffd-6193-11e5-8ac5-06603eb7f303" (adj. p-value = "1.01E-03") and activation of T cells by APCs like "CD40/CD40L signaling_Homo sapiens_1971792f-6190-11e5-8ac5-06603eb7f303" (adj. p-value = 5.05E-04) (Table S5I). Interestingly, also terms that are related to survival and apoptotic processes have been found significantly enriched for this cluster like "BCL-2_complex" (adj. p-value = 2.13E-13) and "BAX_complex" (adj. p-value = 7.99E-10). Moreover, the same analysis highlighted a correlation of this cluster of genes with autoimmune diseases by GWAS association studies like "Rheumatoid arthritis" (adj. p-value = 2.04E-05) and "Inflammatory bowel disease" (adj. p-value = 3.35E-05).

Collectively, this analysis revealed a pattern of gene expression of pathogenic myelin reactive Th17 cells. This pattern of gene expression reflects the differences found between the MS+ group and the others. This is visible also by Principal Component Analysis and k-means clustering (Figure 18).

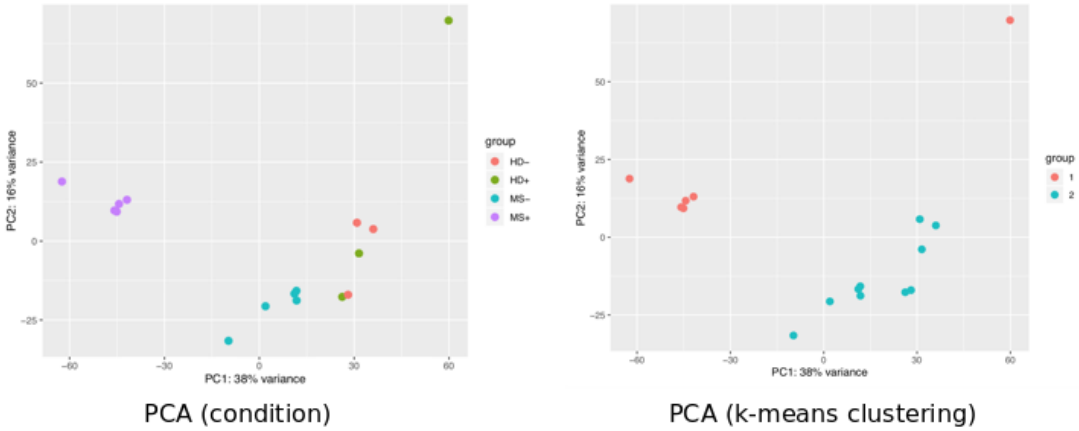

Figure 18. Principal component analysis revealed more similarity between MS-, HD+, HD- groups respect to the MS+ group.

Finally, we visualized the gene expression variability of our IFN-β signature of 23 genes in pathological and healthy Th17 cells (Figure 19).

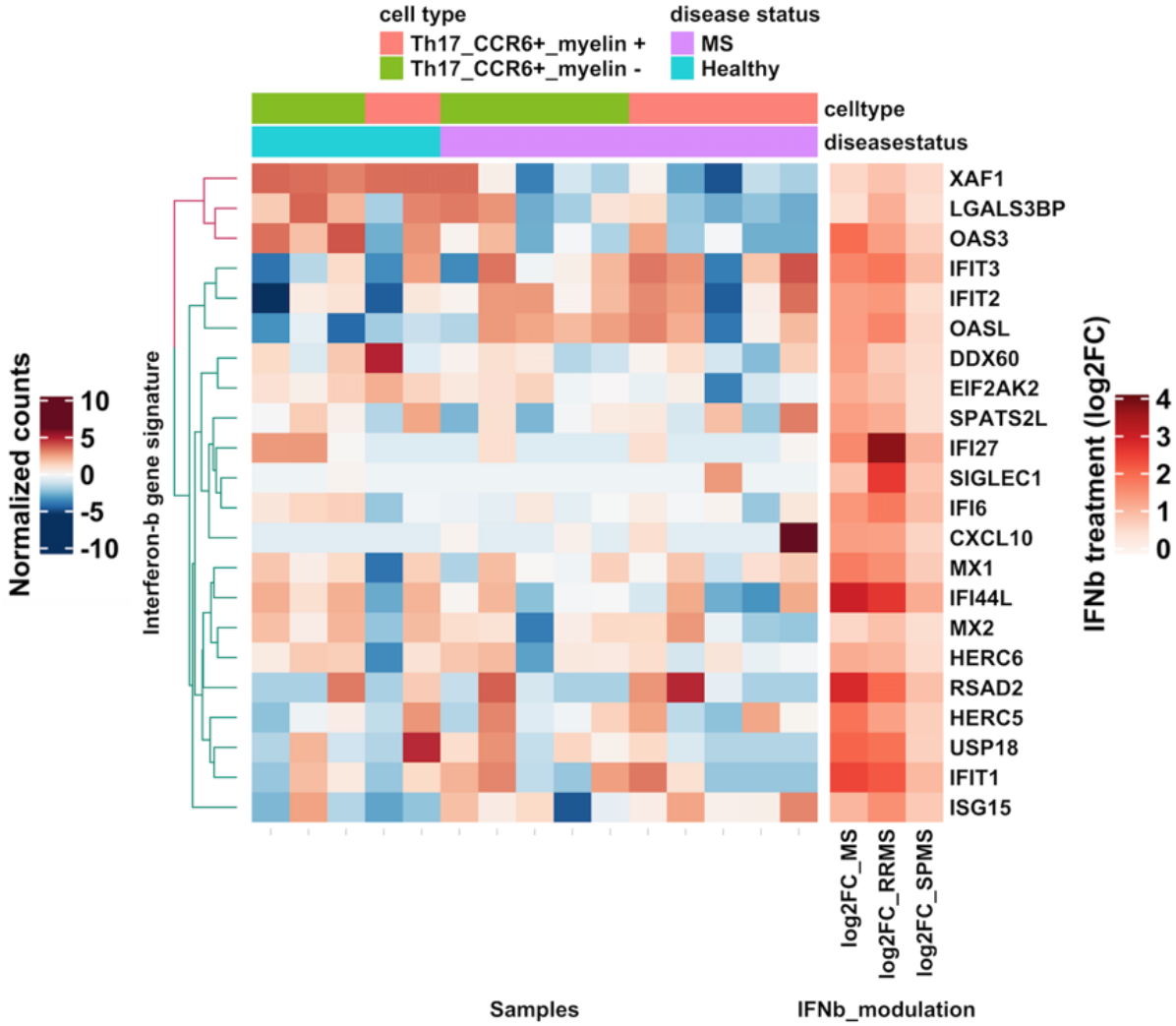

Figure 19. Left heatmap shows gene expression variability of IFN-β signature genes in MS+, MS-, HD+ and HD- groups. Right heatmap represent the log2 fold change expression between treated/untreated MS subjects in each dataset considered in our previous analysis.

We observed that LGALS3BP gene, encoding for the Galectin-3-binding protein is downregulated in Th17 cells from MS+ group respect to MS- (log2FC -6.778868833, adjusted p-value 0.000487098) and HD+ (log2FC -8.313005039, adjusted p-value 7.02E-05) groups. The same for XAF1 gene, encoding for XIAP-associated factor 1, that resulted to be downregulated in MS+ group respect to HD+ group (log2FC -6.300465486, adjusted p-value 2.05E-05). Interestingly both LGALS3BP and XAF1 gene expression can be restored by IFN-β treatment since they are both upregulated by IFN-β therapy in MS patients. Moreover, XAF1 is a pro-apoptotic factor, suggesting an impairment of pathogenic Th17 cells apoptosis that could be restored by IFN-β treatment.

4.2.3. *Apoptosis pathway analysis suggests TNF-α induced apoptosis pathway downregulation and NFKB1 upregulation in Th17 cells in MS*

To investigate the potential action of the pro-apoptotic factor XAF1 in pathological Th17 cells we retrieved the list of genes involved in human apoptosis from KEGG 2019 database. 13 out of 136 genes resulted to be DEG in Th17 cells between MS+ and HD+ groups (Table S5J, Table 2).

Gene	description	function	log2FC	adjusted p-value
CSF2RB	colony stimulating factor 2 receptor beta common subunit [KO:K04738]	survival	20.6	3.20E-05
BAD	BCL2 associated agonist of cell death [KO:K02158]	apoptotic	20.5	2.68E-09
FASLG	Fas ligand [KO:K04389]	apoptotic	10.1	3.64E-29
CTSL	cathepsin L [KO:K01365] [EC:3.4.22.15]	apoptotic	8.6	0.00012426
EIF2AK3	eukaryotic translation initiation factor 2 alpha kinase 3 [KO:K08860] [EC:2.7.11.1]	apoptotic	8.0	1.07E-06
BCL2A1	BCL2 related protein A1 [KO:K02162]	survival	4.8	1.22E-07
TNFRSF10B	TNF receptor superfamily member 10b [KO:K04722]	apoptotic	4.5	1.11E-06
TRAF1	TNF receptor associated factor 1 [KO:K03172]	survival	3.3	5.38E-05
PIK3R1	phosphoinositide-3-kinase regulatory subunit 1 [KO:K02649]	survival	3.3	9.57E-06
NFKB1	nuclear factor kappa B subunit 1 [KO:K02580]	survival	2.5	4.49E-05
CFLAR	CASP8 and FADD like apoptosis regulator [KO:K04724]	survival	1.6	1.35E-05
JUN	Jun proto-oncogene, AP-1 transcription factor subunit [KO:K04448]	apoptotic	-2.5	2.60E-05
CTSS	cathepsin S [KO:K01368] [EC:3.4.22.27]	apoptotic	-3.5	2.58E-05

Table 2. Listed genes correspond to DEGs in Th17 cells between MS+ and HD+ groups (DESeq2 adjusted p-value <0.05) that are included in the KEGG 2019 pathway hsa04210 - Apoptosis - Homo sapiens.

Some pro-apoptotic genes show clear upregulation in pathogenic Th17 (i.e. FASL, TNFRSF10B, BAD, CTSL). Conversely, JUN, CTSS are downregulated in the same cells. Regarding genes that instead promote cell survival BCL2A1, PI3KR1, TRAF1 and NFKB1 are upregulated in pathogenic Th17 cells (Table 2). However, no one of these genes overlap with the previously identified IFN-β signature. Anyway, the modulation of XAF1 could participate in the process of Th17 cell apoptosis by its most known function of inhibitor of the inhibitors of apoptosis protein (IAP) family [175]. XAF1 is frequently inactivated in multiple human cancers and a recent function of XAF1 has been described in tumor suppressor gene activity [176]. XAF1 interacts and stabilize IRF1 increasing stress-induced apoptosis in cancer [176]. The mechanism beyond XAF1 modulation and activity in pathogenic Th17 cells could provide interesting insights in the context of MS therapy that are worth to be further investigated.

5. Methods

5.1. Estrogens analysis

5.1.1. Super Enhancers Prediction

H3K27ac ChIP-Seq data of CD4+CD25–CD45RA+ T cells (GSM773004), CD4+CD25– T cells (GSM997239), CD4+CD25–IL17+ T cells (GSM772987), and CD4+CD25+CD45RA+ T cells (GSM1056941) were retrieved from GEO. Significant H3K27ac ChIP-Seq peaks were defined using MACS2 algorithm version 2.1.0 [177] applied in default settings. Input ChIP-Seq datasets were used as background models for enhancer calling. The list of significant ChIP-Seq peaks was used as input for ROSE algorithm. SEs were identified using Rank Ordering of Super Enhancers (ROSE) algorithm [178] in default settings using data of input as background model.

5.1.2. SNPs Analysis

SNPs associated with 41 different diseases were retrieved from GWAS database v2 [179] and overlapped with SEs from earlier analysis. Enrichment scores were computed generating 1,000,000 random regions of the same length and calculated as:

$$p - \text{value} = 1 + \text{n° of times Npermi} \geq \text{Nobs1} + \text{n° of permutations p} - \text{value} = 1 + \text{n° of times Npermi} \geq \text{Nobs1} + \text{n° of permutations}$$

with:

Nobs = Number of trait-associated SNPs overlapping SEs datasets
Npermi = Number of trait-associated SNPs overlapping the randomly generated datasets (n = 1,000,000).

5.1.3. Chromatin States Analysis

Genome segmentation data from Roadmap Epigenomics Project [180] were retrieved from the project website (http://egg2.wustl.edu/roadmap/web_portal) considering the 25-chromatin states model defined on imputed epigenomic data from 127 different cell types. The model was predicted using ChromHMM and 12 epigenetic marks (H3K4me1, H3K4me2, H3K4me3, H3K9ac, H3K27ac, H4K20me1, H3K79me2, H3K36me3, H3K9me3, H3K27me3, H2A.Z, and DNase accessibility) as input data [181]. The output of the model reported a 200 bp genomic

segmentation for each cell type with the predicted chromatin state. Segmentation data related to "E039—Primary CD25– CDRA45+ Naive T cells," "E043—Primary CD25– Th cells," "E042—Primary IL17+ PMA-I stimulated Th cells," "E044—Primary CD25+ regulatory T cells" (CD4+ T cells segmentation data) were extracted from the initial dataset and subjected to further analyses. Regulatory regions were identified by considering the chromatin states with an emission parameter of H3K27ac and H3K4me1 ≥75 (2_PromU, 9_TxReg, 10_TxEnh5', 13_EnhA1, 14_EnhA2, 15_EnhAF). The set of regulatory regions for each CD4+ T cells subtype was obtained by extracting from the CD4+ T cells segmentation data the genome segments classified in these states using an in-house Python script and, if consecutive, merging them. To distinguish regulatory regions according to their level of activity among CD4+ T cells subtypes, the chromatin state predicted in each 200 bp fragment composing regulatory regions was compared among CD4+ T cell subtypes. If more than half of the fragments within a merged region were classified as active regulatory regions in a specific CD4+ T cell subtype only, the entire region was classified as ARRs in that specific CD4+ T cell subtype. SE-ARRs were obtained overlapping ARRs and SEs using the *intersect* function of Bedtools suite [182].

5.1.4. Histone Marks Enrichment Analysis

Histone marks enrichment within ARRs and SE-ARRs was computed overlapping the regions with ChIP-Seq data in Table S1G using the *intersect* function of Bedtools suite [182] and computed as the mean of replicates over the mean of input datasets in each cell subtype.

5.1.5. Gene Ontology Analysis

Gene Ontology term enrichment of genes mapped in proximity of SEs and SE-ARRs was performed using the Genomic Regions Enrichment of Annotations Tool (GREAT) in default mode [183].

5.1.6. RNA-Seq Analysis (E-MTAB-2319)

PolyA+ RNA-Seq data of each the five biological replicates for CD4+ Naïve cells (CD4+CCR7+CD45RA+CD45RO–), CD4+ Th1 cells (CD4+CXCR3+), CD4+ Th2 cells (CD4+CRTH2+CXCR3–), CD4+ Th17 (CD4+CCR6+CD161+CXCR3–), and CD4+ Treg cells (CD4+CD127–CD25+) isolated from healthy donors were retrieved from ArrayExpress (E-MTAB-2319) [184]. Reads were mapped using TopHat v2 [185] using the hg19 human genome assembly as a reference genome and Gencode v19 as a reference set for gene annotation. FeatureCounts algorithm was used to compute read count and DESeq2 package was used to normalize count tables [186,187]. Normalized read counts were converted to fragments per kilobase of exons per million fragments mapped (FPKM) considering the length of the longest

isoform of each gene. A threshold of FPKM > 1 in all five biological replicates of each CD4+ T cell subtype was applied for further analyses. SEs were annotated to genes whose TSS was mapped within a distance of 100 Kbp from the center of the nearest SE. Differential expression analysis was performed using DESeq2 package [187]. A threshold of FDR adjusted $p < 0.001$ was used to detect significant differentially expressed genes between two CD4+ subtypes. Z-score transformation was performed computing the average expression across the five RNA-Seq replicates of each CD4+ subtype and then the mean and the standard deviation across the five CD4+ subtypes.

5.1.7. Network Reconstruction

For each CD4+ T cell subtype, network nodes represent expressed SE-ARR associated genes. A gene was classified as TF using a list of experimentally validated TFs from the Animal Transcription Factor Database v2 [188]. Network edges represent regulatory interactions predicted by motif finding analysis performed on SE-ARR sequences using Find Individual Motif Occurrences software (FIMO) of the MEME suite [189]. A non-redundant list of human Positional Weight Matrices (PWMs) was obtained from the integration of the four public PWM databases HOCOMOCO v10, jolma 2013, CISBP v1.02 and Jaspar vertebrates 2016 [190–193]. PWM were selected based on species and quality attributes. Human- or mouse-derived PWMs were selected favoring human-TF related matrices coming from experimental evidence respect to mouse-derived matrices or matrices obtained by computational inference. A significance threshold of 0.001 on the *p*-value score has been applied for this analysis. Node inward links connect a node/target gene with its TF regulators whose binding is predicted at node/target gene SE-ARRs. Conversely, outward links represent regulatory interaction of a node/TF with its target genes by SE-ARRs binding. We called CSRs the subset of SE-ARRs associated with highly differentially expressed TFs between Th17/Treg cells and Naive T cells (DESeq2 FDR adjusted $p < 1.0 \times 10^{-7}$). Thus, we filtered networks for CSRs, obtaining core regulatory subnetworks. Pairwise gene expression correlation analysis was performed using the 25 FPKM values from CD4+ RNA-Seq analysis (E-MTAB-2319) [184]. Pearson linear correlation on each pair of genes was computed. An absolute Pearson coefficient > 0.3961 was considered statistically significant for positive or negative correlations (two-tailed *t*-test, $p < 0.05$). Positive and negative correlations were used to represent activatory and inhibitory network links, respectively. For network visualization, Cytoscape version 3.4.0 was used [194]. Network analyzer was applied to compute network statistics [195].

5.1.8. PBMCs, Treg, and Th17 Cells Isolation

PBMCs were isolated from whole blood samples by a Ficoll-Paque TM PLUS (GE Healthcare, Milan, IT) density-gradient centrifugation.

5.1.9. In vitro Th17 Cells Polarization

Isolated PBMCs from female healthy donors (HD) (18–45 years old) were cultured in RPMI 1640 medium containing 10% estrogen deprived Fetal Bovine Serum (FBS), 2% HEPES, 1% Glutamax, and 1% Gentamicin. They were activated with plate-coated anti-CD3 (10 μg/ml) and soluble anti-CD28 monoclonal antibodies (mAbs) (1μg/ml; BD Biosciences, San Diego, CA) for 3 days in the presence of IL-23 (50 ng/ml; R&D Systems) plus anti–IFNγ (100 ng/μl; Biolegend, San Diego, CA) as previously described (*3*). At day 0, cells were treated with 17β-estradiol (E2) 35 ng/mL or vehicle (ethanol) in concomitance with Th17 polarizing cytokines.

5.1.10. Chromatin Immunoprecipitation Assay

PBMCs were incubated with 1% formaldehyde (Formaldehyde solution, Sigma-Aldrich 252549) in 1ml of RPMI 1640 medium containing 10% estrogen deprived Fetal Bovine Serum (FBS), 2% HEPES, 1% Glutamax, and 1% Gentamicin for 10 min at 37°C. Cross-linking was stopped by addition of glycine solution to a final concentration of 0.125 M for 5 min on a shaker at room temperature. Cell pellets were subjected to lysis on ice for 10 min with Lysis Buffer 1 (5 mM Pipes pH 8, 85 mM KCl, 0.5 % NP40) supplemented with complete protease inhibitors cocktail. Subsequently, nuclei pellets, obtained by a 5 min spin cycle at 4 ◦C (4000 rpm) were exposed once again to 10 min lysis in Lysis Buffer 2 (1% SDS, 5 mM EDTA, 50 mM Tris-HCl pH 8.1) supplemented with complete protease inhibitors cocktail. A small fraction of chromatin was decrosslinked with 50 μg of Proteinase K (Thermo Fisher Scientific) and DNA was purified with phenol chloroform (Ambion, Applied Biosystems), followed by ethanol precipitation. Chromatin fragmentation was checked by electrophoretic separation of DNA on a 1.2% agarose gel. Total extracted chromatin was sonicated to an average size of 250–500 bp by using an immersion sonicator device. The desired fragments size was checked on 1.2% agarose-gel and quantified by Nanodrop spectrophotometer at 280 nm, in order to use 50 μg of chromatin per IP. 10 μL (1%) of chromatin extracts was recovered as input normalization-control for each experimental condition. 10 μg (for histone modifications) or 30 μg (for ER*α*) of chromatin extracts were diluted with IP-buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl of pH 8.1, 150 mM NaCl, supplemented with complete protease inhibitors) and incubated overnight with specific antibody or IgG at 4◦ C on a rotating platform. Protein A and G sepharose-beads (GE Healthcare Life Sciences; Little Chalfont, UK; 17-5280-01 and 17-0618-01 respectively) were pre-coated with IP buffer supplemented with 5% BSA, in order to reduce nonspecific antibody binding. Upon 2 h of Protein A or G sepharose-beads incubation (depending on antibody source, i.e., rabbit or mouse

respectively), samples were washed sequentially for 5 min, on a rotating platform with 1 mL of three different Washing Buffer (Washing buffer 1: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl pH 8, 150 mM NaCl; Washing buffer 2 : 0.1 SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl pH 8, 500 mM NaCl; Washing buffer 3: 0.25 M LiCl, 1% NP40, 1% Na DOC, 1 mM EDTA, 10 mM Tris HCl pH8) and twice with TE buffer (10 mM Tris HCl pH8, 1 mM EDTA). After complexes elution at RT with elution buffer (1% SDS, 0.1 M NaHCO3), DNA fragments were de-crosslinked at 65 ◦C overnight with NaCl 5 M and by 1 h of proteinase K treatment. DNA purification was achieved with Phenol:Chloroform:IAA (25:24:1 UltraPure™ formulation, Ambion AM9730) according to the manufacturer's instructions. Quantitative Real-time PCR was carried out on ChIP-enriched DNA using SYBR Green Master Mix. ChIP enrichment was normalized on input samples (1% of total chromatin used per IP) and expressed as fold-enrichment of specific binding over the control nonspecific IgG binding. Antibodies against human ERα (Santa Cruz Biotechnology, Dallas, TX, USA; sc543X, sc7207X), human H3K4me3 (Diagenode), human H3K27me3 (Active Motif), H3K27ac (Active Motif) and normal rabbit and mouse IgG (Merk-Millipore, Burlington, MA, USA; 12-370 and 12-371 respectively) were used in this assay. Custom ChIP primer pairs are reported in Table 1.

5.1.11. Quantitative PCR

Real Time PCR was performed using 7300 Real Time PCR System (Applied Biosystems) and the iTaq Universal SYBR Green Supermix (Biorad) in 96-wells multiwell plates (Applied Biosystems). Gene expression was determined using QuantiTect Primer Assays (QIAGEN, Hilden, Germany). Relative quantification of cDNA was normalized on 18s cDNA level. Primers for ChIP-qPCR analysis of promoter and enhancers were designed using Primer3Plus software (Table 3).

Primer name	Nucleotidic sequence
RORCprom_fw	5′ - CAAGAGCAGCAAGGGTTAGG - 3′
RORCprom_rv	5′ - TTGGGGGACTGTGTCTCTTC - 3′
MAFprom_fw	5′ - TTACACCAAACTTTGCGCCG - 3′
MAFprom_rv	5′ - TTCGGAGCTGTCAATCAGGG - 3′
MAFenh_fw	5′ - AGGACAGGCTCCTCGGTAGT - 3′
MAFenh_rv	5′ - GTCGAATGGCTGCTGAATGC - 3′
FOXP3 promoter fw	5′ - TAATGCATCCATCCTCACGA - 3′
FOXP3 promoter rv	5′ - ATGATGGCGGATATTTGGAA - 3′
SATB1prom_fw	5′ - TGCTTTATAGAGTCCCTGCTGT - 3′
SATB1prom_rv	5′ - AAAGGTCAGGTGACAGGCAC - 3′
SATB1enh_fw	5′ - ACACAGTGGTTGATTCGACTTT - 3′
SATB1enh_rv	5′ - TGGAATTGTGGTCTCGGCAT - 3′

Table 3. Primers designed for ChIP-qPCR experiments on RORC, MAF, FOXP3 and SATB1 CSRs.

ChIP-qPCR signals were normalized on input samples (10% of total chromatin used per IP) and expressed as enrichment of specific binding over the control non-specific IgG binding.

5.1.12. Total RNA Extraction

RNA was extracted using using Purezol™ reagent (Bio-Rad) according to the manufacturer protocol. All total isolated RNAs were subjected to DNase treatment to remove contaminating genomic DNA (ezDNase™ Enzyme, Invitrogen, Carlsbad, CA, USA; 11766051). cDNA synthesis from 500 ng of total RNA template was performed with SensiFAST cDNA Synthesis Kit (Bioline, London, UK; BIO-65054) followed by SYBR-green qRT-PCR amplification (iTaq UniverSYBR Green, Bio-Rad 1725124). Real-time PCR primers for human 18S, RORC, MAF, SATB1, FOXP3 were purchased from Qiagen (Hilden, Germany; QuantiTect™ Primer Assay).

5.2. Interferon-β analysis

5.2.1. Transcriptome analysis of Interferon-beta treated MS patients (GSE73608, GSE16214 and GSE41850).

Differential gene expression was computed using GEO2R tool. GEO2R performs comparisons on original submitter-supplied processed data tables using the GEOquery [196] and limma R package [197] from the Bioconductor project [198]. Bioconductor is an open source software project based on the R programming language that provides tools for the analysis of high-throughput genomic data. The

GEOquery R package parses GEO data into R data structures that can be used by other R packages. The Limma (Linear Models for Microarray Analysis) R package performs statistical tests for identifying differentially expressed genes [197]. It handles a wide range of experimental designs and data types and applies multiple-testing corrections on P-values to help correct for the occurrence of false positives [199]. DEGs were detected applying a threshold of 0.05 for the corrected p-value for multiple testing (Benjamin-Hotchberg) and -0.5/0.5 for the fold change (log2) of expression between compared conditions.

5.2.2. Transcriptome analysis of CCR6+ T cells from MS patients and HD

RNA-seq count tables at the gene level for the study SRP056049 were extracted in the format of RangedSummarizedExperiment object using recount R package [174,200]. Recount2 is an analysis-ready RNA-Seq database that provide pre-computed count matrices at gene level useful to researchers to avoid the computationally process of reads processing in cross-study analyses [174]. SRP056049 study contains four conditions of purified Th17 cells with between 3 and 5 replicates per condition. Precisely, CCR6+ T cells myelin tetramer-positive and negative cells from HD or MS patients. Counts and data referring to MS patients were extracted from the dataset and used for further analysis. Read counts were scaled for a target library size of 40 million reads with the recount function scale_counts and submitted to further analysis. Ensembl ID to gene symbol conversion has been performed using the org.Hs.eg.db R package [201].
Differentially gene expression analysis between conditions was performed using DESeq2 R package [187]. A threshold of count > 1 in all samples was used to define expressed genes. The variance between samples has been computed using the variance stabilizing transformation function of DESeq2 R package. Variance stabilizing transformation function transformed counts (normalized by division by the size factors or normalization factors), yielding a matrix of values which are approximately homoscedastic (having constant variance along the range of mean values). Obtained values were subtracted to the mean of values of each row obtaining the z-score. Data has been plotted using the R package complex heatmap [202].

5.2.3. Gene Set Enrichment analysis

Gene Set Enrichment analysis have been performed using EnrichR software [203,204].

5.2.4. Data Mining

Data mining was performed using the machine learning workbench Wakaito Environment for Knowledge Analysis (Weka) version 3.9.3 amazon's corrected for MacOSX [205]. GSE16214 and GSE41850 expression matrices were retrieved from GEO and formatted to be used as input for Weka. The expression of genes was used as numeric attributes, patients were used as instances and IFN-β treatment constituted the nominal class to test classifiers. The "Weka Explorer" was used to read input files and to engineer data. The expression matrices were normalized for inter-experiment cross-validation using the "Normalize" function within the section of "unsupervised attribute filtering" of the "Weka Explorer". Algorithm comparisons were performed using the Weka Experimenter. Input matrices were filtered using the lists of DEGs in GSE16214 and GSE41850 and the list of 23 ISGs listed in Table S3A-B, D. In this step the performance of 9 different algorithms ("rules.ZeroR", "rules.OneR", "trees.J48", "bayes.NaiveBayes", "functions.SMO", "meta.Bagging", "meta.AdaBoostM1", "lazy.IBk", "trees.RandomForest") were tested in 10-fold cross validation with 20 iterations to minimize overfitting. The performance of the test was expressed in percentage of correctly classified instances and statistically significant differences among algorithms was computed with the "Analyze" section (corrected paired T-test <0.05). Algorithms were ranked according to the results of this test. In the last part of the analysis GSE41850 dataset was engineered removing males and splitting "1-year follow-up" data and "2-year follow up" data. The importance of attributes in the performance of "trees.RandomForest" classifier was tested using the "Ranker search method" and the "InfoGainAttributeEvaluator". Finally, the same classifier was used for inter-experiment cross-validation removing one by one the attributes ranked in Table S4D and to identify the minimal set of attributes that best performed in inter-experiment classification. "PairedCorrectedTTester" function was applied to compute statistics.

5.2.5. Apoptosis

KEGG 2019 pathway hsa04210 - Apoptosis - Homo sapiens (human) gene list was retrieved and expression data integrated and visualized on the corresponding apoptosis pathway map using the Pathview Bioconductor package [206].

5.2.6. Software

All the analyses have been performed in R version 3.6.0 (2019-04-26) and R packages were retrieved with the Bioconductor version 3.9 (2019-05-03) [198].

6. Discussion

Multiple Sclerosis progression and therapy are strictly connected in a continuous balance between monitoring of demyelinating lesions, symptoms, side effects and response to DMTs [20]. In RRMS, DMTs aim to reduce the rate of relapses and preventing or at least delaying disease progression. One of the main issues connected with MS is the progressive accumulation of neurological damage, due to therapy inefficacy. DMT switch is due in case of lack of benefit, intolerable adverse effects, or availability of more effective therapy. Years of clinical experience demonstrated the efficacy of early intervention in MS to delay disease progression [21]. However, to date, the evaluation of the efficacy of therapy is possible only by the evaluation of clinical parameters (CNS lesions, new relapses) since the molecular bases underneath disease progression are still unknown [20]. The diffusion of NGS techniques opened the way to genome-wide based research. Integrative approaches of the large amounts of data, produced by NGS techniques, represent useful tools in the understanding of complex disorders as MS, leading to the identification of new hypotheses to explain disease and therapeutic mechanisms connected to it and more beyond, towards personalized stratification [164].
This thesis followed the common thread of systems biology approach as a tool to produce a vision of the molecular mechanisms involved in two different processes related to Th17 cells in MS pathology: estrogen and interferon-β induced transcriptional activity in Th17 cells.

In the first example, we retrieved the epigenetic and the transcriptome profile of different T helper subtypes and developed an integration pipeline aimed to identify specific targets for the epigenetic action of estrogens on Th17 cells. Pregnancy protection from relapses has been demonstrated by several clinical studies [24–27]. The high level of circulating estrogens (estradiol and estriol) during pregnancy contribute to immunotolerance, inducing Treg cells proliferation [37,38,103]. On the contrary, the postpartum phase is characterized by a strong drop in estrogens levels [37]. Thus, pregnancy and postpartum represent a unique opportunity for comparison, as pregnancy maximizes the differences in immune cell subtypes. As occurs in the postpartum period the female body is characterized by an estrogen drop during menopause [207]. The decline of estrogens' concentration during menopause correlates with a reduced number of B and T cells and an increased secretion of pro-inflammatory cytokines [208]. Moreover, estrogen deficiency in postmenopausal women is associated with increased IL-17A levels [209]. A retrospective questionnaire-based study on menopausal and premenopausal women with MS [210] showed that 82% of menopausal women reported that the severity of their symptoms worsened during the premenstrual

period. Among postmenopausal women, 54% reported worsening symptoms after menopause, and 75% of postmenopausal women who tried hormone replacement therapy reported disease improvement [210]. Clinical observations associated estrogen signaling and the progression of MS disease, such that estrogens entered in use as MS therapy in clinical trials [29–32]. In this context, evidence showed also how this protective effect is mediated and bases on the activity of estrogen receptors. Studies in Experimental Autoimmune Encephalomyelitis (EAE), the murine model of MS, showed that ERα expression and signaling in encephalitogenic CD4+ T cells is required for sustained pregnancy protection [112] and estrogen signaling results in an inhibition of encephalitogenic Th17 cells expansion [211], in an induction of tolerogenic dendritic cells and regulatory B cells [89] and regulatory T cells [40]. Similar to the EAE model and previous MS studies [40,212], we showed that in the third trimester of pregnancy, when circulating estrogens reach the highest level, Th17 population is reduced while Treg cells expanded [102].

We investigated the epigenetic effects of estradiol at pregnancy levels on the Th17 / Treg balance, since it is considered a mirror of MS disease [118] and the protection from relapses during pregnancy has been associated with an expansion in the Treg population [40]. Estrogens act on T helper cells by modulating the production of cytokines and interleukins and influencing the inflammatory environment [33]. The chromatin landscape is important in the context of cellular plasticity as the chromatin state influences TF binding and transcriptional regulation of target genes [113]. Th17 and Treg cells are strictly connected and plasticity has been observed between these two antagonistic cell types. Th17-like Treg cells and FoxP3+ Treg cells that express IL-17 have been reported [213,214]. Moreover, Th17-Treg plasticity shown a dependence on epigenetic modifications produced by histone deacetylase activity [215].

The mechanism of the recruitment of chromatin remodeling complexes by ERα have been largely studied in breast cancer [55–57,59,216]. The formation of functional chromatin loops between ERα binding sites at the enhancers and promoters of target genes that are activated [59–62] or repressed [63]. However, ERα activity is influenced by the tissue-specific presence of coactivators and transcriptional corepressors and their differential interaction with ERα in the presence of estrogens or anti-estrogens [64,65]. In this way, ERα could act on T helper epigenome as a cooperative transcription factor and orchestrate T helper gene expression toward immunotolerance [117].

On these bases, we reconstructed a regulatory network of human Th17 and Treg cells, highlighting CSR-associated TFs that cooperate in cell identity specification. Network reconstruction has been used as a tool to visualize data coming from combining -omics data integration pipelines, or innovative perturbation tools and reveal the molecular bases of the cell identity of Th17 cells of mice [121,122]. Our network exploited PWMs as predictive information of TF binding, including ERα, and chromatin states as a filter for the transcriptional regulation by TFs. The

combination of these two information was useful in the absence of genome-wide data on the gene modulation by estrogens and ERα binding profile in T helper cells. The occurrence of EREs in genomic regions with high transcriptional activity in the specification of cellular identity served to identify the candidates for a validation of the epigenetic action of ERα on polarizing T helper 17 cells.

RORC, MAF, and HSF4 stood out as candidates of estrogen-mediated regulation in Th17 cells. It has been shown yet that estradiol activated ERα was capable to recruit the repressor of estrogen receptor activity (REA) and form the ERα/REA complex on the RORC promoter, thus inhibiting RORC expression and Th17 differentiation. This effect could have been pursued by regulating the recruitment of histone deacetylases [137]. MAF has been proposed as one of the early regulators of T cell- associated diseases [217]. It resulted to be in the top three TFs belonging to the Th1/Th2 gene regulatory network and enriched in autoimmunity-associated polymorphisms. The targets of MAF were also shown to be differentially expressed during MS relapses [217]. Moreover, another study showed that the MAF-associated long intergenic non-coding RNA (linc)-MAF-4 regulated MAF transcription by exploiting a chromosome loop with the promoter of MAF gene and its expression shifted Th cells differentiation alternately toward Th1 or Th2 subtype [184]. Another study proposed c-Maf as important for the establishment of memory Th1 and Th17 cells [218]. Finally, the less studied HSF4 could represent a novel molecular target in the field of MS disease and stress-response in T helper cells.

Regarding Treg cells, FOXP3, IKZF2, IKZF4, SATB1, PRDM1 and MZF1 stood out as candidates of estrogen-mediated regulation in Treg cells. It is known that Foxp3 is the lineage-determining transcription factor of Treg cells, but is not necessary nor sufficient to determine the characteristic Treg cell signature [219]. In particular IKZF2, IKZF4 SATB1 act in synergy with Foxp3 to activate expression of the Treg cell signature and enhance the Foxp3 occupancy at its genomic targets [219]. In particular Satb1 importance have been demonstrated in Treg Super Enhancers activation and its perturbation is causative of autoimmune and other immunological diseases [132].

PRDM1, also known as Blimp1, role have been recently investigated in the epigenetic regulation of Foxp3. The methylation of Foxp3 conserved non-coding sequence 2 (CNS2) was shown to be heavily methylated when Blimp1 was ablated, leading to a loss of Foxp3 expression and severe disease [135]. Interestingly, the FOXP3-CSR that arised from our analyses overlap the conserved non-coding sequences known from EAE studies. Foxp3-CNS are three intronic enhancers identified at Foxp3 gene locus. The epigenetic status of these regulatory regions have been associated with different Treg functions [126]. Finally, MZF1 has been recently counted in key TFs that mediating transcriptional changes between Th1 and Treg cells [220].

Supported by the evidence we selected RORC, MAF, FOXP3 and SATB1-CSRs to be validated for ERα mediated regulation during Th17 polarization in presence of

E2. The epigenetic changes observed and the experimental validation of an increased ERα binding at the regulatory regions of these genes suggested a switch toward immune tolerance upon E2 treatment. The transcriptional effect could be ascribed to the recruitment of a regulatory complexes that involve ERα. However, a direct validation of how this machinery works in T helper cells is still lacking. Of particular interest was the observation that epigenetic modifications occur at their Super Enhancers. This is an important aspect since the non-coding genome, including SEs worth to be deeply investigated as might be used as "epigenetic drugs" for MS. Evidence showed that SEs are vulnerable to various inhibitors of transcriptional activation [221–224]. CD4+ T cells treated with JAK inhibitor tofacitinib selectively targeted rheumatoid arthritis risk genes controlled by SEs [223]. The exposure of CD4+ T cells from Juvenile idiopathic arthritis (JIA) patients to the Bromodomain and Extra-Terminal motif (BET) protein inhibitor JQ1 preferentially inhibited JIA-specific super-enhancer driven gene expression [224]. Other important limitations of this study include the lack of NGS data of epigenetic and transcriptional modulation by E2 in T helper cells. A genome-wide approach could reveal all the changes connected to the effects of estrogens in T helper cells and MS disease.

In the second example, we addressed another issue connected with MS disease. Given the nature of RRMS, the risk of adverse effects and considerable costs for therapy, there is a need to predict success before the start of IFN-β therapy or to monitor its efficacy before any adverse event comes (e.g. relapse, new CNS lesion) and avoid accumulation of disability over time. Many ISGs have been identified during years that could help in the monitoring of response to treatment [27 - 30]. Among them, the myxovirus-resistance protein A (MxA) started entering in the clinical practice and some other promising molecules are under investigation [34-35-36]. The baseline expression level of these genes in whole blood had not the predictive capability of a biomarker for IFNβ response, thus it has been indicated to enlarge cohorts or focus on the mechanisms related to IFN-β in a specific cell type connected with MS [23 - 26].
On these bases, we performed an integrative analysis of the gene expression profile of PBMC in MS patients treated with beta interferons identifying a set of interferon-modulated genes. Then, we speculated on the action of the drug on the transcriptome of pathogenic T helper cells exploiting a public RNA-Seq dataset of autoreactive T cell clones from MS patients. We identified 23 ISGs with a consistent overlap with literature [162,167,168,171,172]. HERC5, IFI44L, IFIT1, MX1, RSAD2, SIGLEC1 [167,168], EIF2AK2, HERC6, IFI6, IFIT3, LGALS3BP, OASL [168] and IFI27, USP18 [167] have been indicated as ISGs in previous studies of transcriptional response to IFN-β in MS patients. Moreover, some of our genes have not been detected by these studies but their use as blood biomarkers for IFN-β therapy in MS is currently under investigation (CXCL10, IFIT2, ISG15, OAS3, XAF1) [162,171,172]. EIF2AK2 has been recently detected as one of the deregulated

molecules in MS using a system biology approach [165]. Its physically interaction with STAT1 and ISG15 downstream IRF regulation has been demonstrated. ISG15 and IFIT2:IFIT3 complex have been indicated in the same study. Recent quantitative proteomic studies detected DDX60 together with other proteins related to antiviral pathways (e.g. MX1, OAS, OAS3, and OASL) as upregulated in Th17 differentiation suggesting the role of IFN signaling in Th17 polarization [225].
In the second part of the analysis we identified two downregulated genes in myelin reactive T helper cells. These two genes are ISGs and this feature could represent important molecular targets for MS therapy. LGALS3BP has been recently indicated as a deregulated gene in MS using a system biology approach [165]. The immunoinhibitory function of LGALS3BP is currently under investigation in the immune evasion of tumor cells during cancer progression [226,227].
XAF1 was downregulated in pathological Th17 cells in MS and its expression could be restored by IFN-β treatment. XAF1 induces apoptosis by inhibiting XIAP, which is one of the most important members of the IAP family. XAF1 acts as a proapoptotic tumor suppressor that is frequently inactivated in multiple human cancers, however the molecular basis for the XAF1-mediated growth inhibition remains largely undefined. Recently, a positive feedback loop of XAF1 with interferon regulatory factor-1 (IRF-1) has been reported. XAF1 can function as a transcriptional coactivator of IRF-1 and suppress tumorigenesis [176].
Moreover, XAF1-IRF1 complex inhibit gene expression of matrix metalloproteases 9 and 2 (MMP9-2), Fibronectin1 (FN1), Vascular cell adhesion molecule 1 (VCAM1), Tenascin C (TNC) regulating growth, migration and invasion. Recently it has been shown that Selective inhibition of MMP9 in CD4+ T-cells reduced clinical severity in the murine model of Multiple Sclerosis (Onwuha-Ekpete 2017) [228]. XAF-1 mRNA has been recently tested together with MxA and TNF-related apoptosis-inducing ligand (TRAIL) in MS to assess IFN-β treatment efficacy [162].
The apoptotic process have been demonstrated to be significantly and selectively greater in Th17 cells rather than Th1 cells upon IFN-β treatment [2]. Further experiments could validate the involvement of XAF1 in the process of apoptosis in myelin reactive Th17 cells and clarify the role IFN-β modulation in this process. XAF1 interacts with IRF1 in tumor suppressor gene activity suggesting a potential mechanism for immune regulation in MS upon IFN-β treatment. The inhibition of MMP9 and MMP2, FN1, VCAM1 and TNC gene expression by XAF1-IRF1 could also regulate the processes behind Th17 invasion in the CNS.
A caveat of this work is the partial or complete unavailability of genetic data about patients. Indeed, some of these genes have already been associated with MS susceptibility or tested for IFN-β modulation in MS [153]. Moreover, the unavailability of data about the production of neutralizing anti-IFN-beta antibodies (Nabs) with affinity for IFNAR was a limitation for our analysis. One of the main reasons for the reduction of IFN-β efficacy is Nabs production. Nabs block the downstream IFN-β signalling leading to alteration of ISGs transcription which

reduce the clinical effect of IFN-β [162]. Nabs could be an interesting parameter to stratify patient's gene expression.
In conclusion, this thesis followed the common thread of the use of NGS data in the field of systems biology to reveal interesting molecular features of Th17 cells and address the therapeutic aspects connected to their involvement in the progression of RRMS disease.

7. References

1. Dendrou, C.A.; Fugger, L.; Friese, M.A. Immunopathology of multiple sclerosis. *Nat. Rev. Immunol.* **2015**, *15*, 545–558.
2. Durelli, L.; Conti, L.; Clerico, M.; Boselli, D.; Contessa, G.; Ripellino, P.; Ferrero, B.; Eid, P.; Novelli, F. T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-beta. *Ann. Neurol.* **2009**, *65*, 499–509.
3. Langrish, C.L.; Chen, Y.; Blumenschein, W.M.; Mattson, J.; Basham, B.; Sedgwick, J.D.; McClanahan, T.; Kastelein, R.A.; Cua, D.J. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* **2005**, *201*, 233–240.
4. Kebir, H.; Kreymborg, K.; Ifergan, I.; Dodelet-Devillers, A.; Cayrol, R.; Bernard, M.; Giuliani, F.; Arbour, N.; Becher, B.; Prat, A. Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat. Med.* **2007**, *13*, 1173–1175.
5. Tzartos, J.S.; Friese, M.A.; Craner, M.J.; Palace, J.; Newcombe, J.; Esiri, M.M.; Fugger, L. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am. J. Pathol.* **2008**, *172*, 146–155.
6. Rolla, S.; Bardina, V.; De Mercanti, S.; Quaglino, P.; De Palma, R.; Gned, D.; Brusa, D.; Durelli, L.; Novelli, F.; Clerico, M. Th22 cells are expanded in multiple sclerosis and are resistant to IFN-β. *J. Leukoc. Biol.* **2014**, *96*, 1155–1164.
7. Compston, A.; Coles, A. Multiple sclerosis. *Lancet Lond. Engl.* **2008**, *372*, 1502–1517.
8. Westerlind, H.; Ramanujam, R.; Uvehag, D.; Kuja-Halkola, R.; Boman, M.; Bottai, M.; Lichtenstein, P.; Hillert, J. Modest familial risks for multiple sclerosis: a registry-based study of the population of Sweden. *Brain J. Neurol.* **2014**, *137*, 770–778.
9. Sadovnick, A.D.; Armstrong, H.; Rice, G.P.; Bulman, D.; Hashimoto, L.; Paty, D.W.; Hashimoto, S.A.; Warren, S.; Hader, W.; Murray, T.J. A population-based study of multiple sclerosis in twins: update. *Ann. Neurol.* **1993**, *33*, 281–285.
10. Hollenbach, J.A.; Oksenberg, J.R. The immunogenetics of multiple sclerosis: A comprehensive review. *J. Autoimmun.* **2015**, *64*, 13–25.
11. International Multiple Sclerosis Genetics Consortium; Wellcome Trust Case Control Consortium 2; Sawcer, S.; Hellenthal, G.; Pirinen, M.; Spencer, C.C.A.; Patsopoulos, N.A.; Moutsianas, L.; Dilthey, A.; Su, Z.; et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* **2011**, *476*, 214–219.
12. Belbasis, L.; Bellou, V.; Evangelou, E.; Ioannidis, J.P.A.; Tzoulaki, I. Environmental risk factors and multiple sclerosis: an umbrella review of systematic reviews and meta-analyses. *Lancet Neurol.* **2015**, *14*, 263–273.
13. Handel, A.E.; Williamson, A.J.; Disanto, G.; Handunnetthi, L.; Giovannoni, G.; Ramagopalan, S.V. An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis. *PloS One* **2010**, *5*.
14. Zhang, P.; Wang, R.; Li, Z.; Wang, Y.; Gao, C.; Lv, X.; Song, Y.; Li, B. The risk of smoking on multiple sclerosis: a meta-analysis based on 20,626 cases from case-control and cohort studies. *PeerJ* **2016**, *4*.
15. McDowell, T.-Y.; Amr, S.; Culpepper, W.J.; Langenberg, P.; Royal, W.; Bever, C.; Bradham, D.D. Sun exposure, vitamin D intake and progression to disability among veterans with progressive multiple sclerosis. *Neuroepidemiology* **2011**, *37*, 52–57.
16. Glenn, J.D.; Mowry, E.M. Emerging Concepts on the Gut Microbiome and Multiple

Sclerosis. *J. Interferon Cytokine Res. Off. J. Int. Soc. Interferon Cytokine Res.* **2016**, *36*, 347–357.
17. Kotzamani, D.; Panou, T.; Mastorodemos, V.; Tzagournissakis, M.; Nikolakaki, H.; Spanaki, C.; Plaitakis, A. Rising incidence of multiple sclerosis in females associated with urbanization. *Neurology* **2012**, *78*, 1728–1735.
18. Fish, E.N. The X-files in immunity: sex-based differences predispose immune responses. *Nat. Rev. Immunol.* **2008**, *8*, 737–744.
19. Testicular hypofunction and multiple sclerosis: Cause or consequence? - Voci - 2014 - Annals of Neurology - Wiley Online Library Available online: https://onlinelibrary.wiley.com/doi/full/10.1002/ana.24276 (accessed on Nov 12, 2019).
20. Montalban, X.; Gold, R.; Thompson, A.J.; Otero-Romero, S.; Amato, M.P.; Chandraratna, D.; Clanet, M.; Comi, G.; Derfuss, T.; Fazekas, F.; et al. ECTRIMS/EAN Guideline on the pharmacological treatment of people with multiple sclerosis. *Mult. Scler. Houndmills Basingstoke Engl.* **2018**, *24*, 96–120.
21. Dobson, R.; Dassan, P.; Roberts, M.; Giovannoni, G.; Nelson-Piercy, C.; Brex, P.A. UK consensus on pregnancy in multiple sclerosis: 'Association of British Neurologists' guidelines. *Pract. Neurol.* **2019**, *19*, 106–114.
22. Angelis, F.D.; John, N.A.; Brownlee, W.J. Disease-modifying therapies for multiple sclerosis. *BMJ* **2018**, *363*, k4674.
23. Korn-Lubetzki, I.; Kahana, E.; Cooper, G.; Abramsky, O. Activity of multiple sclerosis during pregnancy and puerperium. *Ann. Neurol.* **1984**, *16*, 229–231.
24. Confavreux, C.; Hutchinson, M.; Hours, M.M.; Cortinovis-Tourniaire, P.; Moreau, T. Rate of pregnancy-related relapse in multiple sclerosis. Pregnancy in Multiple Sclerosis Group. *N. Engl. J. Med.* **1998**, *339*, 285–291.
25. Vukusic, S.; Hutchinson, M.; Hours, M.; Moreau, T.; Cortinovis-Tourniaire, P.; Adeleine, P.; Confavreux, C.; Pregnancy In Multiple Sclerosis Group Pregnancy and multiple sclerosis (the PRIMS study): clinical predictors of post-partum relapse. *Brain J. Neurol.* **2004**, *127*, 1353–1360.
26. Finkelsztejn, A.; Brooks, J.B.B.; Paschoal, F.M.; Fragoso, Y.D. What can we really tell women with multiple sclerosis regarding pregnancy? A systematic review and meta-analysis of the literature. *BJOG Int. J. Obstet. Gynaecol.* **2011**, *118*, 790–797.
27. Hughes, S.E.; Spelman, T.; Gray, O.M.; Boz, C.; Trojano, M.; Lugaresi, A.; Izquierdo, G.; Duquette, P.; Girard, M.; Grand'Maison, F.; et al. Predictors and dynamics of postpartum relapses in women with multiple sclerosis. *Mult. Scler. Houndmills Basingstoke Engl.* **2014**, *20*, 739–746.
28. Javadian, A.; Salehi, E.; Bidad, K.; Sahraian, M.A.; Izad, M. Effect of estrogen on Th1, Th2 and Th17 cytokines production by proteolipid protein and PHA activated peripheral blood mononuclear cells isolated from multiple sclerosis patients. *Arch. Med. Res.* **2014**, *45*, 177–182.
29. Sicotte, N.L.; Liva, S.M.; Klutch, R.; Pfeiffer, P.; Bouvier, S.; Odesa, S.; Wu, T.C.J.; Voskuhl, R.R. Treatment of multiple sclerosis with the pregnancy hormone estriol. *Ann. Neurol.* **2002**, *52*, 421–428.
30. Voskuhl, R.R.; Wang, H.; Wu, T.C.J.; Sicotte, N.L.; Nakamura, K.; Kurth, F.; Itoh, N.; Bardens, J.; Bernard, J.T.; Corboy, J.R.; et al. Estriol combined with glatiramer acetate for women with relapsing-remitting multiple sclerosis: a randomised, placebo-controlled, phase 2 trial. *Lancet Neurol.* **2016**, *15*, 35–46.
31. Vukusic, S.; Ionescu, I.; El-Etr, M.; Schumacher, M.; Baulieu, E.E.; Cornu, C.; Confavreux, C.; Prevention of Post-Partum Relapses with Progestin and Estradiol in Multiple Sclerosis Study Group The Prevention of Post-Partum Relapses with Progestin and Estradiol in Multiple Sclerosis (POPART'MUS) trial: rationale, objectives and state of advancement. *J.*

Neurol. Sci. **2009**, *286*, 114–118.
32. Pozzilli, C.; De Giglio, L.; Barletta, V.T.; Marinelli, F.; Angelis, F.D.; Gallo, V.; Pagano, V.A.; Marini, S.; Piattella, M.C.; Tomassini, V.; et al. Oral contraceptives combined with interferon β in multiple sclerosis. *Neurol. Neuroimmunol. Neuroinflammation* **2015**, *2*.
33. Maglione, A.; Rolla, S.; Mercanti, S.F.D.; Cutrupi, S.; Clerico, M. The Adaptive Immune System in Multiple Sclerosis: An Estrogen-Mediated Point of View. *Cells* **2019**, *8*, 1280.
34. Watson, C.S.; Alyea, R.A.; Cunningham, K.A.; Jeng, Y.-J. Estrogens of multiple classes and their role in mental health disease mechanisms. *Int. J. Womens Health* **2010**, *2*, 153–166.
35. Wend, K.; Wend, P.; Krum, S.A. Tissue-Specific Effects of Loss of Estrogen during Menopause and Aging. *Front. Endocrinol.* **2012**, *3*.
36. Robinson, D.P.; Klein, S.L. Pregnancy and pregnancy-associated hormones alter immune responses and disease pathogenesis. *Horm. Behav.* **2012**, *62*, 263–271.
37. Patas, K.; Engler, J.B.; Friese, M.A.; Gold, S.M. Pregnancy and multiple sclerosis: feto-maternal immune cross talk and its implications for disease activity. *J. Reprod. Immunol.* **2013**, *97*, 140–146.
38. Somerset, D.A.; Zheng, Y.; Kilby, M.D.; Sansom, D.M.; Drayson, M.T. Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset. *Immunology* **2004**, *112*, 38–43.
39. Santner-Nanan, B.; Peek, M.J.; Khanam, R.; Richarts, L.; Zhu, E.; Fazekas de St Groth, B.; Nanan, R. Systemic increase in the ratio between Foxp3+ and IL-17-producing CD4+ T cells in healthy pregnancy but not in preeclampsia. *J. Immunol. Baltim. Md 1950* **2009**, *183*, 7023–7030.
40. Sánchez-Ramón, S.; Navarro A, J.; Aristimuño, C.; Rodríguez-Mahou, M.; Bellón, J.M.; Fernández-Cruz, E.; de Andrés, C. Pregnancy-induced expansion of regulatory T-lymphocytes may mediate protection to multiple sclerosis activity. *Immunol. Lett.* **2005**, *96*, 195–201.
41. Björnström, L.; Sjöberg, M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol. Endocrinol. Baltim. Md* **2005**, *19*, 833–842.
42. Webb, P.; Nguyen, P.; Valentine, C.; Lopez, G.N.; Kwok, G.R.; McInerney, E.; Katzenellenbogen, B.S.; Enmark, E.; Gustafsson, J.A.; Nilsson, S.; et al. The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. *Mol. Endocrinol. Baltim. Md* **1999**, *13*, 1672–1685.
43. Safe, S. Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. *Vitam. Horm.* **2001**, *62*, 231–252.
44. Caizzi, L.; Ferrero, G.; Cutrupi, S.; Cordero, F.; Ballaré, C.; Miano, V.; Reineri, S.; Ricci, L.; Friard, O.; Testori, A.; et al. Genome-wide activity of unliganded estrogen receptor-α in breast cancer cells. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 4892–4897.
45. Zhao, C.; Dahlman-Wright, K.; Gustafsson, J.-Å. Estrogen receptor β: an overview and update. *Nucl. Recept. Signal.* **2008**, *6*.
46. Reid, G.; Denger, S.; Kos, M.; Gannon, F. Human estrogen receptor-alpha: regulation by synthesis, modification and degradation. *Cell. Mol. Life Sci. CMLS* **2002**, *59*, 821–831.
47. Flouriot, G.; Brand, H.; Denger, S.; Metivier, R.; Kos, M.; Reid, G.; Sonntag-Buck, V.; Gannon, F. Identification of a new isoform of the human estrogen receptor-alpha (hER-α) that is encoded by distinct transcripts and that is able to repress hER-α activation function 1. *EMBO J.* **2000**, *19*, 4688–4700.
48. Denger, S.; Reid, G.; Kos, M.; Flouriot, G.; Parsch, D.; Brand, H.; Korach, K.S.; Sonntag-Buck, V.; Gannon, F. ERalpha gene expression in human primary osteoblasts:

evidence for the expression of two receptor proteins. *Mol. Endocrinol. Baltim. Md* **2001**, *15*, 2064–2077.
49. Wang, Z.; Zhang, X.; Shen, P.; Loggie, B.W.; Chang, Y.; Deuel, T.F. Identification, cloning, and expression of human estrogen receptor-alpha36, a novel variant of human estrogen receptor-alpha66. *Biochem. Biophys. Res. Commun.* **2005**, *336*, 1023–1027.
50. Phiel, K.L.; Henderson, R.A.; Adelman, S.J.; Elloso, M.M. Differential estrogen receptor gene expression in human peripheral blood mononuclear cell populations. *Immunol. Lett.* **2005**, *97*, 107–113.
51. Pierdominici, M.; Maselli, A.; Colasanti, T.; Giammarioli, A.M.; Delunardo, F.; Vacirca, D.; Sanchez, M.; Giovannetti, A.; Malorni, W.; Ortona, E. Estrogen receptor profiles in human peripheral blood lymphocytes. *Immunol. Lett.* **2010**, *132*, 79–85.
52. Murphy, A.J.; Guyre, P.M.; Wira, C.R.; Pioli, P.A. Estradiol regulates expression of estrogen receptor ERalpha46 in human macrophages. *PloS One* **2009**, *4*, e5539.
53. Lin, A.H.Y.; Li, R.W.S.; Ho, E.Y.W.; Leung, G.P.H.; Leung, S.W.S.; Vanhoutte, P.M.; Man, R.Y.K. Differential Ligand Binding Affinities of Human Estrogen Receptor-α Isoforms. *PLOS ONE* **2013**, *8*, e63199.
54. Schmiedel, B.J.; Singh, D.; Madrigal, A.; Valdovino-Gonzalez, A.G.; White, B.M.; Zapardiel-Gonzalo, J.; Ha, B.; Altay, G.; Greenbaum, J.A.; McVicker, G.; et al. Impact of Genetic Polymorphisms on Human Immune Cell Gene Expression. *Cell* **2018**, *175*, 1701-1715.e16.
55. Perissi, V.; Rosenfeld, M.G. Controlling nuclear receptors: the circular logic of cofactor cycles. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 542–554.
56. Métivier, R.; Penot, G.; Hübner, M.R.; Reid, G.; Brand, H.; Kos, M.; Gannon, F. Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* **2003**, *115*, 751–763.
57. Cicatiello, L.; Scafoglio, C.; Altucci, L.; Cancemi, M.; Natoli, G.; Facchiano, A.; Iazzetti, G.; Calogero, R.; Biglia, N.; De Bortoli, M.; et al. A genomic view of estrogen actions in human breast cancer cells by expression profiling of the hormone-responsive transcriptome. *J. Mol. Endocrinol.* **2004**, *32*, 719–775.
58. Habib, P.; Dreymueller, D.; Rösing, B.; Botung, H.; Slowik, A.; Zendedel, A.; Habib, S.; Hoffmann, S.; Beyer, C. Estrogen serum concentration affects blood immune cell composition and polarization in human females under controlled ovarian stimulation. *J. Steroid Biochem. Mol. Biol.* **2018**, *178*, 340–347.
59. Le Dily, F.; Beato, M. Signaling by Steroid Hormones in the 3D Nuclear Space. *Int. J. Mol. Sci.* **2018**, *19*.
60. Fullwood, M.J.; Liu, M.H.; Pan, Y.F.; Liu, J.; Xu, H.; Mohamed, Y.B.; Orlov, Y.L.; Velkov, S.; Ho, A.; Mei, P.H.; et al. An oestrogen-receptor-alpha-bound human chromatin interactome. *Nature* **2009**, *462*, 58–64.
61. Quintin, J.; Le Péron, C.; Palierne, G.; Bizot, M.; Cunha, S.; Sérandour, A.A.; Avner, S.; Henry, C.; Percevault, F.; Belaud-Rotureau, M.-A.; et al. Dynamic estrogen receptor interactomes control estrogen-responsive trefoil Factor (TFF) locus cell-specific activities. *Mol. Cell. Biol.* **2014**, *34*, 2418–2436.
62. Bretschneider, N.; Kangaspeska, S.; Seifert, M.; Reid, G.; Gannon, F.; Denger, S. E2-mediated cathepsin D (CTSD) activation involves looping of distal enhancer elements. *Mol. Oncol.* **2008**, *2*, 182–190.
63. Hsu, P.-Y.; Hsu, H.-K.; Singer, G.A.C.; Yan, P.S.; Rodriguez, B.A.T.; Liu, J.C.; Weng, Y.-I.; Deatherage, D.E.; Chen, Z.; Pereira, J.S.; et al. Estrogen-mediated epigenetic repression of large chromosomal regions through DNA looping. *Genome Res.* **2010**, *20*, 733–744.
64. Lavinsky, R.M.; Jepsen, K.; Heinzel, T.; Torchia, J.; Mullen, T.M.; Schiff, R.; Del-

Rio, A.L.; Ricote, M.; Ngo, S.; Gemsch, J.; et al. Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 2920–2925.
65. Rosenfeld, M.G.; Lunyak, V.V.; Glass, C.K. Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev.* **2006**, *20*, 1405–1428.
66. Khan, D.; Ansar Ahmed, S. The Immune System Is a Natural Target for Estrogen Action: Opposing Effects of Estrogen in Two Prototypical Autoimmune Diseases. *Front. Immunol.* **2016**, *6*.
67. Navarro, F.C.; Herrnreiter, C.; Nowak, L.; Watkins, S.K. Estrogen Regulation of T-Cell Function and Its Impact on the Tumor Microenvironment. *Gend. Genome* **2018**, *2*, 81–91.
68. Kovats, S. Estrogen receptors regulate innate immune cells and signaling pathways. *Cell. Immunol.* **2015**, *294*, 63–69.
69. Cunningham, M.; Gilkeson, G. Estrogen receptors in immunity and autoimmunity. *Clin. Rev. Allergy Immunol.* **2011**, *40*, 66–73.
70. Laffont, S.; Seillet, C.; Guéry, J.-C. Estrogen Receptor-Dependent Regulation of Dendritic Cell Development and Function. *Front. Immunol.* **2017**, *8*, 108.
71. Kim, H.-R.; Lee, J.-H.; Heo, H.-R.; Yang, S.-R.; Ha, K.-S.; Park, W.S.; Han, E.-T.; Song, H.; Hong, S.-H. Improved hematopoietic differentiation of human pluripotent stem cells via estrogen receptor signaling pathway. *Cell Biosci.* **2016**, *6*.
72. Staples, J.E.; Gasiewicz, T.A.; Fiore, N.C.; Lubahn, D.B.; Korach, K.S.; Silverstone, A.E. Estrogen receptor alpha is necessary in thymic development and estradiol-induced thymic alterations. *J. Immunol. Baltim. Md 1950* **1999**, *163*, 4168–4174.
73. Erlandsson, M.C.; Ohlsson, C.; Gustafsson, J.A.; Carlsten, H. Role of oestrogen receptors alpha and beta in immune organ development and in oestrogen-mediated effects on thymus. *Immunology* **2001**, *103*, 17–25.
74. Tiwari-Woodruff, S.; Morales, L.B.J.; Lee, R.; Voskuhl, R.R. Differential neuroprotective and antiinflammatory effects of estrogen receptor (ER)α and ERβ ligand treatment. *Proc. Natl. Acad. Sci.* **2007**, *104*, 14813–14818.
75. Tay, T.L.; Hagemeyer, N.; Prinz, M. The force awakens: insights into the origin and formation of microglia. *Curr. Opin. Neurobiol.* **2016**, *39*, 30–37.
76. Villa, A.; Rizzi, N.; Vegeto, E.; Ciana, P.; Maggi, A. Estrogen accelerates the resolution of inflammation in macrophagic cells. *Sci. Rep.* **2015**, *5*, 15224.
77. Villa, A.; Vegeto, E.; Poletti, A.; Maggi, A. Estrogens, Neuroinflammation, and Neurodegeneration. *Endocr. Rev.* **2016**, *37*, 372–402.
78. Kim, R.Y.; Mangu, D.; Hoffman, A.S.; Kavosh, R.; Jung, E.; Itoh, N.; Voskuhl, R. Oestrogen receptor β ligand acts on CD11c+ cells to mediate protection in experimental autoimmune encephalomyelitis. *Brain* **2018**, *141*, 132–147.
79. Wu, W.; Tan, X.; Dai, Y.; Krishnan, V.; Warner, M.; Gustafsson, J.-Å. Targeting estrogen receptor β in microglia and T cells to treat experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 3543–3548.
80. Benedek, G.; Zhang, J.; Nguyen, H.; Kent, G.; Seifert, H.; Vandenbark, A.A.; Offner, H. Novel feedback loop between M2 macrophages/microglia and regulatory B cells in estrogen-protected EAE mice. *J. Neuroimmunol.* **2017**, *305*, 59–67.
81. Verthelyi, D.I.; Ahmed, S.A. Estrogen increases the number of plasma cells and enhances their autoantibody production in nonautoimmune C57BL/6 mice. *Cell. Immunol.* **1998**, *189*, 125–134.
82. Jones, B.G.; Sealy, R.E.; Penkert, R.R.; Surman, S.L.; Maul, R.W.; Neale, G.; Xu, B.; Gearhart, P.J.; Hurwitz, J.L. Complex sex-biased antibody responses: estrogen receptors bind

estrogen response elements centered within immunoglobulin heavy chain gene enhancers. *Int. Immunol.* **2019**, *31*, 141–156.
83. Grimaldi, C.M.; Cleary, J.; Dagtas, A.S.; Moussai, D.; Diamond, B. Estrogen alters thresholds for B cell apoptosis and activation. *J. Clin. Invest.* **2002**, *109*, 1625–1633.
84. Verthelyi, D.; Klinman, D.M. Sex hormone levels correlate with the activity of cytokine-secreting cells in vivo. *Immunology* **2000**, *100*, 384–390.
85. Benedek, G.; Zhang, J.; Bodhankar, S.; Nguyen, H.; Kent, G.; Jordan, K.; Manning, D.; Vandenbark, A.A.; Offner, H. Estrogen induces multiple regulatory B cell subtypes and promotes M2 microglia and neuroprotection during experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **2016**, *293*, 45–53.
86. Fettke, F.; Schumacher, A.; Costa, S.-D.; Zenclussen, A.C. B Cells: The Old New Players in Reproductive Immunology. *Front. Immunol.* **2014**, *5*.
87. Molnarfi, N.; Schulze-Topphoff, U.; Weber, M.S.; Patarroyo, J.C.; Prod'homme, T.; Varrin-Doyer, M.; Shetty, A.; Linington, C.; Slavin, A.J.; Hidalgo, J.; et al. MHC class II-dependent B cell APC function is required for induction of CNS autoimmunity independent of myelin-specific antibodies. *J. Exp. Med.* **2013**, *210*, 2921–2937.
88. Lehmann-Horn, K.; Kinzel, S.; Weber, M.S. Deciphering the Role of B Cells in Multiple Sclerosis-Towards Specific Targeting of Pathogenic Function. *Int. J. Mol. Sci.* **2017**, *18*.
89. Bodhankar, S.; Wang, C.; Vandenbark, A.A.; Offner, H. Estrogen-induced protection against experimental autoimmune encephalomyelitis is abrogated in the absence of B cells. *Eur. J. Immunol.* **2011**, *41*, 1165–1175.
90. Matsushita, T.; Yanaba, K.; Bouaziz, J.-D.; Fujimoto, M.; Tedder, T.F. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J. Clin. Invest.* **2008**, *118*, 3420–3430.
91. Zhang, J.; Benedek, G.; Bodhankar, S.; Lapato, A.; Vandenbark, A.A.; Offner, H. IL-10 producing B cells partially restore E2-mediated protection against EAE in PD-L1 deficient mice. *J. Neuroimmunol.* **2015**, *285*, 129–136.
92. Fox, H.S.; Bond, B.L.; Parslow, T.G. Estrogen regulates the IFN-gamma promoter. *J. Immunol. Baltim. Md 1950* **1991**, *146*, 4362–4367.
93. Arellano, G.; Ottum, P.A.; Reyes, L.I.; Burgos, P.I.; Naves, R. Stage-Specific Role of Interferon-Gamma in Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis. *Front. Immunol.* **2015**, *6*, 492.
94. Grasso, G.; Muscettola, M. The Influence of Beta-Estradiol and Progesterone on Interferon Gamma Production in Vitro. *Int. J. Neurosci.* **1990**, *51*, 315–317.
95. Gilmore, W.; Weiner, L.P.; Correale, J. Effect of estradiol on cytokine secretion by proteolipid protein-specific T cell clones isolated from multiple sclerosis patients and normal control subjects. *J. Immunol. Baltim. Md 1950* **1997**, *158*, 446–451.
96. Karpuzoglu, E.; Phillips, R.A.; Gogal, R.M.; Ansar Ahmed, S. IFN-gamma-inducing transcription factor, T-bet is upregulated by estrogen in murine splenocytes: role of IL-27 but not IL-12. *Mol. Immunol.* **2007**, *44*, 1808–1814.
97. Haghmorad, D.; Salehipour, Z.; Nosratabadi, R.; Rastin, M.; Kokhaei, P.; Mahmoudi, M.B.; Amini, A.A.; Mahmoudi, M. Medium-dose estrogen ameliorates experimental autoimmune encephalomyelitis in ovariectomized mice. *J. Immunotoxicol.* **2016**, *13*, 885–896.
98. Matalka, K.Z. The effect of estradiol, but not progesterone, on the production of cytokines in stimulated whole blood, is concentration-dependent. *Neuro Endocrinol. Lett.* **2003**, *24*, 185–191.
99. Piccinni, M.P.; Giudizi, M.G.; Biagiotti, R.; Beloni, L.; Giannarini, L.; Sampognaro, S.; Parronchi, P.; Manetti, R.; Annunziato, F.; Livi, C. Progesterone favors the development

of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established Th1 cell clones. *J. Immunol. Baltim. Md 1950* **1995**, *155*, 128–133.
100. Druckmann, R.; Druckmann, M.-A. Progesterone and the immunology of pregnancy. *J. Steroid Biochem. Mol. Biol.* **2005**, *97*, 389–396.
101. Polanczyk, M.J.; Carson, B.D.; Subramanian, S.; Afentoulis, M.; Vandenbark, A.A.; Ziegler, S.F.; Offner, H. Cutting edge: estrogen drives expansion of the CD4+CD25+ regulatory T cell compartment. *J. Immunol. Baltim. Md 1950* **2004**, *173*, 2227–2230.
102. Iannello, A.; Rolla, S.; Maglione, A.; Ferrero, G.; Bardina, V.; Inaudi, I.; De Mercanti, S.; Novelli, F.; D'Antuono, L.; Cardaropoli, S.; et al. Pregnancy Epigenetic Signature in T Helper 17 and T Regulatory Cells in Multiple Sclerosis. *Front. Immunol.* **2019**, *9*.
103. Prieto, G.A.; Rosenstein, Y. Oestradiol potentiates the suppressive function of human CD4 CD25 regulatory T cells by promoting their proliferation. *Immunology* **2006**, *118*, 58–65.
104. Polanczyk, M.J.; Hopke, C.; Huan, J.; Vandenbark, A.A.; Offner, H. Enhanced FoxP3 expression and Treg cell function in pregnant and estrogen-treated mice. *J. Neuroimmunol.* **2005**, *170*, 85–92.
105. Polanczyk, M.J.; Hopke, C.; Vandenbark, A.A.; Offner, H. Treg suppressive activity involves estrogen-dependent expression of programmed death-1 (PD-1). *Int. Immunol.* **2007**, *19*, 337–343.
106. Kebir, H.; Ifergan, I.; Alvarez, J.I.; Bernard, M.; Poirier, J.; Arbour, N.; Duquette, P.; Prat, A. Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. *Ann. Neurol.* **2009**, *66*, 390–402.
107. McClain, M.A.; Gatson, N.N.; Powell, N.D.; Papenfuss, T.L.; Gienapp, I.E.; Song, F.; Shawler, T.M.; Kithcart, A.; Whitacre, C.C. Pregnancy suppresses experimental autoimmune encephalomyelitis through immunoregulatory cytokine production. *J. Immunol. Baltim. Md 1950* **2007**, *179*, 8146–8152.
108. Gatson, N.N.; Williams, J.L.; Powell, N.D.; McClain, M.A.; Hennon, T.R.; Robbins, P.D.; Whitacre, C.C. Induction of pregnancy during established EAE halts progression of CNS autoimmune injury via pregnancy-specific serum factors. *J. Neuroimmunol.* **2011**, *230*, 105–113.
109. Ito, A.; Bebo, B.F.; Matejuk, A.; Zamora, A.; Silverman, M.; Fyfe-Johnson, A.; Offner, H. Estrogen treatment down-regulates TNF-alpha production and reduces the severity of experimental autoimmune encephalomyelitis in cytokine knockout mice. *J. Immunol. Baltim. Md 1950* **2001**, *167*, 542–552.
110. Wang, C.; Dehghani, B.; Li, Y.; Kaler, L.J.; Vandenbark, A.A.; Offner, H. Oestrogen modulates experimental autoimmune encephalomyelitis and interleukin-17 production via programmed death 1. *Immunology* **2009**, *126*, 329–335.
111. Polanczyk, M.; Zamora, A.; Subramanian, S.; Matejuk, A.; Hess, D.L.; Blankenhorn, E.P.; Teuscher, C.; Vandenbark, A.A.; Offner, H. The Protective Effect of 17β-Estradiol on Experimental Autoimmune Encephalomyelitis Is Mediated through Estrogen Receptor-α. *Am. J. Pathol.* **2003**, *163*, 1599–1605.
112. Lélu, K.; Laffont, S.; Delpy, L.; Paulet, P.-E.; Périnat, T.; Tschanz, S.A.; Pelletier, L.; Engelhardt, B.; Guéry, J.-C. Estrogen receptor α signaling in T lymphocytes is required for estradiol-mediated inhibition of Th1 and Th17 cell differentiation and protection against experimental autoimmune encephalomyelitis. *J. Immunol. Baltim. Md 1950* **2011**, *187*, 2386–2393.
113. DuPage, M.; Bluestone, J.A. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease. *Nat. Rev. Immunol.* **2016**, *16*, 149–163.
114. Yosef, N.; Regev, A. Writ large: Genomic dissection of the effect of cellular

environment on immune response. *Science* **2016**, *354*, 64–68.
115. Mukasa, R.; Balasubramani, A.; Lee, Y.K.; Whitley, S.K.; Weaver, B.T.; Shibata, Y.; Crawford, G.E.; Hatton, R.D.; Weaver, C.T. Epigenetic instability of cytokine and transcription factor gene loci underlies plasticity of the T helper 17 cell lineage. *Immunity* **2010**, *32*, 616–627.
116. Wei, G.; Wei, L.; Zhu, J.; Zang, C.; Hu-Li, J.; Yao, Z.; Cui, K.; Kanno, Y.; Roh, T.-Y.; Watford, W.T.; et al. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity* **2009**, *30*, 155–167.
117. Schmidl, C.; Delacher, M.; Huehn, J.; Feuerer, M. Epigenetic mechanisms regulating T-cell responses. *J. Allergy Clin. Immunol.* **2018**, *142*, 728–743.
118. Noack, M.; Miossec, P. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. *Autoimmun. Rev.* **2014**, *13*, 668–677.
119. Kleinewietfeld, M.; Hafler, D.A. The plasticity of human Treg and Th17 cells and its role in autoimmunity. *Semin. Immunol.* **2013**, *25*, 305–312.
120. Rudra, D.; deRoos, P.; Chaudhry, A.; Niec, R.E.; Arvey, A.; Samstein, R.M.; Leslie, C.; Shaffer, S.A.; Goodlett, D.R.; Rudensky, A.Y. Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nat. Immunol.* **2012**, *13*, 1010–1019.
121. Ciofani, M.; Madar, A.; Galan, C.; Sellars, M.; Mace, K.; Pauli, F.; Agarwal, A.; Huang, W.; Parkhurst, C.N.; Muratet, M.; et al. A validated regulatory network for Th17 cell specification. *Cell* **2012**, *151*, 289–303.
122. Yosef, N.; Shalek, A.K.; Gaublomme, J.T.; Jin, H.; Lee, Y.; Awasthi, A.; Wu, C.; Karwacz, K.; Xiao, S.; Jorgolli, M.; et al. Dynamic regulatory network controlling T_H17 cell differentiation. *Nature* **2013**, *496*, 461–468.
123. Guan, H.; Nagarkatti, P.S.; Nagarkatti, M. CD44 Reciprocally regulates the differentiation of encephalitogenic Th1/Th17 and Th2/regulatory T cells through epigenetic modulation involving DNA methylation of cytokine gene promoters, thereby controlling the development of experimental autoimmune encephalomyelitis. *J. Immunol. Baltim. Md 1950* **2011**, *186*, 6955–6964.
124. Coquet, J.M.; Middendorp, S.; van der Horst, G.; Kind, J.; Veraar, E.A.M.; Xiao, Y.; Jacobs, H.; Borst, J. The CD27 and CD70 costimulatory pathway inhibits effector function of T helper 17 cells and attenuates associated autoimmunity. *Immunity* **2013**, *38*, 53–65.
125. Feng, Y.; Arvey, A.; Chinen, T.; van der Veeken, J.; Gasteiger, G.; Rudensky, A.Y. Control of the inheritance of regulatory T cell identity by a cis element in the Foxp3 locus. *Cell* **2014**, *158*, 749–763.
126. Zheng, Y.; Josefowicz, S.; Chaudhry, A.; Peng, X.P.; Forbush, K.; Rudensky, A.Y. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* **2010**, *463*, 808–812.
127. Li, X.; Liang, Y.; LeBlanc, M.; Benner, C.; Zheng, Y. Function of a Foxp3 cis-element in protecting regulatory T cell identity. *Cell* **2014**, *158*, 734–748.
128. Yue, X.; Trifari, S.; Äijö, T.; Tsagaratou, A.; Pastor, W.A.; Zepeda-Martínez, J.A.; Lio, C.-W.J.; Li, X.; Huang, Y.; Vijayanand, P.; et al. Control of Foxp3 stability through modulation of TET activity. *J. Exp. Med.* **2016**, *213*, 377–397.
129. Ohkura, N.; Hamaguchi, M.; Morikawa, H.; Sugimura, K.; Tanaka, A.; Ito, Y.; Osaki, M.; Tanaka, Y.; Yamashita, R.; Nakano, N.; et al. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity* **2012**, *37*, 785–799.
130. Polansky, J.K.; Kretschmer, K.; Freyer, J.; Floess, S.; Garbe, A.; Baron, U.; Olek, S.; Hamann, A.; von Boehmer, H.; Huehn, J. DNA methylation controls Foxp3 gene expression. *Eur. J. Immunol.* **2008**, *38*, 1654–1663.

131. Samstein, R.M.; Arvey, A.; Josefowicz, S.Z.; Peng, X.; Reynolds, A.; Sandstrom, R.; Neph, S.; Sabo, P.; Kim, J.M.; Liao, W.; et al. Foxp3 exploits a pre-existent enhancer landscape for regulatory T cell lineage specification. *Cell* **2012**, *151*, 153–166.
132. Kitagawa, Y.; Ohkura, N.; Kidani, Y.; Vandenbon, A.; Hirota, K.; Kawakami, R.; Yasuda, K.; Motooka, D.; Nakamura, S.; Kondo, M.; et al. Guidance of regulatory T cell development by Satb1-dependent super-enhancer establishment. *Nat. Immunol.* **2017**, *18*, 173–183.
133. Morikawa, H.; Ohkura, N.; Vandenbon, A.; Itoh, M.; Nagao-Sato, S.; Kawaji, H.; Lassmann, T.; Carninci, P.; Hayashizaki, Y.; Forrest, A.R.R.; et al. Differential roles of epigenetic changes and Foxp3 expression in regulatory T cell-specific transcriptional regulation. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 5289–5294.
134. Nakatsukasa, H.; Oda, M.; Yin, J.; Chikuma, S.; Ito, M.; Koga-Iizuka, M.; Someya, K.; Kitagawa, Y.; Ohkura, N.; Sakaguchi, S.; et al. Loss of TET proteins in regulatory T cells promotes abnormal proliferation, Foxp3 destabilization and IL-17 expression. *Int. Immunol.* **2019**, *31*, 335–347.
135. Garg, G.; Muschaweckh, A.; Moreno, H.; Vasanthakumar, A.; Floess, S.; Lepennetier, G.; Oellinger, R.; Zhan, Y.; Regen, T.; Hiltensperger, M.; et al. Blimp1 Prevents Methylation of Foxp3 and Loss of Regulatory T Cell Identity at Sites of Inflammation. *Cell Rep.* **2019**, *26*, 1854-1868.e5.
136. Schmidl, C.; Hansmann, L.; Andreesen, R.; Edinger, M.; Hoffmann, P.; Rehli, M. Epigenetic reprogramming of the RORC locus during in vitro expansion is a distinctive feature of human memory but not naïve Treg. *Eur. J. Immunol.* **2011**, *41*, 1491–1498.
137. Chen, R.-Y.; Fan, Y.-M.; Zhang, Q.; Liu, S.; Li, Q.; Ke, G.-L.; Li, C.; You, Z. Estradiol inhibits Th17 cell differentiation through inhibition of RORγT transcription by recruiting the ERα/REA complex to estrogen response elements of the RORγT promoter. *J. Immunol. Baltim. Md 1950* **2015**, *194*, 4019–4028.
138. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. I. Clinical results of a multicenter, randomized, double-blind, placebo-controlled trial. The IFNB Multiple Sclerosis Study Group. *Neurology* **1993**, *43*, 655–661.
139. Knobler, R.L.; Greenstein, J.I.; Johnson, K.P.; Lublin, F.D.; Panitch, H.S.; Conway, K.; Grant-Gorsen, S.V.; Muldoon, J.; Marcus, S.G.; Wallenberg, J.C. Systemic recombinant human interferon-beta treatment of relapsing-remitting multiple sclerosis: pilot study analysis and six-year follow-up. *J. Interferon Res.* **1993**, *13*, 333–340.
140. Panitch, H.; Miller, A.; Paty, D.; Weinshenker, B.; North American Study Group on Interferon beta-1b in Secondary Progressive MS Interferon beta-1b in secondary progressive MS: results from a 3-year controlled study. *Neurology* **2004**, *63*, 1788–1795.
141. Edan, G.; Kappos, L.; Montalbán, X.; Polman, C.H.; Freedman, M.S.; Hartung, H.-P.; Miller, D.; Barkhof, F.; Herrmann, J.; Lanius, V.; et al. Long-term impact of interferon beta-1b in patients with CIS: 8-year follow-up of BENEFIT. *J. Neurol. Neurosurg. Psychiatry* **2014**, *85*, 1183–1189.
142. Kappos, L.; Freedman, M.S.; Polman, C.H.; Edan, G.; Hartung, H.-P.; Miller, D.H.; Montalbán, X.; Barkhof, F.; Radü, E.-W.; Bauer, L.; et al. Effect of early versus delayed interferon beta-1b treatment on disability after a first clinical event suggestive of multiple sclerosis: a 3-year follow-up analysis of the BENEFIT study. *The Lancet* **2007**, *370*, 389–397.
143. Kappos, L.; Freedman, M.S.; Polman, C.H.; Edan, G.; Hartung, H.-P.; Miller, D.H.; Montalbán, X.; Barkhof, F.; Radü, E.-W.; Metzig, C.; et al. Long-term effect of early treatment with interferon beta-1b after a first clinical event suggestive of multiple sclerosis: 5-year active treatment extension of the phase 3 BENEFIT trial. *Lancet Neurol.* **2009**, *8*, 987–997.
144. Calabresi, P.A.; Kieseier, B.C.; Arnold, D.L.; Balcer, L.J.; Boyko, A.; Pelletier, J.;

Liu, S.; Zhu, Y.; Seddighzadeh, A.; Hung, S.; et al. Pegylated interferon beta-1a for relapsing-remitting multiple sclerosis (ADVANCE): a randomised, phase 3, double-blind study. *Lancet Neurol.* **2014**, *13*, 657–665.
145. Ebers, G.C. Randomised double-blind placebo-controlled study of interferon β-1a in relapsing/remitting multiple sclerosis. *The Lancet* **1998**, *352*, 1498–1504.
146. Jacobs, L.D.; Cookfair, D.L.; Rudick, R.A.; Herndon, R.M.; Richert, J.R.; Salazar, A.M.; Fischer, J.S.; Goodkin, D.E.; Granger, C.V.; Simon, J.H.; et al. Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). *Ann. Neurol.* **1996**, *39*, 285–294.
147. Vollmer, T.L.; Sorensen, P.S.; Selmaj, K.; Zipp, F.; Havrdova, E.; Cohen, J.A.; Sasson, N.; Gilgun-Sherki, Y.; Arnold, D.L.; On behalf of the BRAVO Study Group A randomized placebo-controlled phase III trial of oral laquinimod for multiple sclerosis. *J. Neurol.* **2014**, *261*, 773–783.
148. PRISMS-4: Long-term efficacy of interferon-β-1a in relapsing MS | Neurology Available online: https://n.neurology.org/content/56/12/1628.long (accessed on Nov 15, 2019).
149. Kieseier, B.C.; Arnold, D.L.; Balcer, L.J.; Boyko, A.A.; Pelletier, J.; Liu, S.; Zhu, Y.; Seddighzadeh, A.; Hung, S.; Deykin, A.; et al. Peginterferon beta-1a in multiple sclerosis: 2-year results from ADVANCE. *Mult. Scler. J.* **2015**, *21*, 1025–1035.
150. Kappos, L. Placebo-controlled multicentre randomised trial of interferon β-1b in treatment of secondary progressive multiple sclerosis. *The Lancet* **1998**, *352*, 1491–1497.
151. Andersen, O.; Elovaara, I.; Farkkila, M.; Hansen, H.; Mellgren, S.; Myhr, K.; Sandberg-Wollheim, M.; Soelberg, S. Multicentre, randomised, double blind, placebo controlled, phase III study of weekly, low dose, subcutaneous interferon beta-1a in secondary progressive multiple sclerosis. *J. Neurol. Neurosurg. Psychiatry* **2004**, *75*, 706–710.
152. Feng, X.; Reder, N.P.; Yanamandala, M.; Hill, A.; Franek, B.S.; Niewold, T.B.; Reder, A.T.; Javed, A. Type I interferon signature is high in lupus and neuromyelitis optica but low in multiple sclerosis. *J. Neurol. Sci.* **2012**, *313*, 48–53.
153. Srinivasan, S.; Di Dario, M.; Russo, A.; Menon, R.; Brini, E.; Romeo, M.; Sangalli, F.; Costa, G.D.; Rodegher, M.; Radaelli, M.; et al. Dysregulation of MS risk genes and pathways at distinct stages of disease. *Neurol. Neuroimmunol. Neuroinflammation* **2017**, *4*, e337.
154. Jakimovski, D.; Kolb, C.; Ramanathan, M.; Zivadinov, R.; Weinstock-Guttman, B. Interferon β for Multiple Sclerosis. *Cold Spring Harb. Perspect. Med.* **2018**, *8*, a032003.
155. Ersoy, E.; Kuş, C.N.S.; Sener, U.; Coker, I.; Zorlu, Y. The effects of interferon-beta on interleukin-10 in multiple sclerosis patients. *Eur. J. Neurol.* **2005**, *12*, 208–211.
156. Lobo-Silva, D.; Carriche, G.M.; Castro, A.G.; Roque, S.; Saraiva, M. Interferon-β regulates the production of IL-10 by toll-like receptor-activated microglia. *Glia* **2017**, *65*, 1439–1451.
157. Trentini, A.; Castellazzi, M.; Cervellati, C.; Manfrinato, M.C.; Tamborino, C.; Hanau, S.; Volta, C.A.; Baldi, E.; Kostic, V.; Drulovic, J.; et al. Interplay between Matrix Metalloproteinase-9, Matrix Metalloproteinase-2, and Interleukins in Multiple Sclerosis Patients. *Dis. Markers* **2016**, *2016*, 3672353.
158. Valado, A.; Leitão, M.J.; Martinho, A.; Pascoal, R.; Cerqueira, J.; Correia, I.; Batista, S.; Sousa, L.; Baldeiras, I. Multiple sclerosis: Association of gelatinase B/matrix metalloproteinase-9 with risk and clinical course the disease. *Mult. Scler. Relat. Disord.* **2017**, *11*, 71–76.
159. Rizzo, F.; Giacomini, E.; Mechelli, R.; Buscarinu, M.C.; Salvetti, M.; Severa, M.; Coccia, E.M. Interferon-β therapy specifically reduces pathogenic memory B cells in multiple sclerosis patients by inducing a FAS-mediated apoptosis. *Immunol. Cell Biol.* **2016**, *94*, 886–

894.
160. Trojano, M.; Tintore, M.; Montalban, X.; Hillert, J.; Kalincik, T.; Iaffaldano, P.; Spelman, T.; Sormani, M.P.; Butzkueven, H. Treatment decisions in multiple sclerosis - insights from real-world observational studies. *Nat. Rev. Neurol.* **2017**, *13*, 105–118.
161. Prosperini, L.; Capobianco, M.; Gianni, C. Identifying responders and nonresponders to interferon therapy in multiple sclerosis. *Degener. Neurol. Neuromuscul. Dis.* **2014**, *4*, 75–84.
162. Bertolotto, A.; Granieri, L.; Marnetto, F.; Valentino, P.; Sala, A.; Capobianco, M.; Malucchi, S.; Di Sapio, A.; Malentacchi, M.; Matta, M.; et al. Biological monitoring of IFN-β therapy in Multiple Sclerosis. *Cytokine Growth Factor Rev.* **2015**, *26*, 241–248.
163. Libertinova, J.; Meluzinova, E.; Matoska, V.; Zajac, M.; Kovarova, I.; Havrdova, E.; Horakova, D.; Tomek, A.; Marusic, P.; Bojar, M. MxA mRNA decrease preceding NAb detection in IFNβ-treated MS patients. *Brain Behav.* **2017**, *7*, e00644.
164. Delhalle, S.; Bode, S.F.N.; Balling, R.; Ollert, M.; He, F.Q. A roadmap towards personalized immunology. *Npj Syst. Biol. Appl.* **2018**, *4*, 1–14.
165. Cervantes-Gracia, K.; Husi, H. Integrative analysis of Multiple Sclerosis using a systems biology approach. *Sci. Rep.* **2018**, *8*, 1–14.
166. Ernst, J.; Kellis, M. ChromHMM: automating chromatin-state discovery and characterization. *Nat. Methods* **2012**, *9*, 215–216.
167. Malhotra, S.; Bustamante, M.F.; Pérez-Miralles, F.; Rio, J.; Ruiz de Villa, M.C.; Vegas, E.; Nonell, L.; Deisenhammer, F.; Fissolo, N.; Nurtdinov, R.N.; et al. Search for specific biomarkers of IFNβ bioactivity in patients with multiple sclerosis. *PloS One* **2011**, *6*, e23634.
168. Ottoboni, L.; Keenan, B.T.; Tamayo, P.; Kuchroo, M.; Mesirov, J.P.; Buckle, G.J.; Khoury, S.J.; Hafler, D.A.; Weiner, H.L.; De Jager, P.L. An RNA profile identifies two subsets of multiple sclerosis patients differing in disease activity. *Sci. Transl. Med.* **2012**, *4*, 153ra131.
169. Nickles, D.; Chen, H.P.; Li, M.M.; Khankhanian, P.; Madireddy, L.; Caillier, S.J.; Santaniello, A.; Cree, B.A.C.; Pelletier, D.; Hauser, S.L.; et al. Blood RNA profiling in a large cohort of multiple sclerosis patients and healthy controls. *Hum. Mol. Genet.* **2013**, *22*, 4194–4205.
170. Gurevich, M.; Miron, G.; Falb, R.Z.; Magalashvili, D.; Dolev, M.; Stern, Y.; Achiron, A. Transcriptional response to interferon beta-1a treatment in patients with secondary progressive multiple sclerosis. *BMC Neurol.* **2015**, *15*.
171. Sellebjerg, F.; Krakauer, M.; Hesse, D.; Ryder, L.P.; Alsing, I.; Jensen, P.E.H.; Koch-Henriksen, N.; Svejgaard, A.; Soelberg Sørensen, P. Identification of new sensitive biomarkers for the in vivo response to interferon-beta treatment in multiple sclerosis using DNA-array evaluation. *Eur. J. Neurol.* **2009**, *16*, 1291–1298.
172. Gilli, F.; Marnetto, F.; Caldano, M.; Sala, A.; Malucchi, S.; Capobianco, M.; Bertolotto, A. Biological markers of interferon-beta therapy: comparison among interferon-stimulated genes MxA, TRAIL and XAF-1. *Mult. Scler. Houndmills Basingstoke Engl.* **2006**, *12*, 47–57.
173. Cao, Y.; Goods, B.A.; Raddassi, K.; Nepom, G.T.; Kwok, W.W.; Love, J.C.; Hafler, D.A. Functional inflammatory profiles distinguish myelin-reactive T cells from patients with multiple sclerosis. *Sci. Transl. Med.* **2015**, *7*, 287ra74.
174. Collado-Torres, L.; Nellore, A.; Kammers, K.; Ellis, S.E.; Taub, M.A.; Hansen, K.D.; Jaffe, A.E.; Langmead, B.; Leek, J.T. Reproducible RNA-seq analysis using recount2. *Nat. Biotechnol.* **2017**, *35*, 319–321.
175. Liston, P.; Fong, W.G.; Kelly, N.L.; Toji, S.; Miyazaki, T.; Conte, D.; Tamai, K.; Craig, C.G.; McBurney, M.W.; Korneluk, R.G. Identification of XAF1 as an antagonist of

XIAP anti-Caspase activity. *Nat. Cell Biol.* **2001**, *3*, 128–133.
176. Jeong, S.-I.; Kim, J.-W.; Ko, K.-P.; Ryu, B.-K.; Lee, M.-G.; Kim, H.-J.; Chi, S.-G. XAF1 forms a positive feedback loop with IRF-1 to drive apoptotic stress response and suppress tumorigenesis. *Cell Death Dis.* **2018**, *9*, 1–16.
177. Zhang, Y.; Liu, T.; Meyer, C.A.; Eeckhoute, J.; Johnson, D.S.; Bernstein, B.E.; Nusbaum, C.; Myers, R.M.; Brown, M.; Li, W.; et al. Model-based analysis of ChIP-Seq (MACS). *Genome Biol.* **2008**, *9*, R137.
178. Whyte, W.A.; Orlando, D.A.; Hnisz, D.; Abraham, B.J.; Lin, C.Y.; Kagey, M.H.; Rahl, P.B.; Lee, T.I.; Young, R.A. Master transcription factors and mediator establish super-enhancers at key cell identity genes. *Cell* **2013**, *153*, 307–319.
179. Li, M.J.; Liu, Z.; Wang, P.; Wong, M.P.; Nelson, M.R.; Kocher, J.-P.A.; Yeager, M.; Sham, P.C.; Chanock, S.J.; Xia, Z.; et al. GWASdb v2: an update database for human genetic variants identified by genome-wide association studies. *Nucleic Acids Res.* **2016**, *44*, D869–D876.
180. Kundaje, A.; Meuleman, W.; Ernst, J.; Bilenky, M.; Yen, A.; Heravi-Moussavi, A.; Kheradpour, P.; Zhang, Z.; Wang, J.; Ziller, M.J.; et al. Integrative analysis of 111 reference human epigenomes. *Nature* **2015**, *518*, 317–330.
181. Ernst, J.; Kellis, M. Large-scale imputation of epigenomic datasets for systematic annotation of diverse human tissues. *Nat. Biotechnol.* **2015**, *33*, 364–376.
182. Quinlan, A.R.; Hall, I.M. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **2010**, *26*, 841–842.
183. McLean, C.Y.; Bristor, D.; Hiller, M.; Clarke, S.L.; Schaar, B.T.; Lowe, C.B.; Wenger, A.M.; Bejerano, G. GREAT improves functional interpretation of cis -regulatory regions. *Nat. Biotechnol.* **2010**, *28*, 495–501.
184. Ranzani, V.; Rossetti, G.; Panzeri, I.; Arrigoni, A.; Bonnal, R.J.P.; Curti, S.; Gruarin, P.; Provasi, E.; Sugliano, E.; Marconi, M.; et al. The long intergenic noncoding RNA landscape of human lymphocytes highlights the regulation of T cell differentiation by linc-MAF-4. *Nat. Immunol.* **2015**, *16*, 318–325.
185. Kim, D.; Pertea, G.; Trapnell, C.; Pimentel, H.; Kelley, R.; Salzberg, S.L. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* **2013**, *14*, R36.
186. Liao, Y.; Smyth, G.K.; Shi, W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **2014**, *30*, 923–930.
187. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **2014**, *15*, 550.
188. Zhang, H.-M.; Liu, T.; Liu, C.-J.; Song, S.; Zhang, X.; Liu, W.; Jia, H.; Xue, Y.; Guo, A.-Y. AnimalTFDB 2.0: a resource for expression, prediction and functional study of animal transcription factors. *Nucleic Acids Res.* **2015**, *43*, D76-81.
189. Grant, C.E.; Bailey, T.L.; Noble, W.S. FIMO: scanning for occurrences of a given motif. *Bioinformatics* **2011**, *27*, 1017–1018.
190. Jolma, A.; Yan, J.; Whitington, T.; Toivonen, J.; Nitta, K.R.; Rastas, P.; Morgunova, E.; Enge, M.; Taipale, M.; Wei, G.; et al. DNA-binding specificities of human transcription factors. *Cell* **2013**, *152*, 327–339.
191. Kulakovskiy, I.V.; Vorontsov, I.E.; Yevshin, I.S.; Sharipov, R.N.; Fedorova, A.D.; Rumynskiy, E.I.; Medvedeva, Y.A.; Magana-Mora, A.; Bajic, V.B.; Papatsenko, D.A.; et al. HOCOMOCO: towards a complete collection of transcription factor binding models for human and mouse via large-scale ChIP-Seq analysis. *Nucleic Acids Res.* **2018**, *46*, D252–D259.
192. Weirauch, M.T.; Yang, A.; Albu, M.; Cote, A.G.; Montenegro-Montero, A.; Drewe, P.; Najafabadi, H.S.; Lambert, S.A.; Mann, I.; Cook, K.; et al. Determination and inference of

eukaryotic transcription factor sequence specificity. *Cell* **2014**, *158*, 1431–1443.
193. Mathelier, A.; Fornes, O.; Arenillas, D.J.; Chen, C.-Y.; Denay, G.; Lee, J.; Shi, W.; Shyr, C.; Tan, G.; Worsley-Hunt, R.; et al. JASPAR 2016: a major expansion and update of the open-access database of transcription factor binding profiles. *Nucleic Acids Res.* **2016**, *44*, D110-115.
194. Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N.S.; Wang, J.T.; Ramage, D.; Amin, N.; Schwikowski, B.; Ideker, T. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Res.* **2003**, *13*, 2498–2504.
195. Doncheva, N.T.; Assenov, Y.; Domingues, F.S.; Albrecht, M. Topological analysis and interactive visualization of biological networks and protein structures. *Nat. Protoc.* **2012**, *7*, 670–685.
196. Davis, S.; Meltzer, P.S. GEOquery: a bridge between the Gene Expression Omnibus (GEO) and BioConductor. *Bioinforma. Oxf. Engl.* **2007**, *23*, 1846–1847.
197. Smyth, G.K. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* **2004**, *3*, Article3.
198. Gentleman, R.C.; Carey, V.J.; Bates, D.M.; Bolstad, B.; Dettling, M.; Dudoit, S.; Ellis, B.; Gautier, L.; Ge, Y.; Gentry, J.; et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **2004**, *5*, R80.
199. Benjamini, Y.; Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J. R. Stat. Soc. Ser. B Methodol.* **1995**, *57*, 289–300.
200. Collado-Torres, L.; Nellore, A.; Jaffe, A.E. recount workflow: Accessing over 70,000 human RNA-seq samples with Bioconductor. *F1000Research* **2017**, *6*, 1558.
201. Marc Carlson *org.Hs.eg.db: Genome wide annotation for Human.*; 2019;
202. Gu, Z.; Eils, R.; Schlesner, M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinforma. Oxf. Engl.* **2016**, *32*, 2847–2849.
203. Chen, E.Y.; Tan, C.M.; Kou, Y.; Duan, Q.; Wang, Z.; Meirelles, G.V.; Clark, N.R.; Ma'ayan, A. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics* **2013**, *14*, 128.
204. Kuleshov, M.V.; Jones, M.R.; Rouillard, A.D.; Fernandez, N.F.; Duan, Q.; Wang, Z.; Koplev, S.; Jenkins, S.L.; Jagodnik, K.M.; Lachmann, A.; et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res.* **2016**, *44*, W90-97.
205. Witten, I.H.; Frank, E.; Hall, M.A.; Pal, C.J. *Data Mining, Fourth Edition: Practical Machine Learning Tools and Techniques*; 4th ed.; Morgan Kaufmann Publishers Inc.: San Francisco, CA, USA, 2016; ISBN 978-0-12-804291-5.
206. Luo, W.; Brouwer, C. Pathview: an R/Bioconductor package for pathway-based data integration and visualization. *Bioinforma. Oxf. Engl.* **2013**, *29*, 1830–1831.
207. Christianson, M.S.; Mensah, V.A.; Shen, W. Multiple sclerosis at menopause: Potential neuroprotective effects of estrogen. *Maturitas* **2015**, *80*, 133–139.
208. Gubbels Bupp, M.R.; Potluri, T.; Fink, A.L.; Klein, S.L. The Confluence of Sex Hormones and Aging on Immunity. *Front. Immunol.* **2018**, *9*, 1269.
209. Molnár, I.; Bohaty, I.; Somogyiné-Vári, É. High prevalence of increased interleukin-17A serum levels in postmenopausal estrogen deficiency. *Menopause N. Y. N* **2014**, *21*, 749–752.
210. Smith, R.; Studd, J.W. A pilot study of the effect upon multiple sclerosis of the menopause, hormone replacement therapy and the menstrual cycle. *J. R. Soc. Med.* **1992**, *85*, 612–613.
211. Guéry, J.-C.; Lélu, K.; Laffont, S.; Garnier, L. Estrogen-mediated protection of experimental autoimmune encephalomyelitis: Lessons from the dissection of estrogen receptor-signaling in vivo. *Biomed. J.* **2015**, *38*, 194.
212. Neuteboom, R.F.; Verbraak, E.; Wierenga-Wolf, A.F.; van Meurs, M.; Steegers,

E.A.P.; de Groot, C.J.M.; Laman, J.D.; Hintzen, R.Q. Pregnancy-induced fluctuations in functional T-cell subsets in multiple sclerosis patients. *Mult. Scler. Houndmills Basingstoke Engl.* **2010**, *16*, 1073–1078.
213. Voo, K.S.; Wang, Y.-H.; Santori, F.R.; Boggiano, C.; Wang, Y.-H.; Arima, K.; Bover, L.; Hanabuchi, S.; Khalili, J.; Marinova, E.; et al. Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc. Natl. Acad. Sci.* **2009**, *106*, 4793–4798.
214. Ayyoub, M.; Deknuydt, F.; Raimbaud, I.; Dousset, C.; Leveque, L.; Bioley, G.; Valmori, D. Human memory FOXP3+ Tregs secrete IL-17 ex vivo and constitutively express the TH17 lineage-specific transcription factor RORγt. *Proc. Natl. Acad. Sci.* **2009**, *106*, 8635–8640.
215. Koenen, H.J.P.M.; Smeets, R.L.; Vink, P.M.; van Rijssen, E.; Boots, A.M.H.; Joosten, I. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17–producing cells. *Blood* **2008**, *112*, 2340–2352.
216. Hah, N.; Danko, C.G.; Core, L.; Waterfall, J.J.; Siepel, A.; Lis, J.T.; Kraus, W.L. A rapid, extensive, and transient transcriptional response to estrogen signaling in breast cancer cells. *Cell* **2011**, *145*, 622–634.
217. Gustafsson, M.; Gawel, D.R.; Alfredsson, L.; Baranzini, S.; Björkander, J.; Blomgran, R.; Hellberg, S.; Eklund, D.; Ernerudh, J.; Kockum, I.; et al. A validated gene regulatory network and GWAS identifies early regulators of T cell–associated diseases. *Sci. Transl. Med.* **2015**, *7*, 313ra178-313ra178.
218. Sato, K.; Miyoshi, F.; Yokota, K.; Araki, Y.; Asanuma, Y.; Akiyama, Y.; Yoh, K.; Takahashi, S.; Aburatani, H.; Mimura, T. Marked Induction of c-Maf Protein during Th17 Cell Differentiation and Its Implication in Memory Th Cell Development. *J. Biol. Chem.* **2011**, *286*, 14963–14971.
219. Fu, W.; Ergun, A.; Lu, T.; Hill, J.A.; Haxhinasto, S.; Fassett, M.S.; Gazit, R.; Adoro, S.; Glimcher, L.; Chan, S.; et al. A multiply redundant genetic switch "locks in" the transcriptional signature of regulatory T cells. *Nat. Immunol.* **2012**, *13*, 972–980.
220. Gao, P.; Uzun, Y.; He, B.; Salamati, S.E.; Coffey, J.K.M.; Tsalikian, E.; Tan, K. Risk variants disrupting enhancers of T_H1 and T_{REG} cells in type 1 diabetes. *Proc. Natl. Acad. Sci.* **2019**, *116*, 7581–7590.
221. Lovén, J.; Hoke, H.A.; Lin, C.Y.; Lau, A.; Orlando, D.A.; Vakoc, C.R.; Bradner, J.E.; Lee, T.I.; Young, R.A. Selective Inhibition of Tumor Oncogenes by Disruption of Super-Enhancers. *Cell* **2013**, *153*, 320–334.
222. Chapuy, B.; McKeown, M.R.; Lin, C.Y.; Monti, S.; Roemer, M.G.M.; Qi, J.; Rahl, P.B.; Sun, H.H.; Yeda, K.T.; Doench, J.G.; et al. Discovery and Characterization of Super-Enhancer-Associated Dependencies in Diffuse Large B Cell Lymphoma. *Cancer Cell* **2013**, *24*, 777–790.
223. Vahedi, G.; Kanno, Y.; Furumoto, Y.; Jiang, K.; Parker, S.C.J.; Erdos, M.R.; Davis, S.R.; Roychoudhuri, R.; Restifo, N.P.; Gadina, M.; et al. Super-enhancers delineate disease-associated regulatory nodes in T cells. *Nature* **2015**, *520*, 558–562.
224. Peeters, J.G.C.; Vervoort, S.J.; Tan, S.C.; Mijnheer, G.; de Roock, S.; Vastert, S.J.; Nieuwenhuis, E.E.S.; van Wijk, F.; Prakken, B.J.; Creyghton, M.P.; et al. Inhibition of Super-Enhancer Activity in Autoinflammatory Site-Derived T Cells Reduces Disease-Associated Gene Expression. *Cell Rep.* **2015**, *12*, 1986–1996.
225. Tripathi, S.K.; Välikangas, T.; Shetty, A.; Khan, M.M.; Moulder, R.; Bhosale, S.D.; Komsi, E.; Salo, V.; De Albuquerque, R.S.; Rasool, O.; et al. Quantitative Proteomics Reveals the Dynamic Protein Landscape during Initiation of Human Th17 Cell Polarization. *iScience* **2019**, *11*, 334–355.
226. Läubli, H.; Alisson-Silva, F.; Stanczak, M.A.; Siddiqui, S.S.; Deng, L.; Verhagen, A.; Varki, N.; Varki, A. Lectin Galactoside-binding Soluble 3 Binding Protein (LGALS3BP) Is a

Tumor-associated Immunomodulatory Ligand for CD33-related Siglecs. *J. Biol. Chem.* **2014**, *289*, 33481–33491.
227. Stanczak, M.A.; Siddiqui, S.S.; Trefny, M.P.; Thommen, D.S.; Boligan, K.F.; von Gunten, S.; Tzankov, A.; Tietze, L.; Lardinois, D.; Heinzelmann-Schwarz, V.; et al. Self-associated molecular patterns mediate cancer immune evasion by engaging Siglecs on T cells. *J. Clin. Invest.* **2018**, *128*, 4912–4923.
228. Onwuha-Ekpete, L.C.; Tokmina-Roszyk, D.; Fields, G.B. Selective inhibition of matrix metalloproteinase-9 in CD4+ T-cells reduces clinical severity in a murine model of Multiple Sclerosis. *J. Immunol.* **2017**, *198*, 219.8-219.8.

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