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Regulation of Interleukin-23 Receptor expression in human T-Lymphocytes

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Sommario

ABBREVIATION LIST

CID: Chronic Inflammatory Diseases

GWAS: Genome-wide association studies

ATAC-seq: Assay for Transposase-Accessible Chromatin using sequencing

TF: Transcription factor

Th: T-Helper

IL: Interleukin

IL-23R: Interleukin-23 Receptor

TGF-β: Transforming growth factor beta

IFN: Interferon

RORC: RAR-related orphan receptor C

STAT: signal transducer and activator of transcription

IRF: Interferon Regulatory Factor

IKZF1: Ikaros family zinc finger protein 1

BATF: Basic leucine zipper transcription factor, ATF-like

BACH1: BTB Domain And CNC Homolog 1

siRNA: Small interfering RNA

CRISPR: clustered regularly interspaced short palindromic repeats

ChIP: chromatin immunoprecipitation

ABSTRACT

Chronic inflammatory diseases (CID) are clinically heterogeneous conditions that share common inflammatory pathways and derive from aberrant immune responses. Genome-wide association studies (GWAS), together with mouse models of autoimmune disease[1], demonstrated that Th17 cells play a pivotal role in the initiation of these diseases. The implication of the IL-23/IL-17 axis in several CID is supported by the finding that several of the non-MHC loci genetically linked to Crohn's disease, psoriasis, and axial spondylarthritis (axSpA), are associated with genes in this pathway [2]. It's known that IL-23 is important for the expansion and the functional activity of the Th17 cell subset. However, several studies have suggested that IL-23 may also regulate the function of IL-17-producing innate immune cells, which express the IL-23 receptor (IL-23R). Indeed, substantially less is known about the biologic function of IL-23 in human inflammatory disease, although GWAS results have pointed to an important role of IL23R in the pathogenesis of several CID [3,4], while the mechanism controlling the expression of this gene in different lymphocyte populations are poorly understood. The goal of this study is to increase our knowledge of the role of IL-23 and the signals necessary to induce IL23R expression in neonatal CD4+ T cells or whether environmental signals are required to induce its expression in these innate T cell populations. To address the molecular mechanisms of IL23R induction, we will identify regions with an open chromatin conformation (which are often regulatory elements) in the IL23R locus, in adaptive T-cells population (naïve cord-blood derived CD4+ T cells) using ATAC-seq, which requires low cell numbers. Through bioinformatic analysis and literature research we aim to define the putative transcription factors with their binding sites, and research a possible molecular mechanisms controlling IL23R expression in CD4+ T cells exploiting TF knock-down by siRNA molecules.

1. INTRODUCTION

1.1 Chronic inflammatory diseases (CID)

1.1.1 Characteristics of chronic and acute inflammation

Inflammation is an immune system physiological reaction that can be triggered by a variety of stimuli, which comprise bacteria, viruses, or toxic substances that can cause severe acute or chronic inflammatory reactions, resulting in tissue damage and illness. By definition, the typical inflammatory response is distinguished by a temporary increase in inflammatory activity that happens when a threat is present and resolves after the threat has passed. [1,5,6]. However, if the factors related to the resolution of acute inflammation happen to be suppressed, a state characterized by systemic chronic inflammation will be activated, in which the immune cells and pathways activated are different from those involved in the acute response. [7] The consequent change from a shorter to longer inflammatory response in time will result in a disruption of the immunological tolerance [5,8] with many alterations in tissues, organs, and cellular physiology. Even if they share a common mechanism of action, the acute and chronic inflammatory response are very different [9](*Table 1*). While many acute inflammation features will still be present as the inflammation becomes chronic, the composition of the white blood cell populations will change rapidly, so that neutrophils will be substituted by macrophages and lymphocytes . Their infiltration in the tissue sites represents one of the major signatures of chronic inflammation, followed by their production of inflammatory cytokines and growth factors that contribute to the progression of tissue damage. [10]

	Acute inflammation	Systemic chronic inflammation
Trigger	PAMPs (infection), DAMPs (cellular stress, trauma)	DAMPs ('exposome', metabolic dysfunction, tissue damage)
Duration	Short-term	Persistent, non-resolving
Magnitude	High-grade	Low-grade
Outcome(s)	Healing, trigger removal, tissue repair	Collateral damage
Age-related	No	Yes
Biomarkers	IL-6, TNF-α, IL-1β, CRP	Silent—no canonical standard biomarkers

***Table 1**: Acute inflammation versus systemic chronic inflammation characteristics. Image taken from: Furman, D., et al. Chronic inflammation in the etiology of disease across the life span. Nat Med 25, 1822–1832 (2019).*

T-lymphocytes have an important role among the cellular players in chronic inflammation. When effector CD4+ T cells are wrongly directed against self-antigens or when they're insufficiently regulated in response to environmental antigen, they become a potential threat, despite their ability to give protection to the host against infections. [11] T helper 17 (Th17) cells, which produce interleukin 17 (IL-17) evolved in response to evolutionary forces to protect against various microorganisms, similar to T helper 1 (Th1) and T helper 2 (Th2). For reasons that will be considered below, the development of chronic immune disease mediated by dysregulated Th17 responses has gained a lot of attention in these years. The identification of Th17 cells as a new effector T cell subset was defined responsible for pathogenesis in different models of autoimmune illness, which had been originally attributed to dysregulated Th1 immunity.[11] The identification of Th17 cells also lead to the discovery that the cytokine interleukine-23 (IL-23) was a vital component in the generation of these T helper cells. Since then, genome-wide association studies (GWAS) have connected the Th17 pathway to an increasing number of chronic inflammatory human illnesses.

Among these there are disorders affecting organs that have large epithelial contacts with the external environment, such as mucosal tissues and the skin, where cells of the Th17 pathway are normally abundant [10].

1.1.2 CID and molecular pathways overview

Chronic inflammatory diseases (CID) are a group of conditions not clinically related that share common inflammatory pathways. They are caused by abnormal immunological responses of the human immune system, and their presence, in percentage, is believed to range between 5% and 7% in western societies [12,13]. Genome-wide association studies (GWAS) conducted in several diseases, like rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis psoriasis, and Crohn's disease (*Figure 1*), have identified disease-associations with multiple loci connected to signaling pathways that were not known to be involved in pathogenesis, opening new possibilities for the study of the disease mechanisms. [3,12,14,15,16] . Significant numbers of these loci are shared by multiple chronic inflammatory disorders, indicating that some of the pathogenic pathways may be also shared by multiple diseases [17]. This is the case of Crohn's disease (CD), which affects the gastro-intestinal tract: genomic data as well as experimental data from mouse models and human tissue has confirmed the implication of the Th17 pathway, although the exact role of Th17 and Th1 cells subset in the pathogenesis of the disease is still unclear. Moreover, increased expression of IL-17 and IL-23 has also been found in CD lesions, while also data from genome-wide association studies has linked the IL-23 receptor gene (*IL23R*) to the disease [18,19]. Another CID presenting an increased amount of skin infiltration of CD4+/CD8+ T-cells, defined as the main cause for the dysregulated immune response is Psoriasis, that affects the skin. In fact, several studies published since the identification of the Th17 pathway indicate that the IL-23/IL-17/Th17 axis drives the pathogenesis of the disease, rather than the conventional IL-12/interferon gamma (IFNg)/Th1 one [20,21,22] . Due to the importance of the IL-23 cytokines and its receptor for the development of CID, a more detailed characterization of its role and structure will be presented in the chapter below.

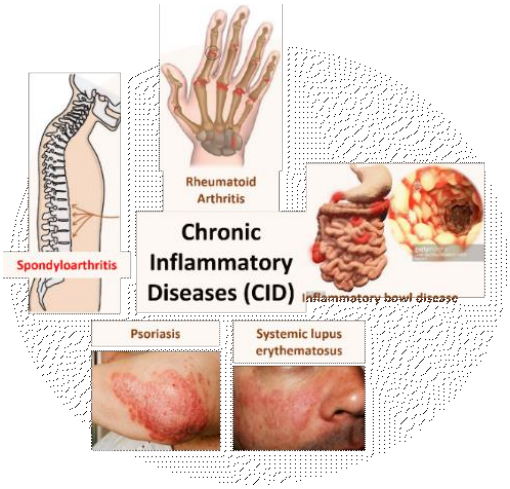


Figure 1: *Overview of major Chronic Inflammatory diseases (CID)*

1.2 The role of the Interleukin-23 Receptor (IL-23R) in CID

As stated above, the cytokine IL-23 and its receptor play a fundamental role in inflammation and autoimmunity [23]The gene encoding the IL-23 receptor (*IL23R*) is located on human chromosome 1, 150 kb upstream of the gene encoding the signaling beta chain of the interleukin-12 receptor (*IL12RB2*). GWAS studies of CIDs have identified several polymorphisms in the *IL23R* gene and in the 50 kb intergenic region between *IL23R* and *IL12RB2*. [24]

GWAS also identified disease associations with variants in additional genes encoding critical molecules in the IL-23/IL-17 pathway, such as variants in the gene encoding the p40 subunit shared between IL-23 and IL-12 (*IL12B*), and variants in *STAT3*, encoding a transcription factor activated by IL-23 signaling [25.]. Furthermore, the study of pre-clinical models of Multiple Sclerosis, and inflammatory bowel disease (IBD) showed that mice deficient for IL-23 signaling were protected from disease development [26,27,28].

1.2.1 IL-23 characterization and signaling

The discovery of IL-23 as a new member belonging to the IL-12 cytokine family, has resulted in a re-determination of the role of Th1 cells in immune-mediated disorders. On a structural level, interleukin-23 is a heterodimer of the p19 subunit and the IL-12p40 subunit, and signals via a receptor constituted by the association of the IL-23R chain and the IL-12Rbeta1 chain. [29,30] (*Figure 2*). Instead, IL-12 is formed by the IL-12p40 subunit connected to the IL-12p35 subunit, and transmits signals via the IL-12 receptor, which is made of the IL-12Rbeta1 and IL-12Rbeta2 subunits [29]. The distinction between IL-12- and IL-23-dependent signaling in mice is partly due to the preferential activation of STAT4 by IL-12 and of STAT3 by IL-23. Given the similarity of the two cytokines and the fact that they have a common receptor subunit, IL-12Rbeta2 [31,32] it was first believed that the activities of IL-12 and IL-23 would overlap. However, subsequent research revealed that IL-23, and not IL-12, stimulated IL-17 production from activated memory T cells [31]. Therefore, it was hypothesized that IL-23 could cause the formation of a unique subset of effector CD4+ T cells (Th17) defined by the release of IL-17. In addition, the significance of IL-23 in autoimmune inflammation was determined by knock-out mice.[32,33]. In various experimental models of autoimmunity, such as experimental autoimmune encephalomyelitis (EAE) and inflammatory bowel disease (IBD), mice with a deletion of the p19 subunit of IL-23 were protected from illness, whereas mice with a deletion of the IL-12p35 subunit were not. Therefore, these data indicate that Th17 cells are a unique subset of CD4+ T lymphocytes that play a crucial role in chronic inflammation and autoimmunity in mice. It is crucial to note, however, that Th17 cells may not be the only T cell subset that promotes immune-mediated inflammation. In fact, subsequent research has revealed that both Th17 and Th1 cells promote illness in the EAE model. [34,35]. In order to have a comprehensive understanding of the different immune cells and stimuli that causes the Th17 subset to be pathological, their discovery and differentiation will be explored in the next chapter.

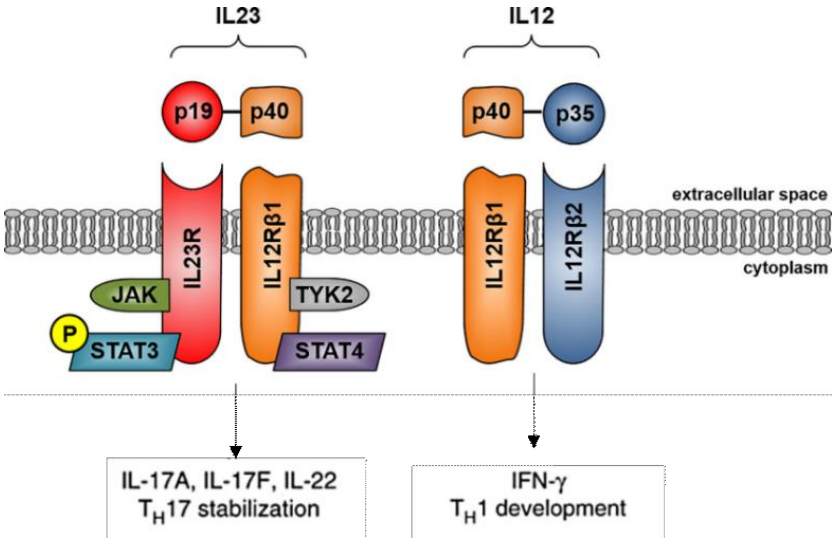


Figure 2: *Structure of IL-23, IL-23R, IL-12 and IL-12Rβ2. Both receptors associate with the kinases JAK2 and TYK2. IL-23 signaling preferentially induces phosphorylation of STAT3, IL-12 signaling of STAT4. IL-23R activation of STAT3 leads to the expression of IL-17A, IL-17F and Il-22 cytokines and to the expansion of the Th17 subset, while IL-12 leads to Th1 development and IFN-γ secretion. Image from Front. Immunol., 30 March 2021 Sec. Cytokines and Soluble Mediators in Immunity https://doi.org/10.3389/fimmu.2021.622934 and Teng, M., Bowman, E., McElwee, J. et al. IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases. Nat Med 21, 719–729 (2015). https://doi.org/10.1038/nm.3895*

1.2.2 T-helper subset differentiation

Many chronic inflammatory diseases are characterized by altered CD4+ T cell responses. CD4+ T cells are crucial to both immunological protection and immune disease due to their essential function in mediating immune responses. Upon activation through the TCR, CD4+ T cells differentiate in distinct effector subsets characterized by the secretion of different patterns of cytokines. [36] The initial subsets to be identified, Th1 and Th2 were based on the analysis of two types of cloned T cell lines from the mouse that were characterized by their differential production of the cytokines IFNγ, for Th1 cells, or IL-4, IL-5 and IL-13, for Th2. (*Figure 3*) [37]. Notably, Th1 cells are important to promote eradication of internal pathogens, whereas Th2 cells had evolved to remove parasitic infections, but little was known about the mechanism through which the extracellular bacterial and fungal infections were eradicated [17]. With the discovery of Th17 cells in mouse models of autoimmunity, such as inflammatory bowel disease and experimental autoimmune encephalomyelitis (EAE), a significant number of disorders were no longer assigned to Th1. The CD4+ T cell effector subset known as "Th17" was given its name after its hallmark cytokine, IL-17. Th17 cells are regarded a separate subgroup of T helper lymphocytes because: they develop from naive T cells in the presence of distinct stimuli, they produce a unique profile of cytokines, and their development is governed by unique transcription factors. Th17 differentiation is driven by the transforming growth factor-b (TGFβ), IL-1β, and IL-6; proliferation, by IL-21; and amplification, by IL-23. Human Th17 cell differentiation has also been obtained in response to IL-1β and IL-23. [38.] Moreover, Th17 cells constitutively express a unique transcription factor, the retinoic acid receptor-related orphan receptor C (RORC), which induces transcription of the IL-17 gene in naïve helper T cells. Its activation contributes to the expression of the IL-23 receptor. IL-23 enhances the expression of IL-17 and IL-22. The development of IL-17 producing cells in the presence of IL-6 and TGF-β, is thought to determine the acquisition of a non-pathogenic phenotype in Th17 subset. Th17 cells produced in the presence of IL-6, IL-

1β, and IL-23, on the other hand, are regarded as highly pathogenic, and cause severe EAE following transfer in mice [3,38,39]. Recent evidence suggests that IL-23 generates mature pathogenic Th17 cells via multiple pathways, including maintenance of Th17 signature genes (RORC and IL-17), induction of effector genes (IL-22, and IFNγ), suppression of repressive factors (Ahr, c-myc), and most importantly by enhancing its own signal strength by upregulating *IL23R* expression.[40,41,42]. Although these have been significant findings, the molecular mechanisms connecting IL-23 to the pathogenic activity of Th17 remains unclear. Because of the importance of IL-23 in Th17 expansion and function, in this thesis we will focalize our attention on the role of the signals involved in inducing *IL23R* expression and the epigenetic and transcriptional mechanisms associated with *IL23R* induction.

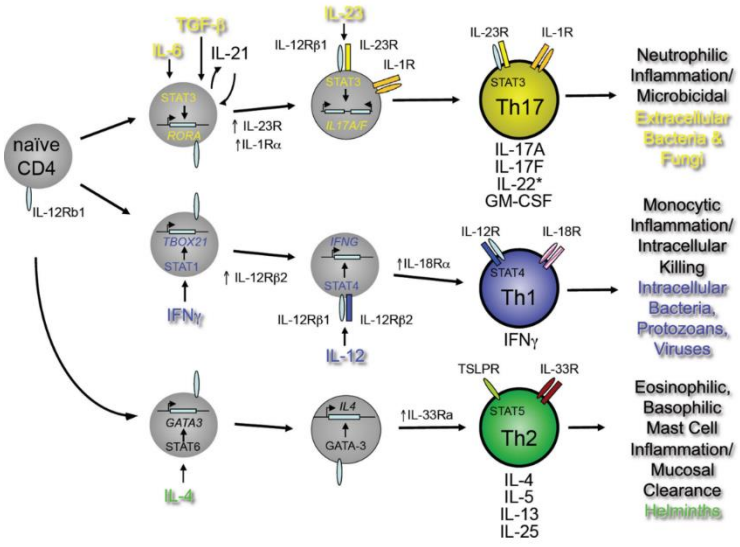


Figure 3: *Development and differentiation of Th17, Th1 and Th2 pathways. Stimuli inducing differentiation, receptors and pathogens to which each response is directed, are reported. Image taken from: Weaver CT, Elson CO, Fouser LA, Kolls JK. The Th17 pathway and inflammatory diseases of the intestines, lungs, and skin. Annu Rev Pathol. 2013 Jan 24;8:477-512. doi: 10.1146/annurev-pathol-011110-130318. Epub 2012 Nov 15. PMID: 23157335; PMCID: PMC3965671.*

1.3 Techniques for epigenetic studies of T-cells differentiation

1.3.1 Identification of putative regulatory regions with assay for transposase accessible chromatin (ATAC-seq)

Mapping changes in cell states is essential to comprehending biological systems. Gene regulatory programs are produced and directed by the activity of transcription factors (TFs) that interpret and alter the chromatin's underlying epigenetic state. The epigenetic state of chromatin can be altered by a number of methods, like the chemical alteration of DNA and histone proteins that affects the chromatin dynamics and its structure. Chromatin, in fact, can exist in a number of distinct states [43,44] that are defined by combinations of epigenetic changes and are associated with specific gene regulation patterns: Silent chromatin is mostly present in a condensed chromosomal state, which is inaccessible to transcription factors, while transcriptionally active chromatin is characterized by a less condensed structure, often associated with histone acetylation. [45] .

Nowadays there are different assays that are available to study the genome and the interactions of transcription factors with the DNA. Among these there are DNA-affinity binding assays and Chromatin immune precipitation and sequencing (ChIP-seq), which allow the identification of transcription factors binding to specific DNA sequences. However, these techniques require an *a priori* knowledge about the mechanism involved into the epigenetic changes [46].

Due to the many complications and time-consuming protocols of the original DNAse-sequencing, the novel assay for transposase accessible chromatin (ATAC-seq) was developed. ATAC-seq is based on the engineered transposase Tn5, characterized for its hyperactivity and ability to insert into nucleosome-free regions [46,47]. Tn5 fragments the DNA and at the same time integrates adapters in the open regions, allowing DNA amplification and the generation of libraries for high through-put sequencing (*Figure 4 A, B).*

The alignment of the obtained fragments to the genome is used to define "peaks" of chromatin accessible regions by Tn5 with putative regulatory function, such as promoters, enhancers or silencers. [48].

Among ATAC-seq major advantages figure the low number of cells required, which is less than fifty thousand, and the short amount of time needed for the overall processing. Hence, ATAC-seq is a powerful tool to identify the putative gene regulatory regions of many type of cells, in relation to different stimuli, developmental stages or disease states. The further use of ATAC-sequencing data to perform a footprinting analysis predicting the possible TFs binding to a specific locus will be examined in the chapter below.

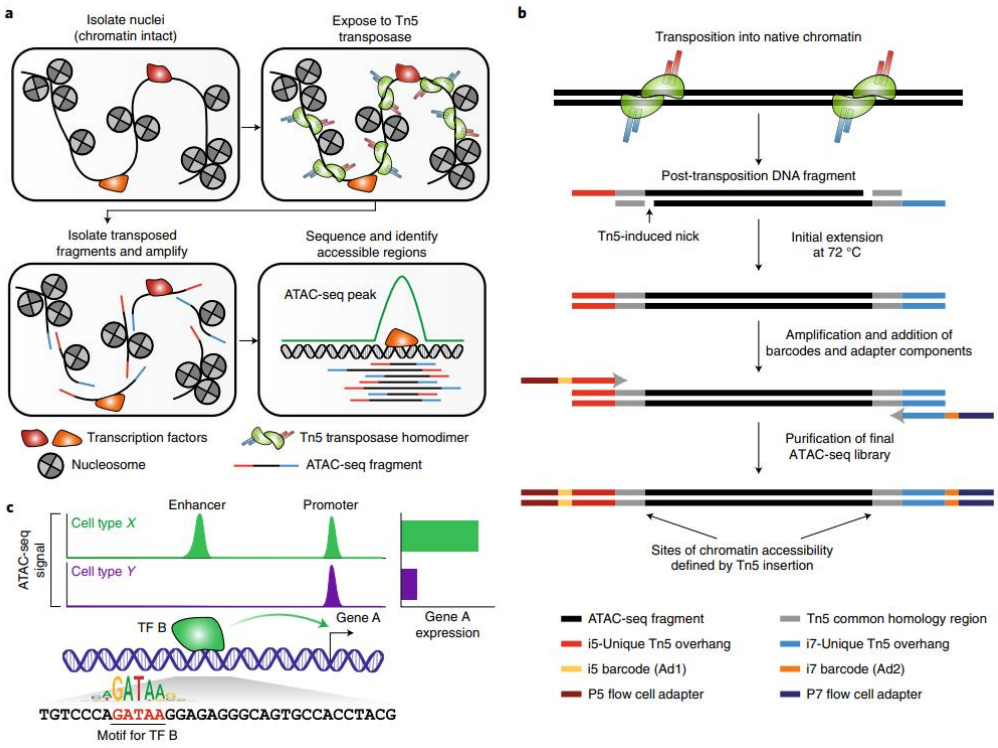


Figure 4: *A summary of the steps in ATAC-seq. Nuclei are isolated from cells while preserving the chromatin structure and DNA-binding proteins, including nucleosomes and transcription factors (TFs). This chromatin is then exposed to the Tn5 transposase, which operates as a homodimer to simultaneously fragment the chromatin and insert sequences carrying PCR handles that allow for further amplification with i5/P5 and i7/P7 ATAC-seq adapters. Only fragments with an i5/P5 adapter at one end and an i7/P7 adaptor at the other will be amplified and sequenced correctly. After sequencing analysis of library fragments, genomic areas enriched for several Tn5 transposition events are classified as ATAC-seq peaks, or peaks of chromatin accessibility. B, Detailed schematic of fragments created by transposition into native chromatin. The nicks left by the transposase after Tn5 insertion of the PCR handles are filled in during the initial 72 °C extension in the first step of the barcoding PCR. The fragments are subsequently barcoded and readied for sequencing. C, Illustration of an enhancer upstream of the transcription start site (TSS) that causes higher levels of gene A expression. This would emerge as a cell-type-specific ATAC-seq peak that is differently accessible between cell types and Y in the ATAC-seq data. The differential accessibility is determined by TF B, which binds to the cell type-specific enhancer and stimulates gene expression. ATAC-seq data can also assist in revealing the TF binding motif linked with these differential peaks, illustrated above Picture and caption taken from:* *Grandi, F.C., Modi, H., Kampman, L. et al. Chromatin accessibility profiling by ATAC-seq. Nat Protoc 17, 1518–1552 (2022). https://doi.org/10.1038/s41596-022-00692-9.*

1.3.2 Transcription factors footprinting analysis

The footprinting analysis in the context of ATAC-seq, aims to the identification of transcription factor binding events to a specific DNA sequence. The binding of a TF in an open region protects the underlying DNA from Tn5 enzyme action, generating a "dip" in the accessible peaks [49]. Consequently, the objective of the footprinting analysis is to identify the protected areas within the accessible peaks and then to determine which transcription factor may have been bound in that location by analyzing the underlying sequence (*Figure 5*). TF footprint identification can be used in the reconstruction of gene regulatory networks and is more specific than motif scanning in detecting the presence of a TF [46]. However, there are numerous obstacles to applying the footprinting analysis to ATAC-seq data, and Tn5's sequence bias can frequently mislead the interpretation, so the subsequent adjustment of the fragments due to Tn5 offset is needed [50,51]. In order to collect adequate data to identify the protected bases, many fragments are demanded in order to map to that particular locus. This requires an adequate depth of sequencing. In conclusion, the implementation of these two techniques, in this study, will help to identify the putative regulatory regions and elements that are involved in *IL23R* gene expression and in the development of Th17 T-cells.

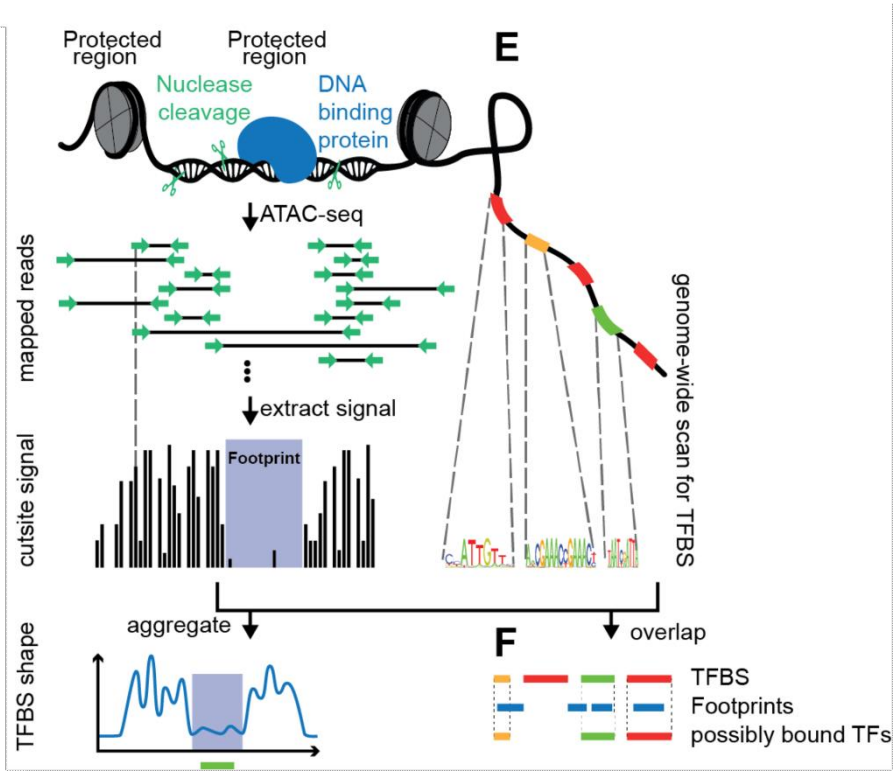


Figure 5: *Schematic picture of ATAC-Seq and footprinting analysis, showing the sequential steps taken to obtain the "dips"of the peaks read, followed by the sequence matching to a TF binding site. Image taken from: Bentsen, M., Goymann, P., Schultheis, H. et al. ATAC-seq footprinting unravels kinetics of transcription factor binding during zygotic genome activation. Nat Commun 11, 4267 (2020). https://doi.org/10.1038/s41467-020-18035-1*

2. AIM OF THE STUDY

The general goal of this project is to improve the understanding of the "IL-23/IL-17 axis". The discovery of the T helper (Th) 17 CD4+ effector cell subset, that showed a different pattern of cytokine expression compared to the already known Th1 and Th2 subsets, brought about a new understanding of immune regulation and of the mechanisms of immune-mediated disease. In fact, many Genome-wide association studies (GWAS) have demonstrated the role of immune cell subsets in chronic inflammatory diseases (CID) and have pointed out the role of several cytokine signaling pathways, including the IL-23/IL-17 pathway. In particular, the IL-23 receptor (*IL23R*) gene has shown a strong association with several CID, including spondyloarthritis, psoriasis and Crohn's disease [1,3]. Consistently, the cytokine IL-23 was shown to be important for the expansion and functional activity of the Th17 subset, but little is known about its biological function in human inflammatory diseases, in which the correspondent receptor IL-23R seems to have an important role.

The first specific goal of this study is to analyze which cells express the IL-23 receptor and are therefore responsive to IL-23, and which signals and stimuli are involved in the expression of IL-23R in naive CD4+ T cells.

The second specific goal of this study is to understand the epigenetic transcriptional mechanisms associated with *IL23R* expression, by identifying putative regulatory regions that show an open chromatin conformation. To this end we have used ATAC-seq to identify accessible regions in adaptive (naïve cord-blood derived CD4+ T cells) . ATAC-seq footprinting analysis allowed us to obtain additional information about the numerous transcription factor and pathways that were activated in the different stimulation conditions.

The 306 transcription factors predicted in the analysis were then evaluated for their expression in the cell types of interest using the Human Protein Atlas and the DICE databases. Other software like Jaspar and String were employed to precisely identify the binding sequence on the IL*23R* locus and to model a possible network of interactions among factors, respectively. From this

evaluation we focused our interest on 7 TFs: IRF1, IRF4, BATF, JUNB, IKZF1, RUNX3 and BACH1.

The further characterization of their role on the locus of interest was assessed by a knock-down experiment employing siRNA molecules directed against the factors. This study lays a foundation for future tests to verify a possible interaction or influence among the proteins in the binding of open chromatin regions on the *IL23R locus* in vitro, and to assess additional factors that may play a role in *IL23R* expression. This could help the overall understanding of the biological pathways involved in chronic inflammation and its related diseases.

3. MATERIALS AND METHODS

3.1 CBMC isolation

Fresh Cord blood from healthy-donors was obtained from the AP-HP Cord Blood Bank (Hôpital Saint Louis, Paris, France), as units discarded following screening for allogeneic transplantation. The blood was diluted 1:3 with dPBS 1X (Gibco™, Life Technologies) and 35mL of blood were distributed into 50mL Falcon tubes prefilled with 14mL RT Ficoll (Lymphocyte separation medium; Eurobio Scientific) and centrifuged at 2400 rpm for 30 min. The ring between the liquid phases was collected and diluted 1:2 with ice-cold PBS and centrifuged at 2000 rpm for 15 min at 4°C. Supernatants were discarded and pellets were joined in a 50mL Falcon tube and centrifuged 1400 rpm for 10 min at 4°C. Cells were re-suspended in 50 mL of Cold dPBS 1X and counted using a 1:50 dilution and Trypan blue in a Neubauer chamber : Number of cells = ((sq 1+sq2)/2) (d *tot volume/100) = tot number of cells (in the total volume).

3.1.1 CD14- CD4+ T cell selection

After determining the cell number, the suspension was centrifuged at 1400 rpm for 10 minutes and the supernatant was aspirated completely. The cell pellet was then resuspended in 80 µl of MACS buffer (cold PBS 1X with 1% FCS, EDTA 0.5M - Accugene®, cat. No.5134), per 10^7 total cells. 20 µL of CD14 MicroBeads conjugated to monoclonal anti-human CD14 antibodies (Miltenyi Biotec, isotype: mouse IgG2a) per 10^7 total cells were added, and the solution was mixed in the refrigerator at 4°C for 15 minutes. A subsequent wash with MACS buffer was done, followed by the complete aspiration of the supernatant. After centrifuging at 1400 rpm for 10 minutes, the CD14- cells were used for CD4+ isolation using the CD4+ T cell positive selection isolation kit (Miltenyi Biotec). After this last isolation, 10^6 cells/mL were plated in RPMI+10% FCS medium and stimulated through the TCR (CD3/CD28), in the presence of Th17 cytokines cocktails the same day of their isolation. (*Table 1*).

3.2 Cytokines and proteins

Human cytokines used throughout all experiments were supplied by Miltenyil Biotec. A list comprehensive of stock concentration and final concentration used for the stimulation is provided down below.

Cytokine		concentrations
CD3/CD28 beads		100 µg/mL
IFN-α		10^6 U/mL
IFN-β		10^6 U/mL
IFN-γ		0.1 mg/mL
Th17 cocktail	IL-1β	10 ng/mL
	IL-21	25 ng/mL
	TGF-β	10 ng/mL
	IL-23	10 ng/mL

***Table 2**: Concentrations of Th17 cocktail of cytokines and interferons. The latter were added into the cell culture to assess their potential role for IL23R gene expression. CD3/CD28 will be referred to as "TCR" stimulation in the results.*

3.2.1 Cell culture

Cells were incubated at 37°C, 4% CO_2 for different times, as specified, and were then harvested for RNA extraction and gene expression analysis, or for ATACseq analysis.

3.3 siRNA delivery

To evaluate the possible effects of the transcription factors selected, we proceeded to knock down their expression using Dharmacon™ Accell™ siRNA delivery method. According to the manufacturer, Accell siRNA is specially modified for use without a transfection reagent and works at a higher concentration than conventional siRNA. To note, each experiment was performed for CD4+ cells that grew in suspension, and it included the following samples in triplicate: 1. Untreated cells plated in Accell Delivery Media 2. Negative control siRNA (Accell Non-targeting siRNA control). 3. siRNA targeting one of the genes of interest. The siRNA Buffer (Cat #B-002000-UB-100) was diluted to 1X by mixing four volumes of sterile RNase-free water with one volume of 5X siRNA Buffer. 100 µM siRNA solution was prepared in 1x siRNA buffer and placed on an orbital mixer/shaker for 70-90 minutes at room temperature. 7.5 µl of the 100 µM siRNA were added in separate tubes, and cells were counted, growth medium was removed after centrifugation, and cells were resuspended in the 750 µL of Accell siRNA Delivery Media (Cat #B-005000), in 1,5 $*10^6$ cells per well in a 96-well plate. 750 µl of the cells plus delivery media mix was then added to 7.5 µl of the 100 µM siRNA so that the final concentration will be 1 µM of Accell siRNA per well in a 96-well plate. After mixing, 100 µl of the delivery mix plus cells was added to each well in a 96-well plate and incubated at 37 °C with 5% CO2 for 72 hours. After this time, cells were collected for RNA extraction and IL23R gene expression analysis. The Table below details the sequences for the transcription factors we tested.

TF:	Concentration	siRNA target sequence
JUN B	20 nmol	UUAUUGAAUCUAUUUAAGU
		CCUUCCACCUCGACGUUUA
		GCCUCUCUCUACACGACUA
		GAGUUUAUUUUAAGACGUG
BATF	20 nmol	GUAUUAAGAAAGAUGCUCA
		GCCCAAUGCAGAAGAGUAU
		GCUCUACGCAAGGAGAUCA
		GAAAGAUGCUCAAGUCCCA
IKZF1	5 nmol	GUCGUGGCCAGUAAUGUUA
		CCGUGAUCCUUUUGAGUGC
		CCUUCAAGAUUAAUGCUAU
		GUUCCAUUUCCAAUUUAGA
SICTRL	50 nmol	UGGUUUACAUGUCGACUAA
		UGGUUUACAUGUUUUCUGA
		UGGUUUACAUGUUUUCCUA
		UGGUUUACAUGUUGUGUGA
IRF1	5 nmol	GGCUCAUCUGGAUUAAUAA
		GGAUGAGGAAGGGAAAUUA
		GCCUUUGCAUUUAUUUAUA
		GGUAUGACUUAAAAUUGGA
IRF4	5 nmol	UUAUCAAGCUUAGUGAGCA
		GAAGUAAGAUGUAAAUUGA
		GCGUAGCUCUCAAAUGUGU
		GCGUAGCUCUCAAAUGUGU
RUNX3	5 nmol	GUCUCAUCCCAGAUUACUA
		GUAGUGGGUACCAAUCUUU
		GCAGCAUGCGGUAUUUUAA
		UUGUCAUGCUGAGGUGUGA

Table 3: *siRNA pool sequences for each transcription factor used for knock-down (Dharmacon™).*

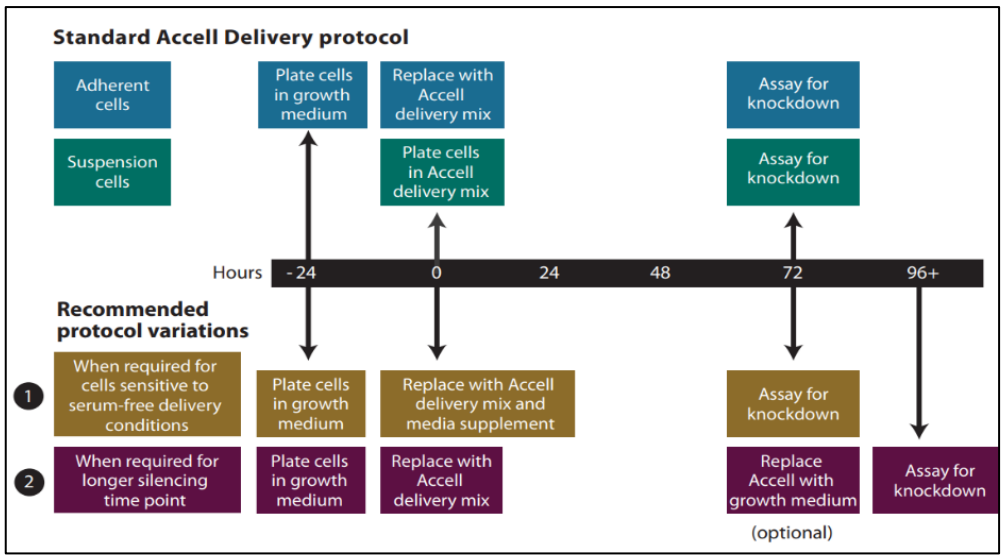


***Figure 6*: *Standard Accel delivery protocol for siRNA pools*.**

3.4 RNA extraction

RNA extraction was performed using Quick-Start Protocol RNeasy® Mini Kit (cat. no. 74004) by QIAGEN®. Cells were harvested at a maximum of 5 x 10^7 cells as a cell pellet, and 350 µL of Buffer RLT were added. The solution was then homogenized and 1 volume of 70% ethanol was added to the lysate and mixed well by pipetting. Each sample, with any precipitate, was transferred to an RNeasy Mini spin column in a 2 ml collection tube, the lid was closed and samples were centrifuged for 30 s at 14000 x g. 700 µL of Buffer RW1 were added into the column and centrifuged 30 s at 14000 x g. The flowthrough was discarded and 500 µL of Buffer RPE was added. After centrifuging at the same time and speed, the flow-through was again discarded and 500 µL of buffer RPE was added. After that the column containing the solution was centrifuged 2 minutes at 14000 x g, followed by a further 1 minute centrifugation to eliminate residual buffer. The column was then placed in a 1,5 collection tube and 30 µL of RNase-free water were added. After centrifuging for 1 minute at 14000 x g, the RNA is eluted. RNA quality was checked using Qubit RNA HS Assay Kit. (Cat. No. Q32855) and then placed on ice or at -80°Cfor further analysis of gene expression by qPCR or Nanostring.

3.5 qRT-PCR

To quantify the gene expression, cDNA conversion from RNA was first performed using TAKARA BIO PrimeScript™ (RT Master Mix TAKARA RR0337A): 2 µL 5X prime script buffer , 0,5µL 5X Prime script RT Enzyme, 0,5µL Oligo primers, 0,5µL Random 6mers and a total of 6,5µL of RNA and water, so that the total final volume is 10 µL for an amount of RNA that is equal or less than 500 ng. Real time PCR was performed using 1,6 µL of 18S primer 4µM, or 1,6 µL of 23S primer mixed respectively with 16 µL of MasterMix SYBR green per sample. To assess the IL23R expression, the respective primers and 18S primers for normalization were used and listed in *Table 2*. Quantification of relative gene expression was performed by the comparative Ct method ($2^{-\Delta\Delta Ct}$).

Target gene	Primer Forward	Primer Reverse
18S	5'-CAGCCACCCGAGAATTATTGAGCA-3'	5'-TAGTAGCGACGGGCGGTGTG-3'
IL23R	5'-CAGGTCACTATTCAATGGGATGC-3'	5'-GCAGTTCTTAATTGCTGCTTGG-3'

***Table 4**:List of Primers forward and reverse for qRT-PCR*

3.6 Nanostring plexset™ Technology

To optimize IL23R gene expression analysis, Nanostring Plexset ™ Technology was exploited. This technology allows multiplexed analysis up to 96 samples, avoiding the conversion of RNA in cDNA, and decreasing the total time and material needed for gene expression, compared to a qRT-PCR assay. The working principle is based on the hybridization of two probes: A Reporter probe, that matches another 50 bases of the target sequence, which ends with a barcode with different fluorescent molecules(Probe A), and one capture probe (Probe B), made of 50 bases that matches the target sequences, with a Biotin molecule at the end , (*Fig. 7 A,B*). mRNA samples are mixed with capture probes and reporter probes to hybridize for 24 hours. The next day purification

is done into the Tape station, and the hybridized probe are then loaded on a cartage. Here they will bind to it by Biotin-streptavidin (on the cartage) interaction. An electrical field is then applied so that all the hybrid complexes will flex in the same direction, exposing the barcode to be hit by the light to be counted and digitally analyzed. In the end there will be a unique barcode for each gene target that goes into each Plexset, enabling an analysis of 12 to 24 genes at the same time. The preparation was performed according to the manufacturer protocol: , Probe A and Probe B Master Stock Pools were diluted with TE-twee to a working concentration of 0.6 nM and 3nM respectively. (*Table 5, Panel A).* Following that, *the* Buffer-probe mix was created by adding probe A and B working pool into the Hybridization buffer. (*Table 5,Panel B*). The creation of the PlexSet master mixes is performed by combining the Buffer/probe mix to a previously thawed PlexSet tubes. Following that, the hybridization reactions were set in a tightly sealed 96 well plate and incubated overnight at 67°C, at the end of which each PlexSet mix was pipetted into each 12 wells of the row and, after that, the samples were added for a total volume of 15 µL . In the last step of post-hybridization processing, a standard nCounter Prep station run with the strip tube containing the pooled reactions was performed and loaded onto the Digital Analyzer. Further data analysis was performed using nSolver ™ software.

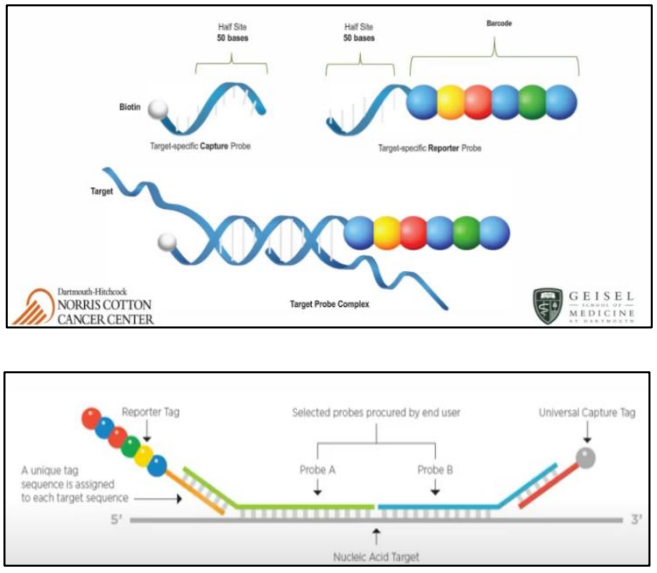


***Figure** 7 : A,B Schematic visuals of the probes A and B and hybridization with the target s equence on the mRNA. Pictures were taken from Labroots Video in nanostring.com website, by Christan Lytle - New multiplexed nCounter® PlexSet™ Reagents- an alternative to qPCR technology*

A

Number of Plexset tubes (A-H) Experimental setup	Total numbers of samples	Aliquote from Probe A or B Master stock (µL)	Te-Tween (µL)	Final volume (µL)
8	96	10	73	83

B

Number of Plexset tubes (A-H) Experimental setup	Volume of hybridization buffer (µL)	Volume 0,6 nM each probe A working pool (µL)	Volume 3 nM each probe B working pool (µL)	Proteinase K 20 ng/mL
8	600	60	60	18

C

Reagent	**Volume per reaction**
Hybridization Buffer	5 µL
Plexset reagent	2 µL
Prob A working pool (0,6 nM)	0,5 µL
Prob B working pool (3 nM)	0,5 µL
RNA sample or crude whole cell lysate	Max 7 µL
Nuclease-free water	Up to 7 µL
Proteinase K (20 mg/mL)	**
Final Hybridization volume	15 µL

Table 5*: A: Dilution volumes for Probe A and B of the Master stock Pools B: Volumes for the creation of a Buffer-pro mix. C:Reagents and respective volumes for Nanostring Plexset Assay.*

3.7 ATAC-Seq

3.7.1 Sample preparation

ATACseq was performed using the kit from Active Motif (Catalog No. 53150), according to the manufacturer's protocol. To prepare the sample for ATACseq, cells aliquot from 50,000 to 100,000 cells were collected into a 1,5 mL tube for each sample and centrifuged at 1000 x g for 10 minutes at 4°C. Supernatant was removed and 100 µL of cold dPBS were added without resuspending, following a 5 minutes spin at 4°C at 500 x g. Supernatant was removed and the pellet was resuspended in 100 µL of ice-cold ATAC lysis buffer. After transferring the resuspended pellet into a PCR tube on ice and spinning down at 500 x g for 10 minutes at 4°C, the Tagmentation mix was prepared according to *Table 4.* After the spin, the supernatant was removed, and the Tagmentation reaction and the following purification steps were performed according to the protocol below.

3.7.2 Tagmentation reaction and purification

50 µL of Tagmentation Master Mix was added to each sample, and the nuclei in the Tagmentation Master Mix were resuspended. The Tagmentation reaction was incubated at 37°C for 30 minutes in a thermomixer set at 800 rpm. Immediately following the tagmentation reaction, each sample was transferred to a clean 1,5 ml microcentrifuge tube, adding 250 µL of DNA Purification Binding Buffer and 5 µL 3 M sodium acetate to each sample. The DNA Purification Binding Buffer has a pH indicator dye, so that the pH of the solution can easily be determined and adjusted by adding 3 M sodium acetate (see figure below). The sample is applied to the column only when the solution is bright yellow (tube on the left), indicating a pH under 7.5. Each sample is mixed by pipetting and transferred into a DNA purification column in a collection tube, centrifuged at 17,000 x g (14,000 rpm) for 1 minute and the flow-through discarded. 750 µl of Wash Buffer were added to the column and centrifuged at 17,000 x g for 1 minute, followed by an additional centrifugation at 17,000 x g for 2 minutes to remove residual Wash Buffer. After transferring

each column to a new tube, 35 µl of DNA Purification Elution Buffer were added to the center of the column matrix, incubated for 1 minute at room temperature and centrifuged to elute the DNA. Purified DNA could either be stored at -20°C, or the following PCR Amplification of Tagmented DNA steps could be performed.

Reagent	Volume
2X Tagmentation Buffer	25 µL
10X PBS	2 µL
0,5% Digitonin	0,5 µL
10% Tween 20	0,5 µL
H_2O	12 µL
Assembled Transposomes	10 µL

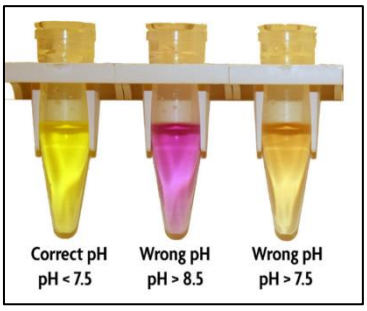


Table 6 (left): *Volumes of reagents for ATAC-seq sample preparation.*

Figure 8 (right): *Solution color as a function of pH.*

3.7.3 PCR Amplification of Tagmented DNA

The PCR reactions is set up by adding the components in the order shown in *Table 5 (A,B)*. Libraries were multiplexed for sequencing on the same flow cell, using unique combinations of i5 and/or i7 indexes. A total of 16 samples were multiplexed (*Table 5*). PCR was performed using the following program on a thermal cycler: 72°C 5 minutes 98°C for 30 seconds 10 cycles of: 98°C for 10 seconds, 63°C for 30 seconds, 72°C for 1 minute Hold at 10°C. After that, DNA was purified with Solid Phase Reversible Immobilization beads. DNA was eluted in 20 µl of DNA Purification Elution Buffer. PCR amplified libraries were analyzed to assess size distribution with a TapeStation (Agilent). Sequencing was performed by Novogene Co (UK).

A

i7 Index	i7 Sequence	i5 Index	i7 Sequence
N701	TCGCCTTA	N501	TAGATCGC
N702	CTAGTACG	N502	CTCTCTAT
N703	TTCTGCCT	N503	TATCCTCT
N704	GCTCAGGA	N504	AGAGTAGA

Table 7 A: Primers for ATAC-seq and relative sequences. B: combination of i7 and i5 primers; C: Reagents and volumes for the Tagmentation Reaction.

B

Use one i7 Indexed Primer	use one i5 Indexed Primer
i7 Indexed Primer 1 = i7 N701	i5 Indexed Primer 1 = i5 N501
i7 Indexed Primer 2 = i7 N702	i5 Indexed Primer 2 = i5 N502
i7 Indexed Primer 3 = i7 N703	i5 Indexed Primer 3 = i5 N503
i7 Indexed Primer 4 = i7 N704	i5 Indexed Primer 4 = i5 N504

C

Reagent	Volume
Tagmented DNA	33,5 µl
i7 Indexed Primer (25 µM)	2,5 µl
I5 Indexed Primer (25 µM)	2,5 µl
dNTPs (10 mM)	1 µl
5X Q5 Reaction Buffer	10 µl
Q5 Polymerase (2U/µl)	0,5 µl

3.8 Statistical analysis

Statistics was analyzed by using GraphPad Prism 9.0 (Graphpad software)

3.9 Bioinformatic tools

Tools used for Transcription factor analysis: Human protein Atlas website, Jaspar 2022 database, IGViewer software. Tools used for gene expression analysis: Nanostring nCounter software.

4. RESULTS

4.1 Induction of IL23R expression in naïve CD4+ cells from cord blood

The general goal of this project is to improve the understanding of the so-called "IL-23/IL-17 axis". Indeed, the major questions to be answered were, at first, what are the cells that respond to IL-23, followed by what the effect of IL-23 stimulation on these cells is. To address the first question, we investigated the regulation of IL-23R expression in innate and adaptive T cell populations.

4.1.1 Kinetics of IL23R expression

Naïve CD4+/CD8+ T lymphocytes were selected from human cord blood and stimulated through the TCR in presence of three different cytokines cocktails, each one known to induce one of the different subsets of T helper cells: Th1, Th2 and Th17 [52] and the subset-characterizing genes were analyzed (*Figure 9*). Cells stimulated in Th17-inducing conditions showed upregulated expression of interleukin 17 (*IL17A*) and of the RORγT (*RORC*) transcription factor, while Th1 cells upregulated the interferon gamma gene (*IFNG*) and expression of the transcription factor Tbet (*TBX21*). *IL23R* was expressed by both these cell subsets at comparable levels, while the beta chain of the IL12 receptor (*IL12RB*) was strongly induced in Th1 cells.

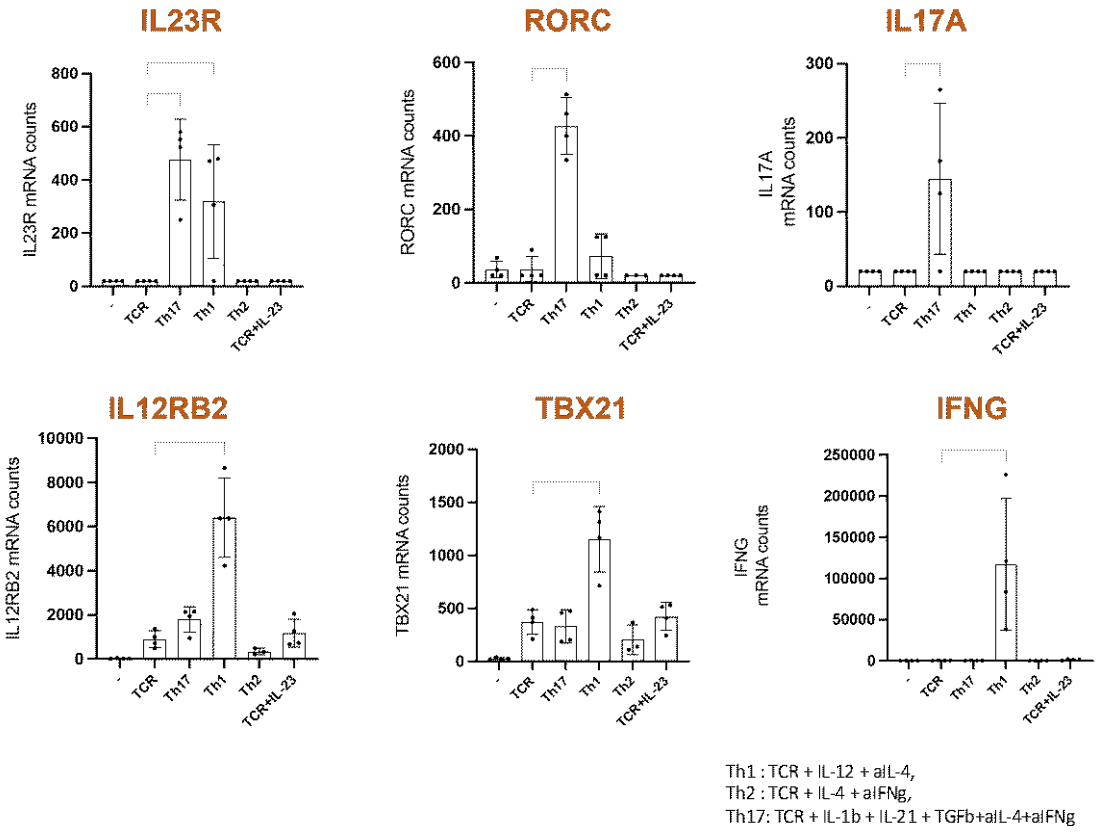


Figure 9: *In vitro differentiation of T helper cell subsets. CD4+ T cells from cord blood were stimulated in the indicated conditions for 96 hours, and gene expression was analyzed by nCounter Plexset technology. TCR: (anti-CD3 and anti-CD28 beads). Th1: CD3/CD28 beads + IL-12, 20ng/ml + anti-IL-4, 1µg/ml; Th2: CD3/CD28 beads + IL-4, 2ng/ml + anti-IFNgamma, 1µg/ml; Th17: CD3/CD28 beads + TGFbeta, 10ng/ml + IL-21, 25ng/ml + IL1beta, 10ng/ml + IL-23, 10ng/ml + anti-IL-4, 1µg/ml + anti-IFNgamma, 1µg/ml). n= 4, Anova test, ** P ≤ 0.01, **** P ≤ 0.0001*

To understand at which time-point it is possible to observe IL23R expression in CD4+ and CD8+ cells, a kinetic experiment was performed, followed by RNA extraction and gene expression analysis of the samples for each time point: 0h, 4h, 18h, 48h, 96h. (*Figure 10*) Thus, it emerged that Th1 and Th17-inducing cytokines stimulate IL23R expression with different kinetics, both in CD4+ and CD8+ T cells, suggesting that the molecular mechanisms of IL23R induction may differ in the two conditions. In particular, the transcription factor RORγt, which is known to contribute to IL-23R expression in Th17 cells, is not induced in Th1 cells, and may not be necessary for IL23R expression induced by IL-12 signaling (*Figure 10, bottom panel)*

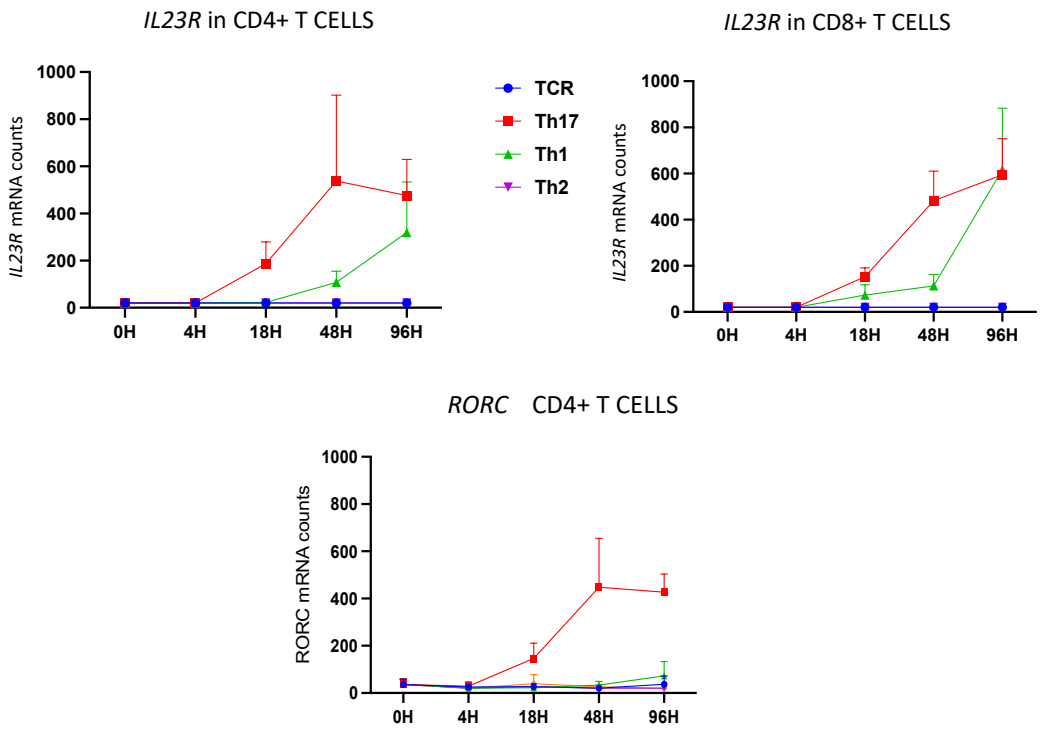


Figure 10 : *Kinetics of IL23R expression at different time points in Th cells subsets. Cord blood CD4+ T cells (left panel) and CD8+ T cells (right panel) were stimulated with different cytokines cocktails: Th1: TCR+IL-12; Th2: TCR+IL-4; Th17: TCR+ IL-1β+ IL-21+ TGF-β+ anti-IL4+anti-IFNγ. Gene expression was assessed at different time points by nCounter Plexset technology. Average and SD of n= 4. From the graph it is possible to observe that the receptor is not expressed on naive CD4+ and CD8+ T cells, while it is expressed at 18h in Th17 cells and at 48h in Th1 cells.*

4.1.2 IL-23R protein expression

Expression of the IL-23R was also confirmed at the protein level by immunofluorescent detection of IL-23R on the surface of cord blood-derived T cell populations stimulated in Th1 or Th17 inducing conditions, or with cytokines of interest for *IL23R* transcription, as detailed in the following section (*Figure 11*).

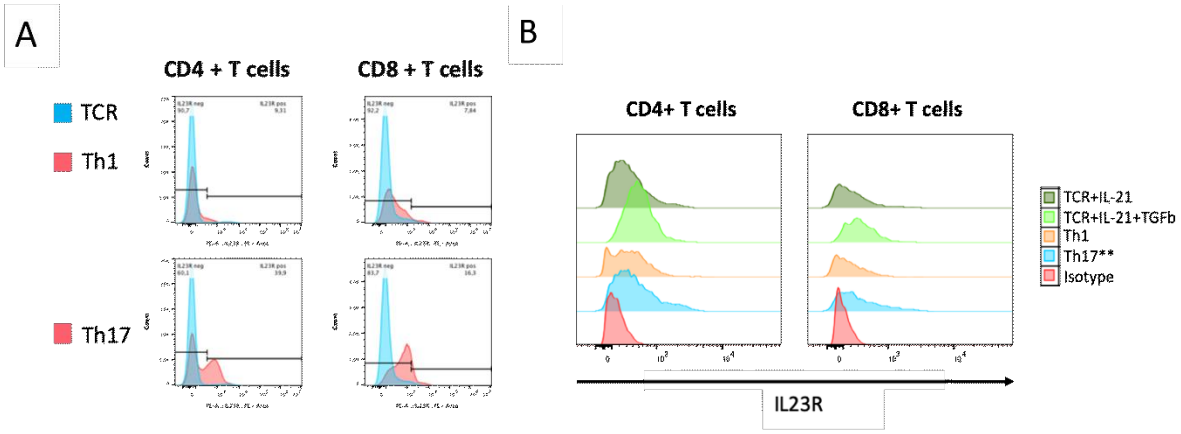


Figure 11*: Induction of IL-23R protein in differentiating T cells subsets. Cord blood derived CD4+ and CD8+ T cells were stimulated in Th1 or Th17 differentiating conditions (A and B) or with anti-CD3/CD28 beads in the presence of the indicated cytokine combination (IL-21, 25ng/ml, TGFbeta, 10ng/ml), and stained with an anti-IL-23R monoclonal antibody followed by biotinylated anti-mouse Ab and Streptavidin PE. Surface expression of IL-23R was analyzed on a FACS Aria cell sorter (BD).*

4.1.3 Cytokine signaling pathways involved in IL23R induction

4.1.3.1 The role of IL-21

To explore in detail the pathways responsible for IL23R induction, we asked what is the role of the different cytokines that constitute the Th17-differentiating condition. To this end, we treated naïve cord blood CD4+ T cells with the Th17-inducing mixture of cytokines, from which single cytokines were omitted (*Figure 12*) While omission of IL-1 beta or IL-23 had no effect on *IL23R* induction at 24 or 48 hours of stimulation, the omission of IL-21 strongly reduced *IL23R* expression at both time points. TGF-beta did not seem to have an effect at early times of stimulation but was necessary at 48 hours to obtain full expression of *IL23R*. Consistent with these results was the findings that IL-21 was sufficient for early induction of *IL23R* in TCR-stimulated CD4+ T cells (*Figure 13 A*)

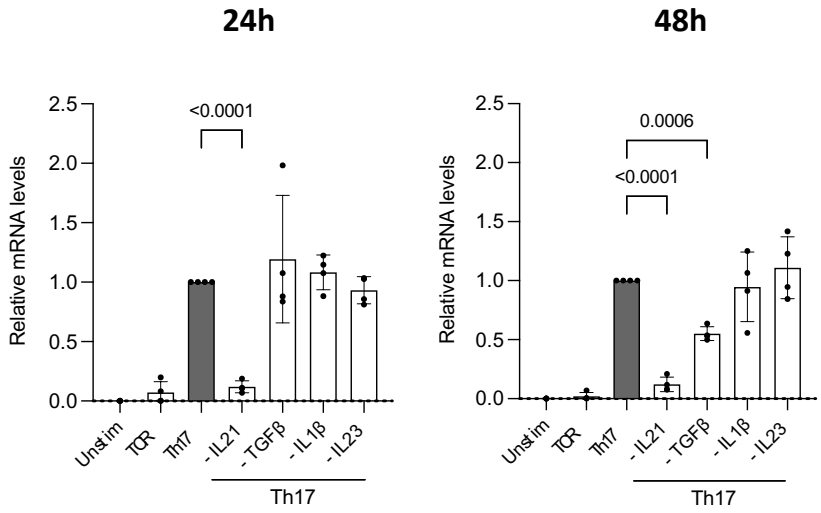


***Figure 12**: Role of individual cytokines for IL23R expression in naïve CD4+ T cells. CD+ T cells isolated from cord blood were stimulated through the TCR alone, in complete Th17-inducing conditions (Th17), or with a mixture of Th17-inducing cytokines from which single cytokines were omitted as indicated. IL23R expression was measured at 24 and 48 hours by Real-Time RT-PCR. Th17: CD3/CD28 + IL-1beta + IL-21 + TGFbeta + anti-IL-4 + anti-IFNgamma. n=4, Anova test.*

Moreover, to understand if the signaling could occur through the common gamma-chain of IL-2, IL-7 and IL-21, the gene expression levels of the *IL23R* were evaluated at 48 hours' time point, according to the previously mentioned data. IL-2 and IL-7 did not induce *IL23R* expression in naïve CD4+ T cells. (*Figure 13B*)

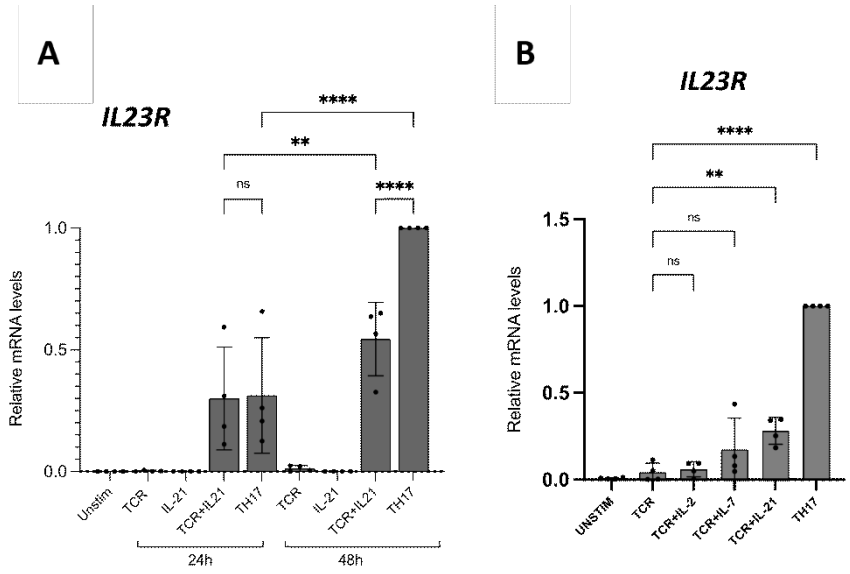


Figure 13: *A: Role of IL-21 in IL23R expression in naïve CD4+ T cells. CD+ T cells isolated from cord blood were stimulated through the TCR alone (CD3/CD28), with IL-21 alone, through the TCR in the presence of IL-21 or in Th17-inducing conditions. IL23R mRNA levels were measured at 24 and 48 hours by Real-Time RT-PCR. Th17: CD3/CD28 + IL-1beta + IL-21 + TGFbeta + anti-IL4 + anti-IFNgamma. n=4, Anova. **, P ≤ 0.001 **** B: Role of gamma chain cytokines in IL23R expression in naïve CD4+ T cells CD+ T cells isolated from cord blood were stimulated through the TCR alone, through the TCR in the presence of the indicated cytokines or in Th17-inducing conditions. IL23R mRNA levels were measured at 24 and 48 hours by nCounter technology using the Nanostring V2 Plus Immune panel. Th17: CD3/CD28 + IL-1beta + IL-21 + TGFbeta + anti-IL4 + anti-IFNgamma. n=4, Anova. P ≤ 0.05 **, P ≤ 0.001 *****

4.1.3.2 The role of IL-6

IL-6 was identified as a key cytokine directing Th17 differentiation in murine animal models. IL-6 signaling induces phosphorylation and activation of the STAT3 transcription factor, which has been reported to bind to the *IL23R* locus. We therefore asked what is the role of IL-6 in the early induction of *IL23R* in naïve CD4+ T cells. In our *in vitro* differentiation system, TCR stimulation in

the presence of IL-6 induced significantly lower levels of *IL23R*, respective to IL-21 stimulation. In addition, replacing IL-21 with IL-6 in the Th17 differentiating cytokine mix yielded significantly reduced levels of *IL23R* (*Figure 14*)

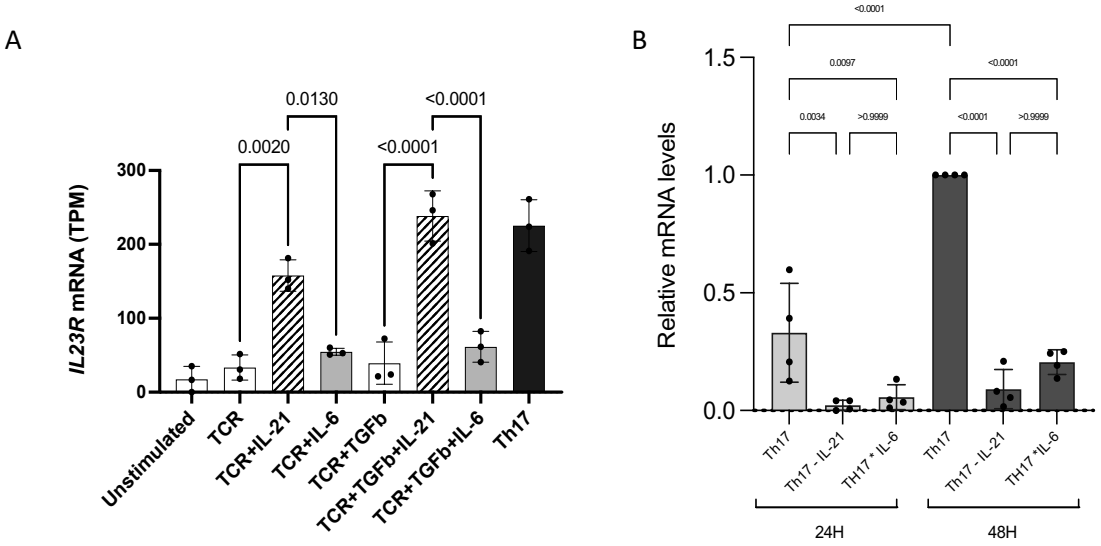


Figure 14: *Role of IL-6 in IL23R expression in naïve CD4+ T cells. Cord blood CD4+ T cells isolated from cord blood were stimulated through the TCR alone, through the TCR in the presence of the indicated cytokines or in Th17-inducing conditions. A: IL23R levels after 24 hours of stimulation were measured by RNAseq. Anova, n=3. B: Cells were stimulated in the following conditions: "Th17" = CD3/CD28 + IL-21 + TGFβ + IL-1β + IL-23; "Th17-IL-21"= CD3/CD28 + TGFβ + IL-1b + IL-23 ; "Th17*IL-6" = CD3/CD28 + IL-6 + TGFβ + IL-1β + IL-23. Friedman test, n = 4.*

4.2 Study of the mechanisms of IL23R expression: identification of putative regulatory regions at the IL23R locus

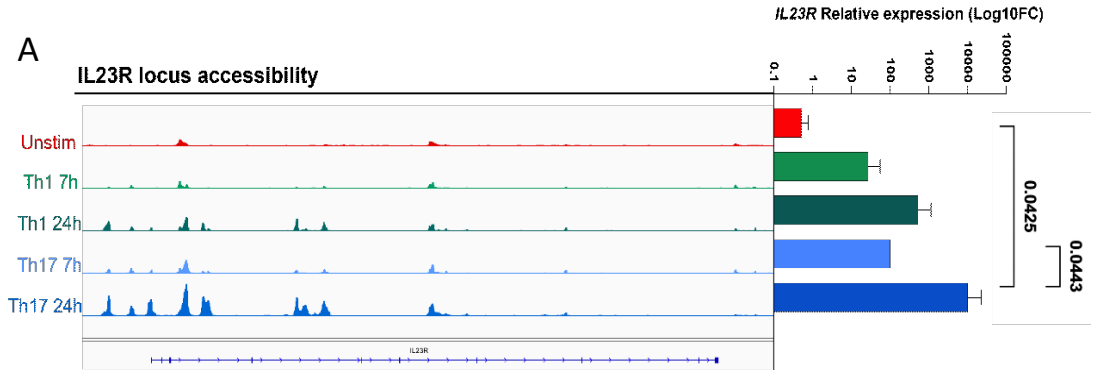
4.2.1 Analysis of accessible regions in differentiating CD4+ T-helper cells at the IL23R locus

To determine the chromatin accessible regions in differentiating CD4+ T cells, ATAC sequencing was performed on cord blood CD4+ T cells from three biological replicates after a 7h and 24h stimulation with the T-helper subset-differentiating cytokines.

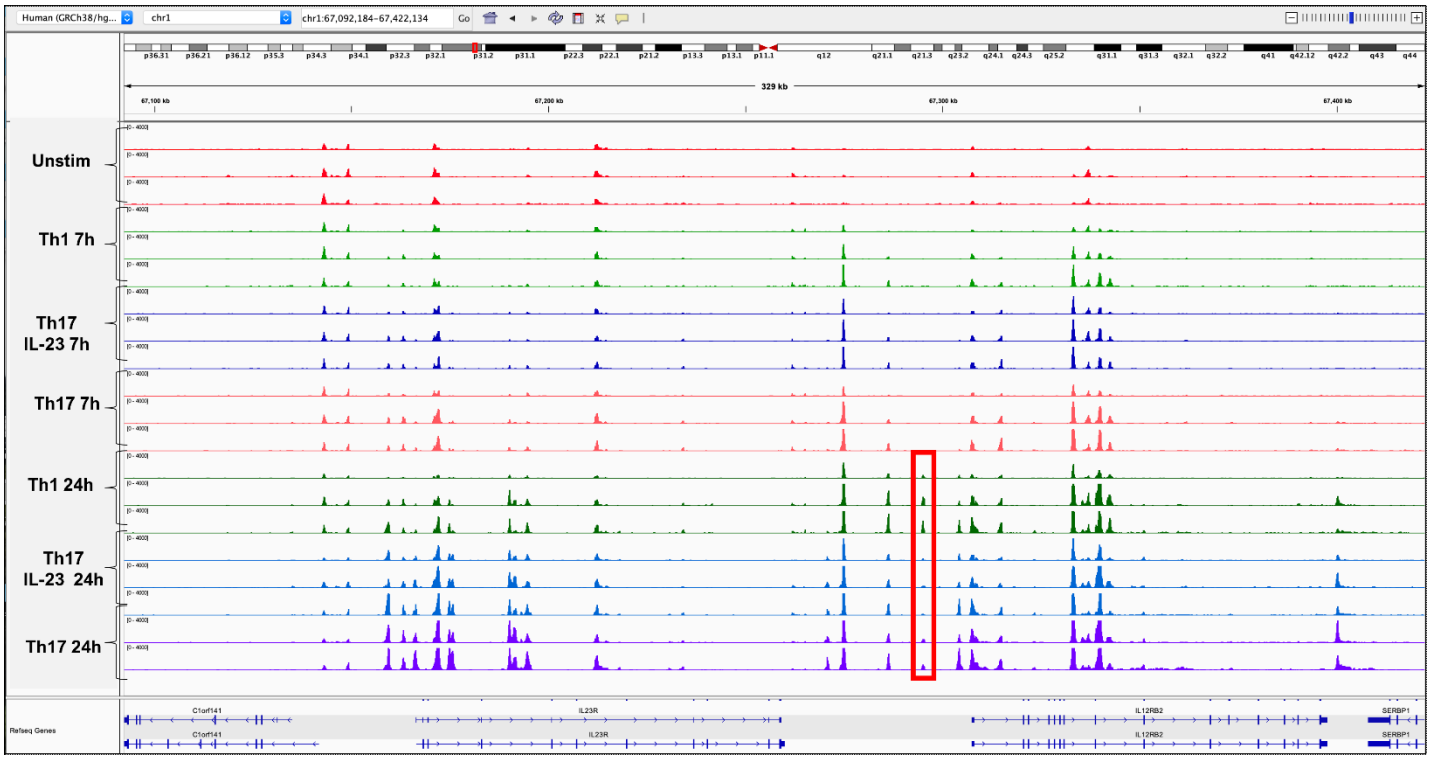
The analysis of these experiments was done in collaboration with bioinformaticians of the Institut Pasteur Bioinformatics Hub (Victoire Baillet, Claudia Chica). From the analysis of the reads it was possible to identify accessible chromatin regions (peaks). Most of the chromatin was shown to be closed, as can be seen also in the unstimulated samples. However, a pattern of increasing number and width of the peaks is seen approaching the 24h stimulation in and around the *IL23R* locus, consistent with previous expression data (*Figure 15,16 A*).

The comparison between Th17 and Th1 conditions also exhibit a great number of peaks that are shared between the two different cells subsets. An example is underlined in *Fig.17 A*, in which a peak close to the *IL12RB* locus, which is known to be expressed mainly in Th1 cells is more accessible in this Th subset.

Taken together, these characteristics allowed us to have a solid base of reproducible data to further characterize our locus of interest. In fact, from now on we will be focusing our attention on the analysis of the 13 peaks belonging to the open chromatin regions detected at the *IL23R* locus, (67,138,897 - 67,283,901) in Chr.1 (*Fig.16 B*), at 24 h of stimulation in the Th17 condition.



A



B

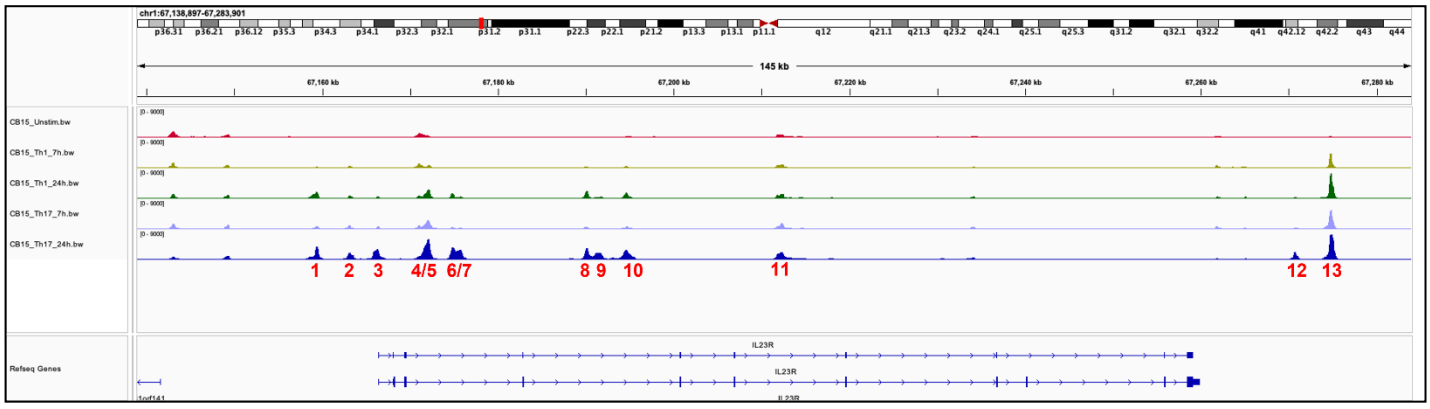


Figure 15: *Accessible regions of CD4+ T-cells at the IL23R locus (left) and IL23R levels after 7 and 24 hours of stimulation (right, measured by qPCR. Fold change set from value of Th17 7h). B. Table with summary of the number of peaks differentially accessible in each comparison. Peaks differentially accessible in Th1 versus Unstimulated are also differentially accessible in Th17 versus unstimulated.*

Figure 16: *A. Differentially accessible region in the IL23R/IL12R2beta locus. The presence of three biological replicates that show similar pattern. indicate a good reproducibility and consistency of the ATAC-seq reads. B. Display of the 13 peaks around the IL23R locus. Both figures have been made from Integrative Genomic Viewer (IGV) software © 2013-2021; peaks were shown according to these parameters: A. Human (GRCh38/hg38) Chr.1 67,092,184 – 67,422,134; B. Human (GRCh38/hg38) Chr.1 67,138,897 - 67,283,901.*

4.2.2 Footprinting analysis on the IL23R locus

To investigate the signals involved in the IL-23 pathway, we sought to map the genome wide accessible regions and at the *IL23R* locus. To do so, the footprinting analysis was chosen because it allowed us to get information from the same ATACseq experiments about the possible transcription factor pathways that are activated in the different stimulation conditions, in addition to the identification of the putative regulatory (accessible) regions. Raw data converted into .bed files were displayed into Integrative Genomic Viewer (IGV) software © (*Figure 9 A*). Moreover, the genome-wide binding score data of the factors predicted in the Th17 condition and in the unstimulated cells were displayed in a Volcano plot. (*Figure 17 C*). The plot indicates that several transcription factors show differential binding in the two stimulation conditions. The proteins that are plotted may also bind in both conditions but have a statistically significant difference in the binding that correlates with a deeper dip in the peak. In fact the Th17 cells have been differentiating respect to the unstimulated ones, and the open chromatin regions have been reflecting this change, especially at the 24 hours' time point. Moreover, the transcription factors (TFs) known to be involved in Th17 differentiation were found to have the highest differential binding score in the Th17 condition, supporting the biological significance of the footprinting identification of the predicted binding.

On the other hand, focusing on the IL23R locus, the preliminary analysis on IGV showed a distinct number of footprinted transcription factors predicted at some accessible region. By zooming into the peak, the software allowed to see the specific binding sequences of these transcription factors (*Figure 17 A, B*), that sometimes were found to be overlapping with each other. This was also due to the fact that multiple predicted factors were identified as members of the same family, or the binding sites were similar or partially overlapping, or the predicted site was not the optimal consensus. Overall, in the Th17 condition at 24 hours after stimulation, a total of 306 TFs were predicted at the *IL23R* locus.

A


Human (GRCh38/hg38)
chr1
chr1:67,070,087-67,358,359
288 kb
Unstim2r_corrected.bw
Th12r_corrected.bw
Th172r_corrected.bw
DA_LOI_Th17Un.bed
chr1:67,189,770-67,190,470
702 bp
Footprinting_3.1_qRL.bed
Refseq Genes

B


chr1:67,165,800-67,166,966
1 167 bp
67 166 000 bp
67 166 200 bp
67 166 400 bp
67 166 600 bp
Th172r_fullmg_footprints.bw
Th12r_fullmg_footprints.bw
Unstim2r_fullmg_footprints.bw
Th172r_corrected.bw
Unstim2r_corrected.bw
Th12r_corrected.bw
Sequence
Gene
DA_LOI_Th17Un.bed
DA_Th17Un_LOI5
DA_Th17Un_LOI6
DA_LOI_Th17Th1.bed
DA_Th17Th1_LOI2
DA_Th17Th1_LOI3
DA_LOI_Th17Th1Un.bed
DA_Th17Th1Un_LOI2
DA_LOI_Th1Un.bed
DA_Th1Un_LOI3
RORG_Th17_bound.bed
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RORG_Unstim_bound.bed
RORG_Unstim_unbound.bed
RORG_HUMAN.H11MO.0.C

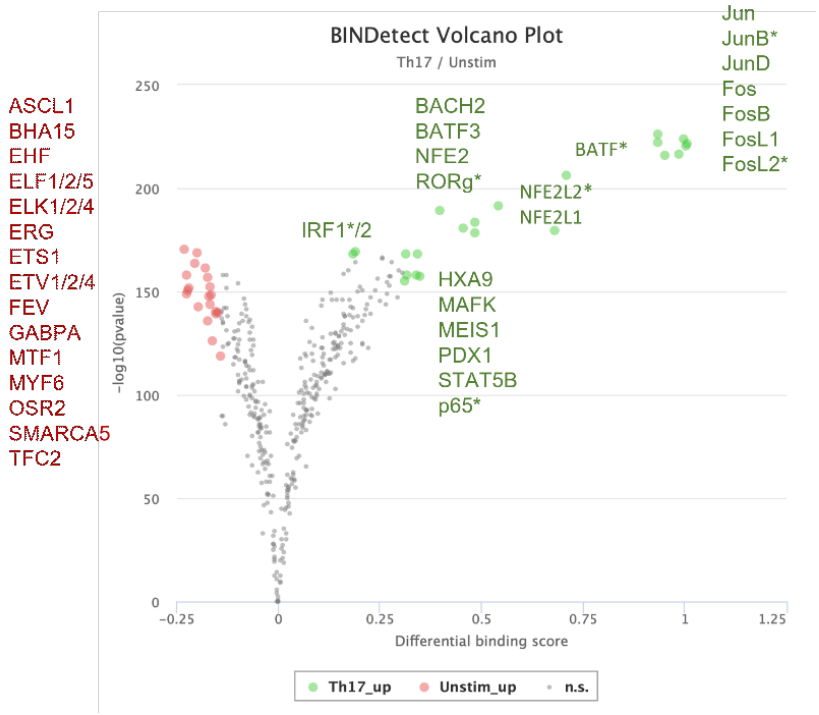

Figure 17: *A, Footprint image from IGV software © showing an example of transcription factors predicted (blue rectangles) in a peak, overlapping with each other in the case of a shared sequence within families; B, Footprint image showing a distinct shape of a peak that presents a protected sequence within an accessible region, sign that a protein was bound in that specific region of the IL23R locus, and interfered with the tagmentation during the ATACseq procedure. In the picture all the Th17 differentiating condition displayed* RORC *as a predicted bound transcription factor, while* RORC *results unbound in the unstimulated sample; C, BINDetect volcano plot of factors predicted to have differential binding in the Th17 (green dots) vs Unstimulated conditions (red dots). The comparison revealed a greater number of TFs in the Th17 condition with a higher binding score.*

4.2.3 Assessing TF gene expression and literature review

The initial footprinting analysis detected 306 Transcription Factors that were predicted to bind the *IL23R* locus. Following this discovery, our task was to prioritize these proteins for further analysis, based on whether they are expressed in the cell types of interest, and a possible coexpression with *IL23R*, as reported in the databases or in the literature. As a first step, all the factors were listed, according to their sequential position in the peaks at the *IL23R* locus. The subsequent analysis of the cell type of interest using the Human Protein Atlas and the DICE database websites showed that some predicted TFs were not expressed in T cells, and were discarded from further analysis. From the Jaspar website it was possible to determine each sequence in the *IL23R* locus where the TF was predicted to bind and check the effective overlapping of some factors. Proceeding with the last step of this analysis, we reviewed all the available data on the role of the TFs in Th17 differentiation in the existent literature, determining which ones were also the most likely to have an effect on *IL23R* expression. By exploiting the String Database, we found a network of possible pathways connections or cooperation between the predicted factors (*Figure 18*). The first factors that were selected to be further tested in this study were: JUNB, BACH1, BATF, IKZF1, RUNX3, IRF1, IRF4.

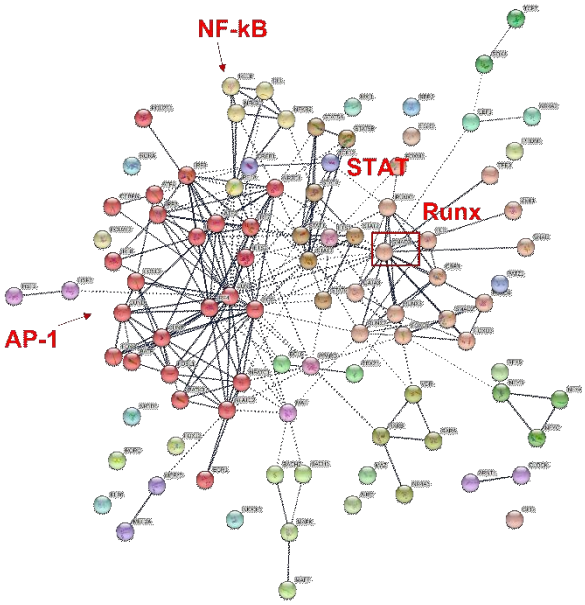

Figure 18 *String database output as network of connections between all the TFs based on: the present literature, Genomic Context Predictions, High-throughput Lab Experiments, (Conserved) Co-Expression and Previous Knowledge in Databases.*

4.3 Functional analysis of the selected Transcription Factors

4.3.1 Preliminary analysis of the role of Interferons

Given the frequent occurrences of IRF transcription factors, we analyzed *IL23R* gene expression by stimulating Naïve CD4+ T cells with Th17 cytokine cocktail in the presence of Type 1, 2 or 3 Interferons. A significant reduction in *IL23R* expression was detected comparing cells stimulated in the Th17 condition to the ones stimulated also with interferons α, β, and γ (*Figure 19*). However, no significance was shown for IFNλ. In conclusion, these data show that the interferon signaling pathway has a role in regulating *IL23R* gene expression.

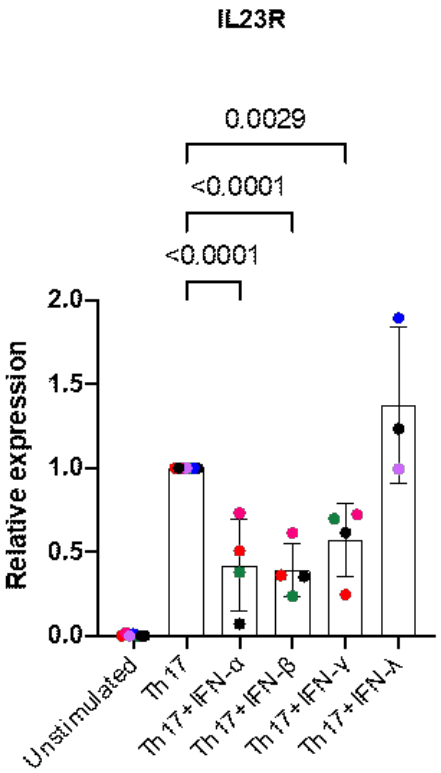

Figure 19: *IL23 relative gene expression assessed by qRT-PCR in CD4+ T cells stimulated in different conditions. Cells were stimulated in the following conditions: "Th17" = CD3/CD28 + IL-21 + TGFβ + IL-1β + IL-23 with IFN type 1, 2 and 3. Statistical significance resulted From Anova test with Bonferroni Correction. n=3-4*

4.3.2 Transcription Factors silencing by siRNA

To further characterize the role of the selected transcription factors on *IL23R* expression, naïve Cord Blood CD4+ T cells were stimulated with Th17 cytokine cocktail and incubated 72 hours with siRNA molecules. The efficacy of siRNA-mediated silencing was measured by the analysis of the expression of the targeted TF (*Figure 20*). A reduction in IL23R expression was observed for all of the TF represented in *Fig.12*, although in some cases (silencing of Runx3) biological variability between samples was elevated, requiring the analysis of additional samples. Moreover, BACH1 knock-down did not have an effect on the receptor expression, although the correspondent siRNA significantly reduced the level of the transcription factor. Overall, the majority of the selected factors can have indeed a possible function on the Th17/IL-23 axis, but further analyses need to be done to assess their mode of interaction or reciprocal influence in binding the open chromatin regions on the *IL23R* locus.

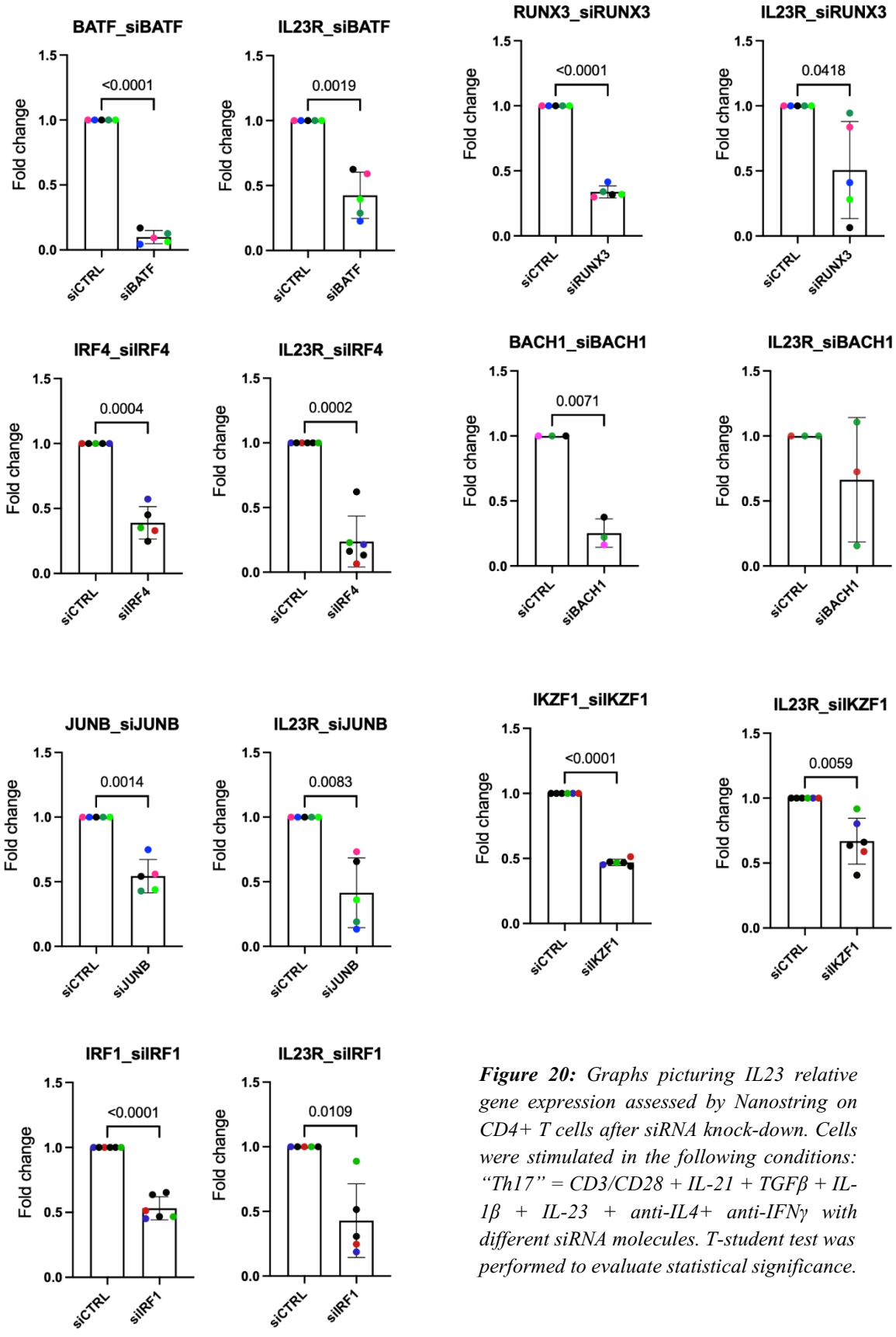

Figure 20: *Graphs picturing IL23 relative gene expression assessed by Nanostring on CD4+ T cells after siRNA knock-down. Cells were stimulated in the following conditions: "Th17" = CD3/CD28 + IL-21 + TGFβ + IL-1β + IL-23 + anti-IL4+ anti-IFNγ with different siRNA molecules. T-student test was performed to evaluate statistical significance.*

5. DISCUSSION

This study aims to improve the understanding of the "IL-23/IL-17 axis". The finding that several of the non-MHC loci genetically linked to Crohn's disease, psoriasis, and axial spondylarthritis (axSpA), are associated with genes in this pathway, has generated a new interest in the characterization of the mechanisms controlling the expression of these genes in immune cell populations that may be involved in the pathogenesis of these chronic inflammatory diseases (CID). In fact, with the discovery of Th17, a novel proinflammatory CD4+ T cell subset that secretes IL-17, it was observed that IL-23 plays a critical role in the differentiation and the functional activity of this subset, which expresses the IL-23R.[53]

The first part of this study was focused on understanding how cells become responsive to IL-23 and which are the pathways involved in IL-23R induction during effector T cell differentiation. To this end, CD4+ naïve T-cells differentiation into Th1, Th2, Th17 was mimicked in vitro by TCR stimulation (CD3/CD28) in the presence of defined cytokines known to promote differentiation of the different subsets. Gene expression analysis showed that cells differentiated in Th17 conditions upregulated expression of the subset-characterizing cytokine interleukin 17 (*IL17A*) and of the RORγT (*RORC*) transcription factor, while Th1 cells upregulated the interferon gamma gene (*IFNG*) and expression of the transcription factor Tbet (*TBX21).* On the other hand, *IL23R* was found to be upregulated by both subsets, while no *IL23R* expression was detected in the Th2 condition. In fact, the kinetic experiments conducted up to 96 hours after stimulation, suggested that there is a different molecular mechanism of induction of the receptor for Th1 and Th17. Also, *IL23R* and IL12Rβ2 have found to be adjacent, spanning 50 kb of intergenic region [54] meaning that they might be co-regulated early during the differentiation process. However, it is possible that, at later time points, when cells are more completely polarized, that the *IL23R* expression will become significantly different in Th17 vs Th1 cells. IL-23 signaling occurs through the same JAK-STAT (signal transducer and activator of transcription (STAT)

protein signaling molecules as IL-12, with IL-23R that associates with JAK2 and STAT3. This may result in different target gene expression in the Th17 versus Th1 cells, which will be object of future tests . In this context the RORγT factor, which is known for its contribution to the production of the cytokines IL-17 A/F in Th17 [55] and was shown to bind to the *IL23R* locus, seemed not to be necessary for *IL23R* expression induced by IL-12 signaling, which does not result in a significant induction of *RORC*. The immunofluorescent detection of IL-23R on the surface of cord blood-derived T cell populations stimulated in Th1 or Th17 inducing conditions confirmed the receptor expression at a protein level, validating the previous findings.

To explore in detail the pathways responsible for IL23R induction, we asked what is the role of the different cytokines that constitute the Th17-differentiating condition. For this reason, we set up a "drop out" experiment at 24h and 48h time points, analyzing the correspondent level of *IL23R* expression. The omission of IL-21 from the cytokine cocktail of induction, abolished the receptor expression at both time points, compared to TGF-beta that seemed to have an effect only at 48h. (*Fig.4*) Consistently, IL-21 was found to be sufficient to induce *IL23R* expression at 24 h (*Fig.5A*). As reported in *Habib T. et al* the common gamma chain (gamma c) is a required signaling component of the IL-21 receptor and supports IL-21-induced cell proliferation via JAK3 [56].

Likewise IL-6 signaling induces phosphorylation and activation of the STAT3 transcription factor, which has been reported to bind to the *IL23R* locus [57] and induce IL23R expression in Th17 cells in mice. So, we asked whether IL-6 could replace IL-21 to induce *IL23R*. Notably, the presence of IL-6 induced significantly lower levels of *IL23R*, respective to IL-21 stimulation, and IL-6 could not substitute for IL-21 in the induction of *IL23R* (*Fig 6 A, B*). Our data also suggest that TGFbeta may be important together with IL-21 to maintain IL-23R expression. IL-6 may not be effective, because IL23R induction requires TCR stimulation, which decreases the levels of *IL6R* expression, making cells unresponsive to this cytokine. In addition, IL-21 signaling may

induce transcription factor complexes that are different from those induced by IL-6 [58].

Moving to the second part of this study, to have a deeper understanding of the mechanism governing the IL-23/Th17 axis, we looked for any chromatin remodeling associated with *IL23R* expression by identifying open chromatin conformation in adaptive T-cells (naïve cord-blood derived CD4+ T cells).

Recent methodological developments, such as the introduction of the assay for transposase-accessible chromatin by sequencing (ATAC-seq), which requires only low cell counts, have allowed to produce reproducible chromatin accessibility profiles for a wide range of cell types [59]. This technique allowed us to observe an increase in the accessible regions in the *IL23R* locus after Th1/Th17 differentiation up to 24 hours from the stimulation, compared to the unstimulated CD4+ T cell sample, while also obtaining a good reproducibility of the results (*Fig 7, 8A*).

Although ATAC-seq is based on the sequencing and alignment of reads generated from Tn5 enzyme fragments [60], the presence of transcription factors (TFs) linked to the DNA results in the enzyme inhibition from cleaving in otherwise nucleosome-free regions. In this study, in fact, we exploited these small regions, known as footprints, where the read coverage is reduced, to predict possible factors binding to our locus of interest and their putative binding sequence (*IL23R*). Using a genome-wide approach at first, our data showed that the differentiation of the Th17 subset could be seen as an increasing number of TFs predicted with higher binding score, compared to an unstimulated sample (*Fig.9 C*).

On the other hand, from our locus-related footprint analysis, 306 factors were predicted to bind. By scanning the literature and reviewing the Human Protein Atlas, Jaspar and String databases, we were able to check their effective expression in the Th17 T-cell subset, find their binding sequence in the *IL23R* locus, generating a possible network of interaction among the factors and downsizing the initial amount predicted.

Interestingly, many Interferons regulatory factors (IRFs) were detected in our TF analysis. The IRF family is characterized by homologous proteins that

regulate interferon (IFN) transcription and IFN-induced gene expression. Their ability to bind DNA to form complexes with STAT factors [61], makes them a good candidate in a possible molecular pathway that is involved in *IL23R* expression in CD4+ T cells.

Brustle et al., in 2007 showed that IRF4 is also critical for the generation of interleukin 17–producing T helper cells (Th17 cells), which are associated with experimental autoimmune encephalomyelitis [62]. From our *IL23R* gene expression study on Th17 cells co-stimulated with type I, II and II Interferons, a reduction of *IL23R* expression in the presence of type I and II interferons was detected, while no significant changes were detected for Type III IFNs.

Conversely, the knock-down experiments by siRNA performed on IRF1 and IRF4 resulted in a decrease of the IL23R expression (*Fig. 12*). A possible explanation could derive from the fact that IFN and IRFs are not acting directly on the *IL23R* locus, but instead they could interact with other factors or engage in a different pathway that can be associated with the IL-23/Th17 axis. In fact, *Ciofani et al., (2012)* discovered that early coordinated binding of BATF and IRF4 affects chromatin accessibility and subsequent recruitment of RORγτ to regulate a subset of Th17-relevant genes during Th17 cell development [63]. Moreover, *Andrea Kröger (2017),* on the other hand, supported the critical role of IRF1 and BATF, where the latter acts as a pioneer factor and prepares the genomic landscape for the binding of additional transcription factors in the Tr1 lineage.[61]

Moreover, the knock-down of the AP-1 transcription factor JUNB had a statistically significant impact on *IL23R* expression. Several studies have shown the importance of JunB for Th17 cell pathogenicity in EAE and colitis models. [64] As reported in *Hasan et al.,(2017),* JunB, which is activated by IL-6, is required for RORγτ and IL-23 receptor expression by enhancing DNA binding of BATF, IRF4, and STAT-3 at the *RORC* gene in IL-23-dependent pathogenic Th17 cells. [64] This allows us to think that this factor has a very important cooperative role that needs to be investigated also *in vivo.*

Another interesting TF that could have an important role in Th17 differentiation and function is IKZF1 (Ikaros). Recent work has suggested that Ikaros is an

important regulator of the Th17 gene program[65]. Specifically, loss of Ikaros expression in in vitro-generated Th17 cells has been linked to decreased expression of Th17 genes such as RORγτ and IL-17. Our knock-down data, in fact, revealed a statistically significant reduction in *IL23R* expression when IKZF1 levels are reduced, suggesting a role for this factor at the *IL23R* locus. Since Ikaros also was demonstrated to negatively regulate expression of IFN-γ, a Th1 effector cytokine, further analysis will be required to address its role in this T-helper subset. Although the majority of knock-down performed seems to have statistically decreased *IL23R* expression, some silencing experiments faced limitations. This was the case of RUNX3, because of the large biological variability in *IL23R* expression in the knocked-down samples, requiring the analysis of additional replicates. To this day, the present study has aimed to clarify the stimuli and mechanisms involved into the IL-23/Th17 axis, determining what happens at an epigenetic level in Th17 cells, supporting the hypothesis that a cooperative TFs binding is required to bind the *IL23R* locus. Future additional experiments focused on investigating a direct effect of these factors on the IL23R locus, by validating their binding *in vitro* by DNA-affinity pull-down assays and *in vivo* with Chip-assays, for the factors for which antibodies active in this assay are available. In addition, a gene- editing strategy that involves the disruption of putative regulatory regions at the locus by CRISPR-Cas9 could be effective to analyze their importance in regulating *IL23R* expression

6. CONCLUSIONS

Many Chronic inflammatory diseases (CID) have been characterized by aberrant CD4+ T cell responses, and in some of these Th17 were found to have a central role. With the discovery of the Th17 CD4+ effector cell subset, which exhibited a distinct pattern of cytokine expression from that of the other Th1/Th2 subsets, a new understanding of immune regulation and immunological-mediated illness has started to develop. The cytokine IL-23 is indeed important in the expansion and functional activity of the Th17 subset, but very little is known about its biological function in human inflammatory diseases, in which the correspondent receptor IL-23R seems to have an important role. This thesis was focused on the improvement of the current knowledge about the IL-23/Th17 axis, especially on the stimuli that make cell responsive to IL-23. The implementation of ATAC-sequencing, a systematic global-scale procedure requiring a low cell number, provided key insights into the epigenetic changes driving a particular cell state without requiring a priori knowledge of the expected mechanism. In particular, ATAC-seq allowed the identification of regions at the *IL23R* locus with an open chromatin conformation in adaptive and innate T-cell populations, which may have a regulatory role for *IL23R* expression. In addition, the footprinting analysis on the locus provided a prediction of the transcription factors potentially involved in *IL23R* expression. Overall, seven TF candidates were selected to be silenced by siRNA and then tested for their activity on *IL23R* expression. The results of thiese studies supports the hypothesis that the TF binding to the locus could be cooperative, and that *IL23R* expression involves multiple transcription factors, like BATF and IRF4, which are known to be important in the development of pathological Th17 cells [66]. Hence, this study lays the foundation for future tests to verify a possible interaction or influence among the proteins in the binding of open chromatin regions on the *IL23R locus* in vitro and *in vivo*, and to assess other factors for a potential role in the IL-23/Th17 axis.

In the past years, research in the immunological field has made great advances in the understanding of the role of cytokines and transcription factors in the

biological pathways responsible for chronic inflammation. Therefore, we believe that understanding what mechanisms make cells responsive to IL-23, and what are the target gene/pathways of IL-23 signaling in these cells may help understand why anti-IL-23 therapy fails in some patients, or some CIDs. It may also help identify additional therapeutic targets.

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