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Dedicated to Uzma, Juveria and Hamza

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MOHD MOIN KHAN: Role of Cancerous Inhibitor of Protein Phosphatase 2A in the Regulation of T-cell Response
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ABSTRACT

T helper (Th) cell subsets with distinct functions are a critical component of the adaptive immune system. Th17 cells secrete interleukin 17 (IL-17) and provide host defense against pathogens. However, dysregulated Th17 response plays an essential role in the development of several autoimmune and inflammatory pathologies. Cancerous inhibitor of protein phosphatase 2A (CIP2A) modulates protein phosphatase 2A (PP2A) activity in cancer cells and neurological disorders. Accordingly, it is a promising target for therapy. However, to exploit the therapeutic potential of CIP2A, a better understanding of the physiological functions of CIP2A in immune cells is required. The doctoral thesis investigated CIP2A functions in regulating CD4+ T-cell activation and differentiation, particularly Th17 cells.

The study showed that T-cell activation induces CIP2A, and its absence hampers T-cell activation. In Th17 cells, CIP2A depletion enhances *IL17A* expression and STAT3 phosphorylation. The transcriptome analysis showed CIP2A siRNA silencing upregulates many Th17-specific genes. The STAT3 interactome indicated CIP2A controls acylglycerol kinase (AGK) interaction with STAT3 and therefore modulates STAT3 phosphorylation and *IL17A* expression in Th17 cells. Furthermore, we performed the CIP2A protein interactome in Th17 cells. In addition to the CIP2A known interactor PP2A, we identified many novel protein interactions of CIP2A. For the first time, we identified CIP2A interaction with protein phosphatase PP1. Moreover, the study suggested the role of CIP2A in many pathways. Hence, this thesis provides an insight into CIP2A's functions in immune cell regulation.

KEYWORDS: Th17 cells, CIP2A, RNAi, RNA Seq., STAT3, interactome, mass spectrometry

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TIIVISTELMÄ

T-auttajasolujen (Th-solujen) alatyypit ovat keskeisiä adaptiivisen immuunijärjestelmän toiminnalle. Interleukiini-17-proteiinia (IL-17) tuottavat tyypin 17 auttaja T-lymfosyytit (Th17-solut) suojaavat elimistöä patogeeneiltä. Th17-solujen toiminnan häiriöt vaikuttavat useiden autoimmuunisairauksien ja tulehduksellisten tautien kehittymiseen. CIP2A-proteiini säätelee PP2A-molekyylin aktiivisuutta syöpäsoluissa ja on potentiaalinen syöpäterapian kohde. CIP2A-proteiinin terapeuttisen potentiaalin hyödyntäminen edellyttää CIP2A:n fysiologisten funktioiden parempaa ymmärtämistä immuunipuolustuksen soluissa. Tässä väitöskirjassa tutkittiin miten CIP2A säätelee CD4+ T-solujen aktivaatiota ja erilaistumista, keskittyen erityisesti Th17-soluihin.

Osoitimme, että CIP2A:n indusoituu T-soluissa aktivaation seurauksena ja sen puuttuminen johtaa heikentyneeseen T-solujen aktivaatioon ja lisääntyneeseen Th17-solujen erilaistumiseen. CIP2A-hiljennetyissä Th17-soluissa *IL17A*-geenin ja monen muun Th17-soluille spesifisen geenin ilmeneminen oli lisääntynyt ja Th17-solujen erilaistumista ohjaava STAT3:n fosforylaatio oli pitkittynyt. Fosforyloituneen STAT3:n interaktomia tutkimalla paljastui, että CIP2A-hiljennetyissä soluissa acylglycerol-kinaasin ja STAT3:n vuorovaikutus säätelee STAT3:n fosforylaatiota ja *IL17A*:n ilmenemistä Th17-soluissa. Identifioimme useita uusia CIP2A-proteiinin kanssa vuorovaikuttavia tekijöitä karakterisoimalla CIP2A-interaktomin. Yksi näistä on PP1-proteiinifosfataasi. Väitöskirja valottaa CIP2A:n merkitystä immuunisolujen säätelyssä.

AVAINSANAT: Th17-solut, CIP2A, RNAi, RNA Seq., STAT3, massaspektometria

Table of Contents

Abbreviations

AGK	acylglycerol kinase
AP1	activator protein 1
APC	antigen presenting cell
BATF	basic leucine zipper ATF-like transcription factor
BCL6	B-cell lymphoma 6
CBP	CRE-binding protein
cDNA	complementary DNA
CFSE	carboxyfluorescein succinimidyl ester
ChIP	chromatin immunoprecipitation
ChIP-seq	chromatin immunoprecipitation (ChIP) with DNA sequencing
CIP2A	Cancerous inhibitor of protein phosphatase 2A
CRE	cAMP response element
CREB	cAMP response element–binding protein
CREM	cAMP response element modulator
CRTH2	chemoattractant receptor homologous molecule on Th2 cells
CSF2	colony stimulating factor 2
DC	Dendritic cell
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EGFR	epidermal growth factor receptor tyrosine kinase
FDR	false discovery rate
FOXO1	forkhead box O1
FOXP3	forkhead box P3
GATA3	GATA binding protein 3
GSEA	gene set enrichment analysis
HIF1α	hypoxia-inducible factor 1α
IBD	inflammatory bowel disease
ICOS	inducible T-cell co-stimulator
IL	interleukin
ILC	innate lymphoid cell
IPA	ingenuity pathways analysis

IRF	interferon regulatory factor
IRF4	interferon-regulatory factor 4
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LCMT1	leucine carboxyl methyl transferase 1
miRNA	micro RNA
mRNA	messenger RNA
MS	multiple sclerosis
mTOR	mammalian target of rapamycin
NFAT	nuclear factor of activated T cells
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PLA	proximity ligation assay
PME-1	protein methyl-esterase 1
PRRs	pattern recognition receptors
PTM	post-translational modifications
PTPA	phosphotyrosyl phosphatase activator
RA	rheumatoid arthritis
RBPJ	recombination signal binding protein for immunoglobulin κJ
RNA	ribonucleic acid
RNAi	RNA interference
RNS	reactive nitrogen species
RORC	RAR-related orphan receptor C
ROS	reactive oxygen species
RPTOR	regulatory associated protein of MTOR complex 1
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
SAM	s-adenosylmethionine
siRNA	small interfering RNA
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
SRM-MS	selected reaction monitoring targeted mass spectrometry
TCR	T cell receptor
TF	transcription factor
TFBS	transcription factor binding sites
Tfh	T follicular helper cell
TFs	transcription factors
TGF-β	transforming growth factor beta
Th	T helper cell
TLR	toll-like receptor

TNBC	triple-negative breast cancer
TNF-α	tumor necrosis factor alpha
Treg	T regulatory cell
TSS	transcription starting site
β-ME	beta mercaptoethanol

List of Original Publications

This thesis is based on the following publications referred to in the text by the Roman numerals I-III.

I Côme C., Cvrljevic A., **Khan M.M.**, Treise I., Adler T., Aguilar P.J.A., Au Y.B., Sittig E., Laajala T.D., Chen Y., Oeder S., Calzada W.J., Horsch M., Aittokallio T., Busch D.H., Ollert M.W., Neff F., Beckers J., Gailus D.V., Fuchs H., Hrabě A.M., Chen Z., Lahesmaa R., and Westermarck J. "CIP2A promotes T-cell activation and immune response to Listeria monocytogenes infection". PLoS One. 2016. Apr 21;11(4): e0152996.

II **Khan M.M.**, Ullah U., Khan M.H., Kong L., Moulder R., Välikangas T., Bhosale S.D., Komsi E., Rasool O., Chen Z., Elo L.L., Westermarck J., and Lahesmaa R. "CIP2A constrains Th17 differentiation by modulating STAT3 signaling" iScience, 2020. Mar 27;23(3):100947

III **Khan M.M.***., Välikangas T*., Khan M.H*., Moulder R., Ullah U., Bhosale S.D., Komsi E., Butt U., Westermarck J., Elo L.L., and Lahesmaa R. "Protein interactome of the cancerous inhibitor of protein phosphatase 2A (CIP2A) in Th17 cells" Current Research in Immunology, 2020. Dec vol 1(10-22) *shared author

1 Introduction

The human immune system protects against pathogens and transformed cancer cells through the coordinated action of immune cells and their ability to distinguish self from non-self (Owen et al. 2018). However, functional impairment of these cells can lead to autoimmune and inflammatory disorders, and therefore, a detailed molecular understanding of immune cells is of prime importance. The immune system is broadly classified into the innate and adaptive immune systems (Owen et al. 2018). The innate part detects the structures on foreign pathogens or transformed host cells and mounts quick destruction of the pathogen via the complement cascade, phagocytosis and more. However, the innate immune response is non-specific and transitional, but importantly, it also activates the adaptive immune response. The adaptive immune response is due to highly specialized immune cells. After the initial encounter with the specific pathogen, cells of the adaptive immune system create immunological memory for long-lasting and enhanced subsequent response to pathogens (Murphy et al. 2017; Owen et al. 2018).

The antibody-secreting B lymphocytes and T lymphocytes, subdivided into CD4+ T helper (Th) cells and CD8+ cytotoxic killer T (Tc) cells, provide an adaptive immune response to foreign pathogens or self-altered cells (Murphy et al. 2017; Owen et al. 2018). Depending upon the nature of the antigen, activation, and the cytokine, the CD4+ Th precursor (Thp) cells differentiate into effector Th1, Th2, Th17, Th9, Th22, and regulatory T (Treg) cells (Basu et al. 2021; Zhu 2018). Each subset of Th cells is characterized by a specific transcription factor (TF) and signature cytokine (Basu et al. 2021; Zhu 2018). Interleukin 17 (IL-17)-producing Th17 cells provide protection against pathogens of bacterial and fungal origin, but they are also critical drivers of pathogenesis in various autoimmune and inflammatory diseases (Stockinger et al. 2017; Stassen et al. 2012). Accordingly, an understanding of the dichotomy of Th17 cells could help in the development of the strategies to mitigate excessive Th17 responses in inflammatory diseases (Stockinger et al. 2017).

Protein phosphorylation affects almost every aspect of cell biology, and therefore, regulation of the activities of kinases and phosphatases is essential for normal cell function (Ardito et al. 2017). Protein phosphatase 2A (PP2A) is one of

the major serine-threonine phosphatases and controls various cell signaling pathways (O'Connor et al. 2018; Kauko et al. 2018; Ohlmeyer et al. 2019). PP2A impairment due to inhibitory mechanisms has been observed in several diseases (Grech et al. 2016; Baldacchino et al. 2014; Velmurugan et al. 2018; Xing 2016; Janssens 2019; Shentu et al. 2018). The inhibition of PP2A in human cancer and neurodegenerative disorders results from the actions of cancerous inhibitor of protein phosphatase 2A (CIP2A) (Junttila et al. 2007; Shentu et al. 2018). Accordingly, CIP2A has been considered as a target for therapeutic development (Khanna et al. 2013). However, the impact of its deletion on immune cells must also be considered before any therapy development.

In the work presented in this thesis, the physiological functions of CIP2A in T-cell activation (Publication **I**) and differentiation (Publication **II**) were investigated. These data revealed that CIP2A is important for T-cell activation but negatively regulates IL-17 expression and Th17 cells differentiation. In addition, the CIP2A protein interactome was characterized for the first time in Th17 cells (Publication **III**). Taken together, the findings presented in this thesis provide insight into the role of CIP2A in immune cells.

2 Review of the Literature

2.1 Overview of the immune system

An interactive network of lymphoid organs, cells, humoral factors, and cytokines, called the immune system, is essential for host defense in eradicating pathogens or altered cells (Marshall et al. 2018; McComb et al. 2019). The immune system protects from a multitude of pathogen intrusions and eliminates host-altered cancer-causing cells. The most remarkable features of the immune system are its recognition and response. Recognition helps to discriminate host cells from foreign infection-causing pathogens and self-altered cancer or apoptotic cells (Marshall et al. 2018; McComb et al. 2019; Owen et al. 2018). The immune response triggers effector cells to eliminate pathogen and tumor-promoting host cells. In addition, immune cells develop memory cells for subsequent quick immune response to detect and remove re-infections (Marshall et al. 2018; McComb et al. 2019; Owen et al. 2018). Hematopoietic stem cells (HSCs) are adult stem cells that can differentiate into all types of blood cells, including immune cells, by a process called hematopoiesis (Owen et al. 2018). The bone marrow is the primary lymphoid organ, where all the HSCs reside and give rise to immune cells (**Figure 1**). Most of the immune cells differentiate and mature in the bone marrow before traveling to peripheral organs via blood. However, T cells mature in the thymus (Owen et al. 2018).

The immune system is broadly divided into the innate immune system and adaptive immune system (McComb et al. 2019; Owen et al. 2018). The innate immune system is the immediate first line of defense, highly conserved in nature, and eliminates infection directly by phagocytosis and the complement cascade or indirectly by activating the adaptive immune system (McComb et al. 2019; Owen et al. 2018).

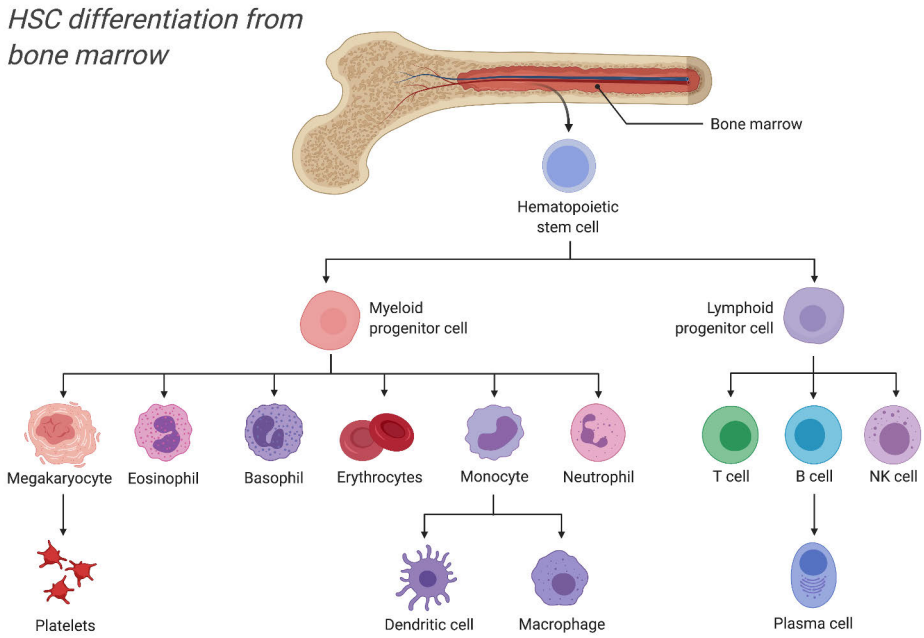

Figure 1: Self-renewing HSCs reside in the bone marrow and give rise to lymphoid and myeloid progenitor cells for the development of blood cells by a process called hematopoiesis (Owen et al. 2018). BioRender.com Illustration.

The adaptive immune response is a hallmark of higher animals. It is antigen-specific, and provides long term immunological memory for response upon re-exposure to the pathogen (McComb et al. 2019; Owen et al. 2018; Murphy et al. 2017). Immunological memory is the ability of the immune cells to recognize previously encountered antigens and build a faster immune response to eliminate the same pathogen reinfection.

2.1.1 Innate immune system

Innate immune system is an essential first line of defense against disease-causing pathogens in vertebrates (Marshall et al. 2018). It comprises physical, chemical, and cellular components. The anatomical or physical barriers of the innate immune system (e.g., skin and mucous membranes) provide external protection against microbial invasion (Marshall et al. 2018; Owen et al. 2018). These also produce active biochemical substances for defense. For example, human skin produces the anti-microbial protein ‘psoriasin’, which has potent antimicrobial activity against *Escherichia coli* to prevent its colonization on human skin (Otto 2010; G. Wang 2014; Marshall et al. 2018; Owen et al. 2018). The antimicrobial biochemical agents

produced by the skin are important to provide protection. However, breaks in the skin from scratches, wounds or other disruption of anatomical barriers, such as insect (e.g., mosquitoes, mites, ticks) bites, can introduce pathogenic organisms into the body (Owen et al. 2018). Innate immune cells, directly eliminate pathogens or apoptotic cells and also activate adaptive immune cells by a process of phagocytosis. Cells of the innate immune response express germline-encoded receptors called pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), to detect pathogen or altered host cells (Marshall et al. 2018; Owen et al. 2018). TLRs are transmembrane receptors on cells of the innate immune system (macrophages and dendritic cells) to sense conserved molecules on pathogens (Delneste et al. 2007). Upon activation, TLRs recruit adaptor proteins to orchestrate inflammatory responses and propagate antigen-induced signal transduction pathway by upregulation or suppression of genes and proteins (Murphy et al. 2017; Owen et al. 2018). PRRs identify two classes of molecules that are pathogen-associated molecular patterns (PAMPs) on pathogens and damage-associated molecular patterns (DAMPs) to detect altered host cells (Murphy et al. 2017; Owen et al. 2018). PAMPS recognize structurally conserved specific molecules on microbes, such as carbohydrates (e.g., lipopolysaccharide, mannose), bacterial or viral nucleic acids (DNA or RNA), peptidoglycans and lipoteichoic acids, and fungal glucans. TLR2s detect peptidoglycans, and TLR4s sense the polysaccharides on the cell walls of Gram-positive bacteria (Sabroe et al. 2003). Innate immune cells recognize PAMPS on pathogens, but antibody or complement protein attachments to the pathogens improve subsequent phagocytosis (Takeda and Akira 2015; Medzhitov 2001; Janeway and Medzhitov 2002; Pasare and Medzhitov 2005; 2004).

Neutrophils, macrophages, monocytes, natural killer cells and dendritic cells (DCs) are the key cells involved in innate immune reactions. They protect the host from pathogens by antimicrobial peptides, inflammation mediators and phagocytosis (Nathan 2006). Neutrophils are the first cells that migrate towards the site of infection and are essential for innate responses against bacteria and fungi (Nathan 2006; Yoo et al. 2011; Owen et al. 2018; Marshall et al. 2018). Macrophages are subclassified as M1 and M2, promoting inflammation or suppressing inflammation and assisting tissue repair, respectively (Mills et al. 2012; Marshall et al. 2018). DCs and macrophages are also antigen-presenting cells (APC). They present antigens by specific glycoproteins on their cell membrane known as major histocompatibility complex (MHC) molecules, of which there are two types, MHC-I and MHC-II, that interact with Tc cells and Th cells, respectively. Immature APCs, recognize pathogens by a variety of PRRs, then undergo a maturation process (Schenten and Medzhitov 2011; Iwasaki and Medzhitov 2010; Dowling and Mansell 2016; Asami and Shimizu 2021; Janeway and Medzhitov 2002). Mature APCs then migrate to the

lymphoid tissue to activate Th and Tc cells and initiate an adaptive immune response (Charles A Janeway et al. 2001).

Additionally, there are oxidative and nonoxidative pathways of defense in neutrophils, macrophages and DCs against pathogens (Owen et al. 2018; Murphy et al. 2017). They employ reactive oxygen species (ROS) and reactive nitrogen species (Fang 2004; Nathan and Ding 2010; Nathan and Cunningham-Bussel 2013). The enzyme NADPH oxidase (also called phagosome oxidase) generates ROS. Mechanistically, during phagocytosis, oxygen consumption of the cell increases several folds via a process called a respiratory burst, and the NADPH oxidase enzyme complex convert oxygen to superoxide (Owen et al. 2018). Also, during phagocytosis, the inducible nitric oxide synthase (iNOS) enzyme oxidizes L-arginine to make L-citrulline and nitric oxide (NO) (Fang 2004; Nathan and Cunningham-Bussel 2013; Nathan and Ding 2010; Owen et al. 2018). NO has strong antimicrobial activity and combines with superoxide to produce more potent antimicrobial nitrogen and oxygen reactive species (Owen et al. 2018). Accordingly, patients with the chronic granulomatous disease due to defective NADPH oxidase enzyme have increased susceptibility to fungal and bacterial infections, thus illustrating the significance of this immune component for the host (Heyworth, Cross, and Curnutte 2003; Owen et al. 2018).

Natural killer (NK) cells constitute 5-10% of lymphocytes in human peripheral blood. The NK cells do not depend on recombination-activating gene proteins and genomic recombination-based receptors but as in innate immune cells, antigenic diversity is due to germline-encoded receptors (O'Leary et al. 2006; Paust, Senman, and Von Andrian 2010; Owen et al. 2018; Murphy et al. 2017). They do not have antigen specific receptors and are characterized based on the NK 1.1 surface marker and the presence of cytotoxic granules (also called large granular lymphocytes). They provide defense against viral infections and transformed tumor cells. The downregulation of MHC class I on virus infected and tumor cells are detected by NK cells (Murphy et al. 2017; Owen et al. 2018). NK cells act through two types of receptors: activating (lectin like) receptors to initiate killing of virus infected or transformed cells, and inhibitory (killer cell immunoglobin-like receptors or KIRs) receptors that abort the NK cell killer function (Owen et al. 2018; Marshall et al. 2018). These receptors determine the cytotoxic activity of NK cells. A deficiency of activating events results in tumor development or viral infection, and a defective inhibitory receptor promotes NK-mediated killing of the host cell. The ligands on the target cell are still not known for most of the activating receptors, but the ligands for the inhibitory NK cell KIR receptor are well studied as the allelic forms of class I MHC molecules (Owen et al. 2018). Thus, NK cells remove cells without MHC-I expression. Also, since both KIR and MHC-I are variable and diverse in nature, their combination sometimes fails and causes enhanced NK cell mediated killing, such as

those associated with autoimmune disease conditions in type 1 diabetes and psoriatic arthritis (Owen et al. 2018). However, NK cells also express receptors for immunoglobulins and can detect pathogens or their proteins on infected cells independent of MHC-I expression. Once these targets are detected, NK cells release granules to induce cell death. They produce immunoregulatory cytokines such as IFN-γ and tumor necrosis factor alpha (TNF-α), which can stimulate the maturation of the DCs (Owen et al. 2018; Murphy et al. 2017). The IFN-γ produced by NK cells also activates macrophages for their phagocytic and microbicidal activities and is an important regulator of Th1 vs Th2 commitment of Th cell populations. IFN-γ stimulates Th1 development as it induces IL12 production by DCs (Owen et al. 2018; Murphy et al. 2017). Some lymphoid cells share characteristics of both T lymphocytes and NK cells called NKT Cells. Like T cells, they have a T-cell receptor (TCR), which recognizes specific lipids presented by the MHC-related molecule CD1. They also have NK cell receptors and cytotoxic granules for target cell killing (Owen et al. 2018).

2.1.2 Adaptive immune system

The adaptive immune system is also referred to as the acquired immune system. It consists of specialized cells and supports the innate immune cells in removing the invading pathogens. The adaptive immune system is characterized by immunological memory, which develops as a result of the primary encounter with a pathogen (Owen et al. 2018; Marshall et al. 2018; McComb et al. 2019). Importantly, this leads to an enhanced response to future encounters with the pathogen. Adaptive immune responses are carried out by B and T lymphocytes, which are much more diverse than innate immune cells in their ability to recognize the pathogens. Genomic recombination hones their antigen receptor.

In mammals, B cells are produced and mature in the bone marrow. The mature cells synthesize and display a membrane-bound immunoglobulin (antibody) molecule as B-cell receptor (BCR) for antigen binding (Owen et al. 2018). Through the process of somatic hypermutation, rearrangements of the BCR immunoglobulin heavy and light chains genes improve pathogen detection and binding. In addition, class switching in B cells generates many classes of functional antibodies to inactivate and degrade pathogens and antigens. Once activated (by antigen encounter), B cells form either plasma cells, for continuous antibody secretion, or memory cells, for rapid response to future infections (Palm and Henry 2019).

2.2 T helper (Th) cells

T cells are subdivided into CD4+ Th and CD8+ Tc cells. T lymphocytes only detect antigens that are loaded onto the MHCs on APCs, such as DCs (Basu et al. 2021; Marshall et al. 2018). Pathogens are broken down to peptides and loaded to MHC I and MHC II molecules for TCR ligation, resulting in the activation of CD8+T cells and CD4+T cells, respectively (Basu et al. 2021). In this way, T cells detect both intracellular and extracellular pathogens. Similar to BCR, the TCR is developed by random gene recombination of α and β chain led to the formation of αβTCR repertoire (Owen et al. 2018; Marshall et al. 2018). In a minority of T cells, rearrangements in γ and δ chains resulted in the development of γδT cells (Owen et al. 2018; Marshall et al. 2018).

Upon TCR activation, naive CD4+ T cells differentiate and expand into various subsets of Th cells. Many factors play significant roles for T-cell activation and Th cell lineage commitment (**Figure 2**). Depending upon the type of APC, the strength of the antigen recognition induced signaling, presence of cytokines, and co-stimulatory signals, Th cells develop into one of the various subsets and promote the eradication of the disease-causing microbe or other intruding factors (Bevington et al. 2017). The nature of the antigen also influences the activation and differentiation of the various Th cell subsets (Bevington et al. 2017; Davis et al. 2003; Pennock et al. 2013). In addition, the activation of T cells by signaling events is also essential for cell proliferation, survival, cytokine production, and differentiation. TCR stimulation upon detection of antigen on MHC, and signaling from costimulatory molecule CD28, as well as cytokines, are required for T-cell activation (Bevington et al. 2017; Marshall et al. 2018; Basu et al. 2021; Riley et al. 2002; Diehn et al. 2002). TCR signaling starts with the cytosolic component of immunoreceptor tyrosine-based activation motifs (ITAMs) phosphorylation by protein tyrosine kinases Lck and Fyn, followed by recruitment of zeta-chain-associated protein kinase (Zap-70) at ITAMs (Wang et al. 2010). The Zap-70 kinase phosphorylates SH2-domain-containing leukocyte phosphoprotein of 76 (SLP-76) (Liu et al. 2010; Wardenburg et al. 1996) and linker for activation of T cells (LAT) to initiate downstream signaling (Wang et al. 2010; W. Zhang et al. 1998). T-cell activation of involves the essential phosphorylation of several key proteins. Phosphorylation of phospholipase Cγ1 (PLCγ1) by inducible T-cell kinase (Itk) results in the generation of secondary messengers' diacylglycerol (DAG) and inositol trisphosphate (IP3). The DAG activation of serine/threonine-protein kinase C (PKCθ) promotes phosphorylation of CARMA1 (Eitelhuber et al. 2011). The activation of adaptor protein CARMA1 promotes the recruitment of BCL10 and MALT1 and initiates signaling of TF nuclear factor-κB (NF-κB) (Eitelhuber et al. 2011). The binding of IP3 to calcium channels on the endoplasmic reticulum initiates the Ca2+ release. The Ca2+ forms a complex with the calcium-binding protein calmodulin and activates

the serine/threonine phosphatase calcineurin. The latter activates TF NFAT (nuclear factor of activated T cells) to induce Il2 for T-cell survival and proliferation (Choi et al. 2018). In addition, TCR–CD3 and CD28 co-stimulation results in the induction of several TFs essential for T-cell activation and differentiation. The co-stimulatory molecule CD28 signaling promotes recruitment of phosphatidylinositol 3-kinase (PI3K), which plays a role in T-cell proliferation (Choi et al. 2018). Signaling due to cytokine receptor binding is required for chromatin remodeling and TF activation, both of which are essential for the transcription of the genes needed to mediate T-cell differentiation and effector functions (Bevington et al. 2017).

Signal transducer and activator of transcription (STAT) proteins play an essential role in transmitting signals from the cytokine receptor and regulating gene expression during the differentiation of the different Th cell subsets (O'Shea et al. 2011; Seif et al. 2017). There are seven STAT proteins (i.e., STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6), and their impact on the differentiation of Th cell subsets (**Figure 2**) has been firmly established from next-generation sequencing analyses such as chromatin immunoprecipitation followed by sequencing (ChIP-Seq) and RNA-Sequencing (RNA-Seq) etc. of gene knock out animals (O'Shea et al. 2011; Seif et al. 2017).

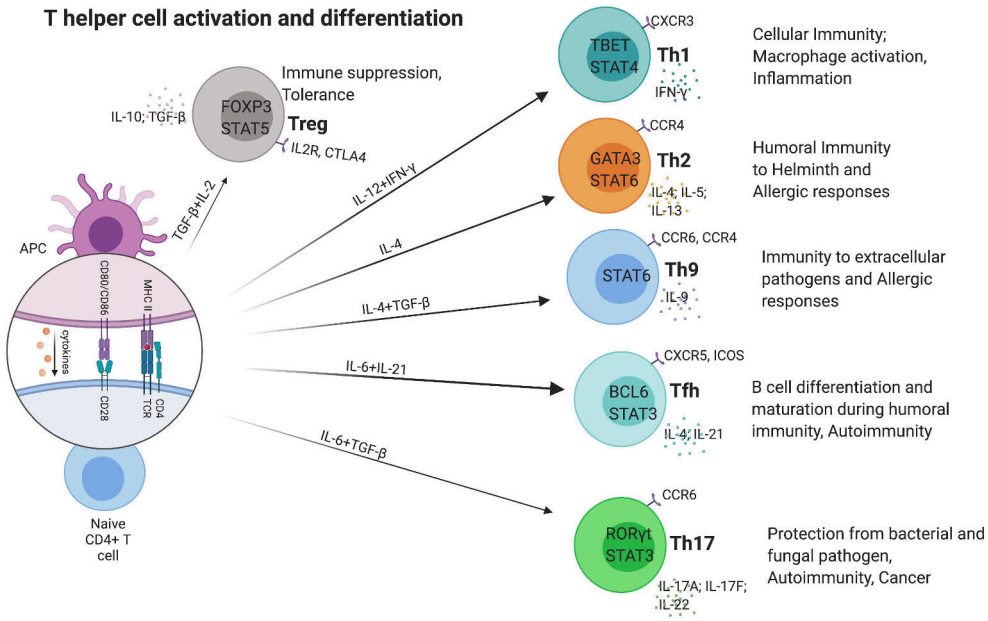

Figure 2: Upon activation by an APC, naive CD4+ T cells differentiate to different Th subsets, characterized by lineage-specific transcription factors, cytokine secretion, and the immune response. Illustration created with BioRender.com

2.2.1 Th1, Th2, and Th9 cells

The role of adaptive immune cells in the pathogenesis of autoimmune diseases was first proposed in the work of Bob Coffman and Tim Mossman (Mosmann et al. 1986; Cherwinski et al. 1987). Three decades ago, they suggested the existence of two subsets of immune cells, Th1 and Th2 cells, based on the cytokine's profiles (Mosmann et al. 1986). They classified CD4+ T cells into IFN-γ producing Th1 and IL4 producing Th2 cells to explain the cellular and humoral effector immune response, respectively (Mosmann et al. 1986; Cherwinski et al. 1987).

The Th type 1 (Th1) cells secrete IFNγ and are characterized by the expression of *Tbet* (*TBX21* in humans) as the lineage-specific TF (Iwata et al. 2017). Th1 cells provide cell-mediated immune responses by activating macrophages, cytotoxic T lymphocytes and producing NO for the phagocytosis of cells infected with intracellular viral and bacterial pathogens (Suzuki et al. 1988; Sallusto 2016). Patients with defective Th1 responses are especially susceptible to *Salmonella* and mycobacterial infections (Sallusto 2016; Santos et al. 2006; Jong et al. 1998; Altare et al. 1998). The surface expression of C-X-C chemokine receptor CXCR3 is specific to Th1 cells (Yamamoto et al. 2000; Sallusto et al. 1998; Sallusto 2016; Langenkamp et al. 2003) and helps Th1 cell recruitment to inflammation sites by recognizing the chemokine ligands CXCL9, CXCL10, and CXCL11(Groom et al. 2012; Groom and Luster 2011). In addition to the clearance of intracellular pathogens, Th1 cells have also been implicated in the development of many autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, type 1 diabetes, and inflammatory bowel disease (Dardalhon et al. 2008; Germann et al. 1996; James et al. 2014; Kamali et al. 2019; Sallusto 2016).

Th2 cells secrete the cytokines IL-4, IL-5, and IL-13 (Cherwinski et al. 1987; Lewis et al. 1988; Mosmann et al. 1986; Wierenga et al. 1990; 1991). Th2 cells provide host protection against helminths (Katona et al. 1988; Mohrs et al. 2003; Vignali et al. 1989; Basu et al. 2021; Sallusto 2016). Dysregulated Th2 responses, however, are responsible for the pathogenesis of allergic reactions, such as asthma (Kotsimbos et al. 1997; Georas et al. 2005; Fallon et al. 2007; Bosnjak et al. 2011). The cytokine IL-4 controls the priming and clonal expansion of Th2 cells and induces class switching of immunoglobulin (Ig) to IgE in B cells (Nelms et al. 1999; A. Basu et al. 2021; Marshall et al. 2018). The IgE cross-linking on mast cells or cells with an Fcε receptor results in the degranulation of inflammatory mediators for parasite removal (Gould et al. 2003). The C-C chemokine receptor (CCR) 4 (Bonecchi et al. 1998; Langenkamp et al. 2003; Sallusto et al. 1998) and chemoattractant G-protein-coupled receptor for chemoattractant receptor homologous molecule expressed on Th2 are both expressed on the surface of Th2 cells, with the latter used as a marker for human Th2 cells (Fanis et al. 2007; Georas et al. 2005; Nagata et al. 1999). IL-4-induced activation of STAT6 and GATA3

expression results in downstream activation of target genes necessary for transcriptional regulation of Th2 cell differentiation (Zheng and Flavell 1997; Takeda et al. 1996; Swain et al. 1990; Shimoda et al. 1996; Ouyang et al. 1998; Gros et al. 1990; Kaplan et al. 1996; A. Basu et al. 2021).

T helper 9 (Th9) cells are named according to their secretion of IL-9 as a signature cytokine. Similarly to Th2, Th9 cells are responsible for the elimination of extracellular pathogens, such as the helminth, *Trichuris muris* (Veldhoen et al. 2008; Darlan, Rozi, and Yulfi 2021; Bouchery et al. 2014). Dysregulation of Th9 cells is responsible for pathogenic allergic reactions, such as asthma and atopy (Wierenga et al. 1991; Wenzel 2012; Temann et al. 1998; Staudt et al. 2010; Stassen et al. 2012; Robinson et al. 1992; Bouchery et al. 2014; Hirahara et al. 2016). However, there has been some controversy concerning the existence of Th9 cells. In a study to identify which cells secrete cytokine IL-9, using the IL9 fate reporter mouse strain during papain-induced lung inflammation indicated the in vivo production of IL-9 is mainly from the innate lymphoid cells (ILC). Only a minor contribution of CD4+T cells was found during fate mapping of cellular sources of IL-9 (Wilhelm et al. 2011), questioning the existence of Th9 cells (Wilhelm et al. 2012). But the bias for non-Th9-mediated inflammation in the experimental setting could be the reason for ILC-specific IL9 expression (Wilhelm et al. 2011; 2012).

2.2.2 Tfh cells

T follicular helper (Tfh) cells are a recently characterized subset of CD4+ T cells mainly localized in secondary lymphoid tissues, such as the lymph node, spleen, and payer patches, and are responsible for T-cell-dependent humoral immune responses (Hirahara et al. 2016; Basu et al. 2021). B-cell CCL/lymphoma 6 (BCL6) is the lineage-determining TF for Tfh cells. Tfh cells are required for B-cell differentiation into plasma cells for high-affinity antibody production against pathogens and immunological memory B cells for quick immune response against encountered antigens (Kräutler et al. 2017).

Tfh cell differentiation starts upon CD4+ T-cell interaction with myeloid APCs, such as DCs in lymphoid tissues. The Tfh cell's primary function is to help the B cells. Thus, they predominately localized in lymphoid tissue, whereas non-Tfh cells, such as Th1, Th2, and Th17 cells, leave the lymphoid tissues and traffic to the sites of infection and inflammation. In mice, differentiation of Tfh cells is programmed by IL-6 and costimulatory molecule inducible T-cell co-stimulator (Vinuesa et al. 2016; Linterman et al. 2009; Crotty 2014). IL2-R signaling and IL-2 are strong inhibitors of Tfh differentiation, through induction of BLIMP-1 and STAT5 (Johnston et al. 2012; Ditoro et al. 2018; Ballesteros-Tato et al. 2012). DC priming is essential but not sufficient for Tfh differentiation (Crotty 2014). Complete Tfh

differentiation is multistage and multifactorial and requires APCs DC and B cells (Crotty 2014). Tfh differentiation requires DC priming, but B-cell interaction is indispensable for Tfh-GC maturation. DC priming of CD4+ T cells results in the upregulation of BCL6, chemokine receptor CXCR5, and repression of CCR7 to allow Tfh migration and interaction with B cells. Therefore, BCL6 and BLIMP1 are antagonists in Tfh differentiation (Vinuesa et al. 2016; Crotty 2014; Johnston et al. 2009).

The gene expression profiles, BCL6 expression, and surface protein receptors are similar between human and mouse GC-Tfh cells, suggesting evolutionary conservation of Tfh biology. However, the cytokines that induce Tfh differentiation are markedly different in the two species. Tfh differentiation is characterized by IL-21 expression and is induced by IL-6 in naive murine CD4+ T cells (Crotty 2014), but by IL-12 with additional factors, such as transforming growth factor beta (TGF-β) and STAT proteins, in naive human CD4+ T cells (Crotty 2014; Schmitt et al. 2014). Human *in vitro* Tfh differentiation is optimized by coculturing IL-12+TGF-β or IL-12+ activin A (Schmitt et al. 2014; Locci et al. 2016) and accomplished by blocking IL-2, a known inhibitor of murine Tfh differentiation (Locci et al. 2016).

The primary function of Tfh cells is to facilitate the antibody-mediated protection against pathogens of viral, fungal, bacterial origin, and parasitic infections. As an example, in the absence of Tfh, the immune response to vaccinia virus infection is reduced (Xiao et al. 2014). Both in humans and mice, Tfh cell associations have been observed in a wide range of autoimmune disorders. These include type 1 diabetes, systemic lupus erythematosus (SLE) (Gensous et al. 2018; Linterman et al. 2009), rheumatoid arthritis, and experimental autoimmune encephalomyelitis (EAE; an animal model for multiple sclerosis)(Quinn et al. 2018). In humans, the role of Tfh in SLE is clear, and a low IL-2 dose has been considered for the treatments for SLE due to the potent inhibition of Tfh cells by IL-2 (He et al. 2016).

2.2.3 Treg cells

Treg cells are a subclass of CD4+ T cells that are characterized by the expression of TF FOXP3 and suppression of immune cells. Therefore, Treg are responsible for the homeostasis of the immune cells and prevention of autoimmunity. The Foxp3+ Treg cells depletion causes severe autoimmunity, allergy, and immunopathology in animals (Bennett et al., 2001; Fontenot et al., 2003). In line with this, reconstituting Treg cells prevents disease development (Sakaguchi et al. 1995). Treg cells can be classified as thymic Treg (tTreg), peripheral Treg (pTreg), and induced Treg (iTreg) cells. In the thymus, T cells that recognize the self-antigens are removed by negative selection. However, a minority of T cells with a TCR repertoire for self-antigen escape and become tTreg cells (Jordan et al. 2001; Hsieh et al. 2006; Wing et al.

2019). The TF Aire, expressed in thymic cells, is necessary for the development of tTreg cells (Shevach and Thornton 2014). CD4+ T cells that gain stable FOXP3 expression in the small intestine, and lamina propria of the colon is called as pTreg cells (Shevach and Thornton 2014; A. Basu et al. 2021). CD4+ T cells differentiated *in vitro* in the presence of IL-2, TGFβ, and retinoic acid are defined as iTreg cells. Although iTregs have been extensively used in the study of the Treg lineage, they are unstable and lack the full repertoire of Treg epigenetic gene expression (Wing et al. 2019).

Foxp3 is indispensable for Treg function (Khattri et al. 2017; Hori at al. 2017; Fontenot et al. 2003). Foxp3 controls the gene expression profile of Treg cells (Gavin et al. 2007; Hill et al. 2007; Morikawa et al. 2014) by exploiting the pre-existing enhancer landscape and Treg transcription factors (Kitagawa and Sakaguchi 2017; Morikawa et al. 2014; Samstein et al. 2012). Treg suppression of effector T cells by promoting constitutive expression of CTLA4 is one of the most important downstream function of FOXP3. However, CD4+ T-cell precursors with CD25+Foxp3- in the thymus differentiated into the Treg lineage, and therefore, FOXP3 is not the initiating factor for Treg development (Lio and Hsieh 2008; Lin et al. 2007; Gavin et al. 2007). Also, iTreg cells with high FOXP3 expression lack Treg-specific DNA hypomethylation, and CD25+CD4+ T cells from scurfy mice display demethylation of Treg specific genes, including Foxp3 (Ohkura et al. 2012; Floess et al. 2007; Polansky et al. 2008). The TF and global chromatin organizer SATB1, together with additional factors, bind enhancer regions of Treg-specific genes, including Foxp3, to provide the epigenomic environment necessary for Treg differentiation (Kitagawa and Sakaguchi 2017). Maintenance of the stable Treg phenotype and function requires DNA demethylation of Treg-specific genes (Polansky et al. 2008; Morikawa et al. 2014; Ohkura et al. 2012), most importantly within the Treg-specific demethylation region (TSDR) of the Foxp3 enhancer CNS2, as its deletion reduced the Foxp3 in Treg cells (Y. Zheng et al. 2010; Yue et al. 2016; Feng et al. 2014; X. Li et al. 2014). The TSDR in other Treg markers, such as CD25, CTLA-4, and Helios, in addition to Foxp3, explain their high constitutive expression in Treg cells, compared to reduced expression in activated T cells, and are therefore instrumental in defining functional Treg cells (Baron et al. 2007; Ohkura et al. 2012).

A fundamental feature of FOXP3+ Treg cells is the high expression of the interleukin-2 (IL-2) receptor and the co-inhibitory molecule CTLA-4 (Takahashi et al. 2000; Salomon et al. 2000; S Sakaguchi et al. 1995; Read, Malmström, and Powrie 2000). The IL-2 receptor on Treg cells quickly senses and consumes IL-2 produced by activated T cells. This helps to expand Treg cells but also acts as negative feedback to suppress reactive T cells and prevent autoimmunity (Shimon Sakaguchi et al. 2006; Takahashi et al. 1998; J. M. Kim, Rasmussen, and Rudensky 2007; O'Gorman et al. 2009; Zhiduo Liu et al. 2015). On the other hand, CTLA-4

acts in an extrinsic manner by binding to its ligands CD80 and CD86 on APCs and makes them unavailable for conventional T cells and leads to their apoptosis (Walker and Sansom 2011; Wing et al. 2008; Onishi et al. 2008; Sakaguchi et al. 2006).

Proinflammatory environments use ubiquitination as the mode of Foxp3 regulation to destabilize the regulatory cells. Proinflammatory cytokines upregulate the E3 ubiquitin ligase, Stub1, which interacts with and promotes FOXP3 degradation (Chen et al. 2013). Overexpression of Stub1 leads to inactivation of Treg cell-mediated suppression of inflammatory response (Chen et al. 2013). On the other hand, deubiquitinase (DUB), USP7 upregulation prevents ubiquitination and degradation of Foxp3 in Treg cells (vanLoosdregt et al. 2013).

2.2.4 Th17 cells

2.2.4.1 Identification and Th17 cell differentiation

Th17 cells secrete IL-17 and are primarily associated with the development of autoimmunity and inflammation. However, they also provide immunity to pathogens of bacterial and fungal origins. Th17 cells were initially identified as a separate subset of Th cells after the discovery of IL-23, an IL-12 cytokine family member (Cua et al. 2003; Murphy et al. 2003). Of note, the IL-12 receptor is a heterodimer composed of p40 and p35 subunits. Mice deficient in the IL-12 receptor p40 subunit gene were resistant to EAE and collagen-induced arthritis (CIA) disease (Rangachari and Kuchroo 2013). Therefore, enhanced Th1 responses were considered responsible for the development of animal models of autoimmunity, EAE and CIA (Rangachari and Kuchroo 2013). The role of Th1 cells in murine models of autoimmunity was questioned after the discovery of IL-23. The IL-23 receptor shares the p40 subunit of the IL-12 receptor but contains additional subunit p19. Seminal studies were conducted to show that, in addition to *IL-12/IL-23p40*, *IL-23p19* gene knockout and not *IL-12p35* causes resistance against EAE and CIA in animals (Cua et al. 2003; C. A. Murphy et al. 2003). This led to the identification of a role for the IL-23 cytokine in autoimmunity. Later, Th17 cells were identified and characterized (Romagnani 2008; Nakae et al. 2003; Stockinger and Omenetti 2017). In addition, before identification of Th17 cells, the existence of IL-17 producing cells was known in various autoimmune diseases (Harrington et al. 2005).

Although IL-23 is required to amplify and stabilize the Th17 phenotype, it is not needed for Th17 commitment. Instead, TGF-β, IL-6, and IL-1β are needed to initiate Th17 differentiation (Veldhoen et al. 2006; Mangan et al. 2006; Bettelli et al. 2006; Chung et al. 2009). *IL6*-deficient animals are resistant to EAE (Samoilova et al. 1998) and CIA (Alonzi et al. 1998). IL-6 signaling activates STAT3 (Yang et al. 2007), which drives the transcription of Th17-specific genes RAR-related orphan

receptor C (RORC), IL17, and IL23r (Durant et al. 2010) and suppresses TGF-β-induced FOXP3 expression. IL-6 inhibits the development of Treg cells to promote Th17 differentiation (Bettelli et al. 2006).

IL-1β is also crucial for the initial phase of Th17 differentiation, and therefore, *IL1r*-deficient animals are resistant to EAE (Sutton et al. 2006). IL-1R signaling promotes interferon regulatory factor (IRF4) to reinforce RORγt (Chung et al. 2009) and activates mammalian target of rapamycin (mTOR) to enhance the metabolic fitness of Th17 cells (Gulen et al. 2010). In addition, intestinal IL-1 suppresses retinoic acid induction of FOXP3 but activates STAT3, which competes with STAT5 and promotes Th17 cell vs iTreg fate (R. Basu et al. 2015). The role of TGF-β is more complex as it also stimulates FOXP3+ Treg cell development. However, mutually exclusive Th17 and Treg development from naive precursors by TGF-β has been reported (Das et al. 2009). In addition, TGF-β promotes Th17 differentiation indirectly by inhibiting alternative IFN-γ- and IL-4-producing cell fates (Das et al. 2009). The TGF-β knock-out animals are characterized by excessive IFN-γ and IL-4 production. In cells lacking Tbet and STAT6 in the absence of TGFβ, IL-6 alone can drive Th17 differentiation, suggesting TGFβ promotes Th17 differentiation by repressing alternative Th1 and Th2 cell fates (Das et al. 2009). Therefore, both IL-1β and TGF-β are essential for Th17 differentiation. However, TGF-β also promotes the development of Treg cells.

Over the past decade, several laboratories independently studied the transcriptome of Th17 cells to understand their regulation at the transcriptional level. But the studies used *in vitro* cultures, restricted by the inherent limitations of incomplete differentiation and heterogeneity of the cell populations (Stockinger and Omenetti 2017; Gaffen et al. 2014). Nevertheless, these studies led to the discovery of genes crucial for Th17 differentiation. A significant difference in the transcriptomes from human and mouse Th17 cells was identified (Tuomela et al. 2016). The results of this comparison indicated that, overall, only the core elements were conserved between human and mice cells. Therefore, the relevance of any novel genes should be first verified in human Th17 cells before further studies using the animal model (Stockinger and Omenetti 2017). Interestingly, the recent use of single-cell RNA-sequencing (scRNA Seq.) for *ex vivo* Th17 cells used to describe the Th17 signature genes involved in EAE development (Gaublomme et al. 2015; Wang et al. 2015). Thus, Th17 cells are a recently identified subset of Th cells, and the factors that develop Th17 cells are characterized.

2.2.4.2 STAT3 transcriptional control of Th17 cells

The critical event in the lineage specification of Th17 cells is the activation of TF STAT3 by IL-6 or IL-23. The relevance of the role of STAT3 in IL17 signaling is

highly apparent in the human disease autosomal dominant hyper-IgE syndrome (AD-HIES; also known as Job's syndrome). The recurrent *Aspergillus*, *Candida* and *Staphylococcus* infections are due to STAT3 mutations and reduced numbers of IL17-producing T cells (Holland et al. 2007; Minegishi et al. 2007; Milner et al. 2008). Studies in human and mice cells indicated that STAT3 directly binds to the specific TFs (e.g., *Rorc*, *Batf* and *Irf4*), cytokines, and their receptor genes (e.g., *Il17a*, *Il17f*, and, *Il23r*), and thus plays a crucial role in Th17 cell differentiation (Durant et al. 2010; Tripathi et al. 2017).

The gene for RORC (RAR-related orphan receptor C) encodes isoforms RORγ and RORγt by alternative promoter selection (Eberl and Littman 2003; Medvedev et al. 1997; Villey, De Chasseval, and De Villartay 1999; Y. W. He et al. 1998). RORγt is considered the master regulator of Th17 differentiation, as it induces essential genes for Th17 differentiation, and mice lacking *Rorc* are resistant to the autoimmune EAE disease (Ivanov et al. 2006). However, RORγt expression is not sufficient for complete Th17 differentiation. Notably, factors such as BATF and IRF4 govern chromatin accessibility for recruitment and binding of *Rorc* and are crucial for Th17 specification (Li et al. 2012; Ciofani et al. 2012). Interestingly, RORγt protein levels increase during the late phase of differentiation of Th17 cells (Yosef et al. 2013b), but Th17 cells cannot maintain the RORγt levels if BATF (Schraml et al. 2009) or IRF4 are deficient (Brüstle et al. 2007). However, RORγt overexpression rescues Th17 cells with BATF and IRF4 deficiency. The activator protein 1 (AP1) family TF BATF is upregulated during TCR activation and is essential for Th17 cell development. Mice lacking *Batf* are resistant to EAE development and have defects in Th17 differentiation (Schraml et al. 2009). BATF forms dimers with JUNB to bind on the promoter of the genes (Il17a, Il17f, Il21) critical for Th17 differentiation (Schraml et al. 2009). The TF IRF4 is essential for the development of both Th2 (Lohoff et al. 2002) and Th17 cells (Brüstle et al. 2007). Th17 differentiation is impaired in Irf4-deficient mice and fails to develop EAE (Brüstle et al. 2007). BATF and IRF4 form a complex and positively regulate each other's binding to target genes (Li et al. 2012; Ciofani et al. 2012). Due to their functional cooperation, chromatin remodeling makes the promoters accessible for RORγt in Th17 cells (Li et al. 2012; Ciofani et al. 2012). Gene promoter co-occupancy by BATF and IRF4 has also been found in TCR-activated cells, suggesting BATF and IRF4 are pioneer factors that make the chromatin accessible for lineage-specific TFs (P. Li et al. 2012; Ciofani et al. 2012). Therefore, RORγt fine tunes and completes the differentiation of Th17 cells, initiated by STAT3 (Ciofani et al. 2012; Yosef et al. 2013a; Li et al. 2012).

In addition, the TF hypoxia-inducible factor 1α (HIF-1α) is induced by TCR activation, and its expression is further upregulated by IL-6-STAT3 signaling and under hypoxia conditions. HIF-1α directly binds to and induces *Rorc* and forms a

complex with RORγt to induce the expression of IL-17 (Dang et al. 2011). Intriguingly, HIF-1α interacts with Foxp3 and target Foxp3 proteasomal degradation (Dang et al. 2011). HIF1α deficiency in mice causes diminished EAE disease and is associated with a defect in Th17 cell differentiation and enhanced Treg development (Dang et al. 2011). Therefore, the metabolic pathways regulate Th17 and Treg cell differentiation, and environmental conditions, such as hypoxia, promote Th17 cell differentiation.

2.2.4.3 Pro-inflammatory and protective Th17 cells

Th17 cells are potent inflammatory cells that are responsible for the pathogenesis of many autoimmune diseases, namely psoriasis, Sjogren's syndrome, inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis (Stockinger and Omenetti 2017; Littman and Rudensky 2010). However, at steady-state, Th17 cells are crucial for the endowment of host immunity against pathogens, such as *Candida albicans* and *Staphylococcus aureus* (Romani et al. 2011; Otto 2010; Stockinger and Omenetti 2017).

The criteria for pathogenic Th17 cells are the use of different cytokine combinations for Th17 differentiation and EAE disease development in animals after adoptive transfer. Initially, the pathogenic and nonpathogenic subsets of Th17 cells were identified by culturing undifferentiated T cells with different combinations of cytokines (Ghoreschi et al. 2010; McGeachy et al. 2007; Veldhoen et al. 2006). In the presence of IL-6 and TGF-β1, naive cells seemed to give rise to Th17 cells that that could not induce the disease after adoptive transfer. However, cells cultured with IL-6, IL-1β, and IL-23 or TGF-β3 were identified as Th17 cells responsible for EAE (Lee et al. 2012; Ghoreschi et al. 2010). Surprisingly, TGF-β3-induced Th17 cells were different from those generated with TGF-β1 and were highly pathogenic in disease development (Lee et al. 2012). Recently, a study showed that a $CD11b^{+}$ Sirpα+ DC is essential for the generation of pathogenic Th17 cells and the encephalitogenic priming of T cells for EAE (Heink et al. 2017). The $CD11b^{+}Sirp\alpha^{+}$ DC-derived IL-6 activates STAT3 designated as "IL-6 cluster signaling" for pathogenic Th17 cells differentiation, while sources of IL-6 other than DC are important for suppression of Foxp3+ Treg cells (Heink et al. 2017).

Thus, IL-23 is indispensable in the regulation of inflammatory Th17 cells and the development of EAE in animals. Interestingly, IL-23R (IL-23 receptor) expression is regulated by Notch signaling and protein C receptor (PROCR) important for pathogenic Th17 cell differentiation (Horste et al. 2016; Kishi et al. 2016). In Notch signaling, recombination signal binding protein for immunoglobulin κJ binds and transactivates the *Il23r* promoter, which is essential for pathogenic Th17 cells (Horste et al. 2016). However, PROCR negatively regulates IL-23R

expression and pathogenic Th17 cells by an as-yet undefined mechanism (Kishi et al. 2016).

MicroRNAs (miRNAs) are small RNAs that regulate the gene expression by direct binding to the untranslated regions of messenger RNA (mRNA), and there are studies reporting their roles in regulating Th17 differentiation and autoimmunity. The miRNA miR-183-96-182 cluster (miR-183C) promotes differentiation of pathogenic Th17 by TF forkhead box O1 (FOXO1) gene repression (Ichiyama et al. 2016). Here, miR-183C prevents the inhibition of IL-1R1 by FOXO1 and, therefore, promotes inflammatory cytokine production by Th17 cells. Similarly, miR-326 and miR-155 cause repression of certain gene expressions of Th17 cells. They are negative regulators ETS1 (Du et al. 2009) and JARID2 (Escobar et al. 2014), respectively, promoting Th17 cell expansion and EAE disease development (Du et al. 2009). Also, mice lacking miR-21 develop EAE and display defective Th17 differentiation. Mechanistically, miR-21 enhances TGF-β signaling and represses IL-2-inhibitory Th17 effects (Murugaiyan et al. 2015). Therefore, Th17 cells play a significant role in autoimmune disease development but also provide immunity against the pathogen. Understanding of the dichotomy of Th17 cells in the regulation of autoimmune disease and immunity to infections is essential.

2.3 Protein phosphatase 2A

Protein phosphorylation is a reversible post-translational modification (PTM) that is an essential component of the regulatory mechanisms in cell signaling. It functions as a molecular switch since it can alter protein charge, global conformation, subcellular localization, activity, and protein interactions essential for protein function. Therefore, a precise balance between kinase and phosphatases that phosphorylate and dephosphorylate proteins, respectively, is required to maintain cell homeostasis. However, the kinases in a cell outnumber phosphatases, with more than 500 vs. less than 200, respectively.

Initially, phosphorylation was considered specific and dephosphorylation non-specific and (therefore) less interesting. This is still reflected by the 10-fold more studies related to kinases than to phosphatases (Clark and Ohlmeyer 2019). The misconception arose as phosphorylation requires energy in the form of ATP consumption, and therefore, researchers considered that kinase activity was specific to conserve energy. However, recent studies have observed inhibition of phosphatase activity as a common signature for the development of many diseases, including cancers, neurological diseases, and autoimmune disorders (Clark and Ohlmeyer 2019). Also, due to the inherent complexity of the diverse regulatory subunits that provide substrate specificity, an increasing number of studies now indicate that the

number of phosphatases is much higher. Therefore, an understanding of phosphatase regulation, in addition to kinases, is now considered equally important.

The phosphatases are classified into four categories: (1) protein tyrosine phosphatases (PTPs), (2) protein serine/threonine phosphatases (PSTPs), (3) dual specificity phosphatases, and (4) histidine phosphatases (Kaur and Westermarck 2016). The protein phosphatase 2A (PP2A) and phosphatases PP1, PP2B, PP2C, PP4, PP5, and PP6 are the major PSTP in the eukaryotic cells (O'Connor et al. 2018; Janssens and Goris 2001; Shi 2009).

Phosphatase PP2A is a heterotrimeric holoenzyme. An active PP2A enzyme contains a 65-KDa scaffolding subunit "A", a 36-KDa catalytic subunit "C" and a substrate-specific regulatory subunit "B" of variable size (Clark and Ohlmeyer 2019; O'Connor et al. 2018; Lambrecht et al. 2013; Kauko and Westermarck 2018). The scaffolding structural A subunit and catalytic C subunit form a dimer of the core enzyme, to which one of the B subunits binds (Xu et al. 2006; Cho and Xu 2007). The association of B subunits to the core enzyme is mutually exclusive. Only one B subunit can interact with core enzyme at a time (Mayer-Jaekel and Hemmings 1994; Janssens and Goris 2001) (**Figure 3**).

The A subunit is characterized by 15 repeats of antiparallel alpha-helices of a structural motif with 39 amino acids, known as the HEAT (**h**untingtin, **e**longation factor, the A subunit of PP2A, and **t**arget of rapamycin) domain. The stacking of antiparallel alpha helices of the HEAT domain is flexible due to a hinge region between HEATS 12 and 13 and a hydrophobic inner ridge that creates a hook-like structure for B and C subunit binding to A subunit (O'Connor et al. 2018; Xu et al. 2006; Groves et al. 1999; Cho and Xu 2007). The A and C subunits each have two isoforms, α and β, subdivided into PPP2R1A or PPP2R1B, also called PP2A Aα or PP2A Aβ and PPP2CA or PPP2CB, also called PP2A Cα or PP2A Cβ, respectively. The two isoforms of PP2A catalytic C subunit, α (alpha) and β (beta) share around 97% homology in their primary sequence, but the gene promoter for *PP2ACA* is 7–10-fold stronger than the gene promoter of *PP2ACB*, and thus, PP2A Cα is about 10 times more abundant than the PP2A Cβ (Khew-Goodall and Hemmings 1988; V. Janssens and Goris 2001; O'Connor et al. 2018). The structure of the catalytic subunit of PP2A has remained remarkably constant throughout evolution.

The regulatory subunit B, encoded by different genes in humans, interacts with the core enzyme of PP2A. The B subunit is further subdivided into four groups: **B** (PPP2R2A, PPP2R2B, PPP2R2C, and PPP2R2D), **B′** (PPP2R5A, PPP2R5B, PPP2R5C, PPP2R5D, and PPP2R5E), **B″** (PPP2R3A, PPP2R3B, and PPP2R3C), and **B‴** (STRN, striatin family) (Kauko and Westermarck 2018; O'Connor et al. 2018; Clark and Ohlmeyer 2019). The alternate names of the various subunits of PP2A are shown in **Table 1 and Figure 3,** taken from Kauko et al. 2018; Clark and Ohlmeyer 2019.

Table 1: Systematic and alternative names of PP2A subunits taken from Kauko and Westermarck, 2018; Clark and Ohlmeyer, 2019. The part of the systematic and alternate names is bold to give an overview of the nomenclature.

PP2A Subunit	Subunit groups	Systematic name	Alternative names
A (scaffolding)		**PPP2R1**A	PR65α, PR65A, **PP2A-A**α, MRD36
		PPP2R1B	PR65β, PR65B, **PP2A-A**β
B (regulatory)	B	**PPP2R2**A	**B**α, PR55α, **B55**α
		PPP2R2B	**B**β, PR55β, **B55**β, SCA12
		PPP2R2C	**B**γ, PR55γ, **B55**γ
		PPP2R2D	**B**δ, PR55δ, **B55**δ, KIAA1541
	B'	**PPP2R5**A	**B'**α, PR61α, B56A, **B56**α
		PPP2R5B	**B'**β, PR61β, B56B, **B56**β
		PPP2R5C	**B'**γ, PR61γ, B56G, **B56**γ
		PPP2R5D	**B'**δ, PR61δ, B56D, **B56**δ, MRD35
		PPP2R5E	**B'**ε, PR61ε, B56E, **B56**ε
	B"	**PPP2R3**A	**B"**α, PR130, PR72
		PPP2R3B	**B"**β, PR70, PR48
		PPP2R3C	**B"**γ, C14orf10, G5PR
	B"'Striatin	**STRN**	**B"'**α, PPP2R6A, PR93, STRN1
		STRN3	**B"'**β, PPP2R6B, PR110, SG2NA
		STRN4	**B"'**γ, PPP2R6C, zinedin, ZIN
C (catalytic)		**PPP2C**A	**PP2A-C**α, PP2CA, PP2Aα
		PPP2CB	**PP2A-C**β, PP2CB, PP2Aβ

The B subunits are distinguished by their diversity and complete lack of sequence similarity, even though they interact with the same PP2A A subunits. The A and C subunits ubiquitously expressed, but the expression levels and cellular localization of the B subunits vary, depending upon the cell types. Therefore, the complexity and diversity of the PP2A holoenzymes are due to the regulatory B subunits, as these provide substrate specificity and subcellular localization of PP2A heterotrimers (Lambrecht et al. 2013; Haesen et al. 2014; Clark and Ohlmeyer 2019). Notably, diverse PP2A functions result from specific PP2A complexes directed by B subunits (Slupe A.M, et al., 2011; Kauko and Westermarck 2018). In mammalian cells, PP2A subunits exist as AC dimers (called core dimers), or ABC trimers (called holoenzyme).

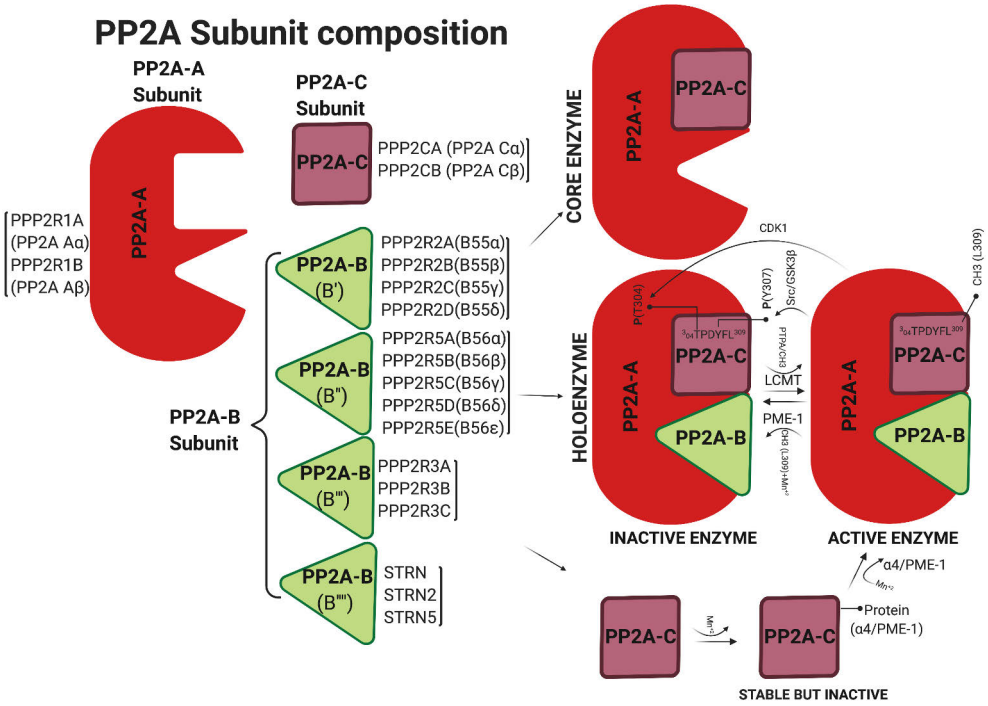

Figure 3: PP2A subunit composition and regulation of PP2A holoenzyme by PTM. Illustration created with BioRender.com.

2.3.1 Regulation of PP2A function

As PP2A has a strong influence on cell biology, it is tightly regulated at several levels to avoid the formation of an active enzyme with impaired substrate specificity (Lambrecht et al. 2013; Haesen et al. 2014; Kauko and Westermarck 2018; O'Connor et al. 2018; Clark and Ohlmeyer 2019). The following mechanism operates in cells to regulate and control the activity of PP2A.

2.3.1.1 Post-translational modifications

The hotspot for PP2A PTMs is on the carboxy terminus of the catalytic C subunit. Modifications there control B-subunit binding and the enzymatic activity of PP2A (Cho and Xu 2007; Xing et al. 2008; Janssens et al. 2008). Through biochemical and mass spectrometric analysis, three modification sites have been identified that include methylation on the carboxy-terminal leucine 309 (L309) residue, and phosphorylation at the tyrosine 307 (Y307) residue and the threonine 304 (T304) residues to regulate PP2A enzymatic function, substrate specificity and composition (**Figure 3**).

PP2A Methylation: One of the best-known PP2A PTMs is methylation of the L309 residue, which is required for the enzyme's activity (Yu et al. 2001; Longin et al. 2007; Kaur and Westermarck 2016). The crystal structural analysis of PP2A heterodimers showed that the negatively charged carboxyl group on the non-methyl L309 prevents the interaction between the subunits. Therefore, methylation of the PP2A L309 residue regulates the interaction of the PP2A core dimer and one of the B-subunits (e.g., the interaction of PPP2R2A (B55α) with PP2A-A-C dimer). Hence, methylation of the L309 residue of the catalytic C subunit is an regulatory modification that determines the holoenzyme composition necessary for PP2A enzymatic functions (Cho and Xu 2007). In other words, the methylation site of the L309 residue of PP2A highlights the importance of PTM regulation in PP2A functions.

Protein methyl-esterase 1 (PME-1) is responsible for demethylation of the L309 residue. In addition, PME-1 directly interacts with PP2Ac and sequesters manganese ions essential for the activity of the PP2Ac catalytic site (Xing et al. 2008). Analysis of the crystal structure of the PME-1-PP2Ac complex, together with mutational analysis, has shown that the R369 site on PME-1 is involved in binding to the catalytic site of PP2Ac (Pokharel et al. 2015; Xing et al. 2008). PME-1 activity is regulated by leucine carboxyl methyl transferase 1 (LCMT1), which adds a methyl group to the free carboxyl group on the L309 residue by utilizing S-adenosylmethionine as the substrate (Kaur and Westermarck 2016; De Baere et al. 1999). The knockdown of LCMT1 also promotes cell transformation, further implicating its association with the tumor-suppressive function of PP2A.

PP2A Phosphorylation: Biochemical assays have shown that tyrosine 307 (Y307) phosphorylation of the PP2A catalytic C subunit inactivates PP2A (Neviani et al. 2013; Rincón et al. 2015; Neviani et al. 2005; Cristóbal et al. 2014). The catalytic subunit C phosphorylation site at T304, however, is not well characterized. Mutational analysis showed that it regulates the association between the regulatory subunit B55 and the catalytic subunit (Longin et al. 2007). The CDK1 is the kinase that phosphorylate this site during mitosis (Schmitz et al. 2010). Importantly, the specificity of the antibodies used for PP2Ac phosphorylation detection has been challenged by various studies.

Free but inactive and stable PP2A catalytic C' subunits have also been reported to interact with proteins PME-1 and α4 (Kremmer et al. 1997; Kaur and Westermarck 2016; Kong et al. 2009). The free catalytic C subunit is either subjected to proteasomal degradation or stabilized and inactivated by interaction with protein α4 or PME-1 (Kremmer et al. 1997; Kaur et al. 2016; Kong et al. 2009). In addition, phosphotyrosyl phosphatase activator and binding of Mn^{2+} ions govern the catalytic activity of catalytic C subunit due to the structural isomer formation (Guo et al. 2014; Xu et al. 2006; Cho and Xu 2007; O'Connor et al. 2018).

2.3.2 PP2A inhibitors

Initially, two endogenous PP2A inhibitors, isolated from the bovine kidney, were characterized. These are acidic nuclear phosphoprotein 32A (ANP32A) and SET, also known as PP2A inhibitor 1 (I1PP2A) and PP2A inhibitor 2 (I2PP2A), respectively (Li et al. 1995; Li et al. 1996). The ANP32A and SET were identified as members of the SET (Su(var)3-9, Enhancer-of-zeste and Trithorax) domain. SET binding inactivates PP2A and its dysregulation has been implicated in leukemia and several other cancers (Apostolidis et al. 2016; O'Connor et al. 2018). CIP2A is another endogenous PP2A inhibitor (Junttila et al. 2007). CIP2A directly interacts with PP2A and inhibits its activity towards oncoprotein c-Myc (Junttila et al. 2007). The c-Myc dephosphorylation by PP2A results in degradation of oncoprotein c-Myc and prevent cell transformation. Overexpression of CIP2A have been reported in several types of cancer (Junttila et al. 2007; Kauko and Westermarck 2018; Khanna and Pimanda 2016). Proteins PME-1 and α4 are also considered as PP2A inhibitors as they impede the heterotrimeric conformations of the phosphatase. Enhanced PME-1 activity and expression has been observed in several cancers and is associated with disease progression (Kaur and Westermarck 2016; Wandzioch et al. 2014; Puustinen et al. 2009). PME-1 inhibition promotes PP2A activity in cancer cells. Okadaic acid is a naturally occurring small molecule that can penetrate cells for broad-spectrum phosphatase inhibitor activity, including PP2A. Okadaic acid is a polyether fatty acid synthesized by the marine dinoflagellates and causes shellfish poisoning (Bialojan and Takai 1988).

2.3.3 PP2A dysregulation in cancer cells

PP2A is a tumor suppressor, and inhibition of its activity in several human cancers is well known (O'Connor et al. 2018; Westermarck and Hahn 2008; Janssens and Rebollo 2012; Haesen et al. 2014; Grech et al. 2016; Carratù et al. 2016). In addition, dysregulated phosphorylation of the PP2A substrates is a hallmark of cancer cells. In line with this, activation of PP2A results in inhibition of cancer cell proliferation and apoptosis. The mechanisms of PP2A inhibition and cell transformation in cancer include: (1) Enhanced expression of PP2A endogenous inhibitors, such as CIP2A and SET (Hung and Chen 2017; Soofiyani, Hejazi, and Baradaran 2017; Kauko and Westermarck 2018). (2) Decreased expression of the positive regulator PPP2R4 of PP2A (Sents et al. 2017). (3) The somatic mutations of PP2A scaffolding subunit PPP2R1A and the associated impact on the recruitment of B subunits (Ruediger, Ruiz, and Walter 2011; Haesen et al. 2016). (4) PP2A function impairment by the dysregulated oncogenic kinases (Zonta et al. 2015; J. Chen, Parsons, and Brautigan 1994). (5) DNA tumor virus, SV40, is characterized, based on proteins small and middle T antigens that inhibit PP2A and other tumor suppressor genes promote cell

transformation (Sablina and Hahn 2008; Chen et al. 2007; Cho et al. 2007; Guergnon et al. 2011). (6) Chromosomal deletion, epigenetic and micro-RNA mediated decrease in expression of specific B subunits results in impaired PP2A activity (Ruvolo 2015). Therefore, PP2A inhibition is well-established in cancer cells. Recently, reports about PP2A inhibition is also observed in other diseases, such as Alzheimer's disease (AD) (Sontag and Sontag 2014; Shentu et al. 2018). Thus, PP2A activation in disease conditions could have therapeutic relevance.

2.3.4 PP2A activation

PP2A activation in the cell is observed either by indirectly inhibiting PP2A inhibitor or directly with compounds that interact and activate PP2A (O'Connor et al. 2018). Induction of methylation on catalytic subunit by xylulose-5-phosphate and chloroethyl nitrosourea activates PP2A. In addition, inhibition of PME-1 by azalactam and sulfonyl acrylonitrile enhances PP2A activity in cells (Kaur and Westermarck 2016; O'Connor et al. 2018). Also, compounds, such as bortezomib and celastrol, activate PP2A by inhibiting CIP2A. Vitamin E analogs (e.g., α-tocopherol succinate, forskolin, and carnosic acid) activate PP2A (O'Connor et al. 2018). Off-target effects of anti-psychotic phenothiazine drugs (e.g., chlorpromazine) also activate PP2A (Clark and Ohlmeyer 2019). Phenothiazines activation of PP2A led to enhanced FOXO1 dephosphorylation and nuclear localization (Kau et al. 2003; L. Yan et al. 2008; Gutierrez et al. 2014). Further processing of phenothiazines led to the development of tricyclic sulfonamide compounds called small-molecule activator of PP2A, such as DBK- 1154, DT-061, or iHAPs (improved heterocyclic activators of PP2A). These interact and activate PP2A by acting as an adhesive for PP2A heterotrimeric holoenzyme assembly (Kastrinsky et al. 2015; Morita et al. 2020; Leonard et al. 2020).

Sphingolipid ceramide activates PP2A in many cell types, including cancer cells and T cells. Mechanistically, ceramide binding to the PP2A inhibitor SET causes de-repression of PP2A activity (Mukhopadhyay et al. 2009; Clark and Ohlmeyer 2019). Metabolism of ceramide by ceramidase enzyme produces sphingosine. Fingolimod (FTY720; 2-amino-2-[2-(4-octylphenyl) ethyl] propane-1,3-diol), a metabolite of fungus *Isaria sinclairii*, is a sphingosine analogue. FTY720, similar to ceramide, functions as a PP2A activator by direct binding to SET and disruption of latter inhibitory interaction to promote PP2A activation. The PP2A activation by FTY720 treatment is promising in anti-tumor effects in pre-clinical cancer models.

When phosphorylated by sphingosine kinases 1 or 2 (Sphk1 and Sphk2), sphingosine becomes sphingosine-1-phosphate (S1P). The latter is well known for its immune cell-related functions. Once S1P is transported out of the cell by the transporter Spinster 2, it binds to its receptor, S1PR, by inside-out signaling. The

regulation of leukocyte traffic by S1P-S1PR signaling is of particular importance. The S1P gradient drive lymphocyte migration from lymphoid tissues, in which it has a lower concentration than the comparatively high S1P concentration in circulating blood/lymph (Spiegel and Milstien 2011; Clark and Ohlmeyer 2019). In multiple sclerosis, autoreactive lymphocytes egress from the lymphoid tissues due to an S1P gradient and move to the central nervous system to cause disease (Chun and Hartung 2010). The auto-aggressive lymphocytes responsible for multiple sclerosis include proinflammatory IL17-producing Th17 cells (Tzartos et al. 2008). Th17 cell enrichment in the active multiple sclerosis lesions implicated in the pathogenesis of multiple sclerosis (Tzartos et al. 2008; Mehling et al. 2010). In *in vitro* models, Th17 cells migrate across the blood-brain barrier and promote inflammation and destruction of neurons by producing IL17 and pro-apoptotic granzyme B, respectively (Kebir et al. 2007).

FTY720 is a commonly used immunosuppressive compound and approved for multiple sclerosis treatment. It binds on S1PR on lymphocytes and results in S1PR internalization and degradation. FTY720 treatments prevent EAE disease development in animals (Chun and Hartung 2010), and the underlining mechanism is due to the lymphocyte's impaired migration and their confinement within the lymph nodes. The immunosuppressive role is due to its phosphorylation. Derivatives of FTY720, AAL(s), MP07-66, OSU-2S, P053, and aza-cyclic were developed by the substitution of the FTY720 phosphorylation site (Kubiniok et al. 2019; McCracken et al. 2017; Kim et al. 2016; Turner et al. 2018; Kiuchi et al. 1998; Omar et al. 2011). These derivatives bind SET and activate PP2A for antitumor properties. However, AAL(s), MP07-66 and OSU-2S, cannot bind the sphingosine receptor for immunosuppressive functions (Mani et al. 2015; Mani et al. 2017; Omar et al. 2016; Roberts et al. 2010; Smith et al. 2016). In addition, AAL(s) treatments failed to reduce EAE symptom severity in animals (Brinkmann et al. 2002), arguing for a significant role of PP2A activation in FTY720 treatment. However, fingolimod treatment also reduced IL17+ Th17 cells and generated more suppressive T cells than untreated patients (Chun and Hartung 2010; Villar et al. 2019). In addition, AAL treatments reduce the disease in an experimental model of rheumatoid arthritis (Ross et al. 2017). Therefore, one cannot rule PP2A activation as a mechanism by which FTY720 modulates autoimmune disease treatment.

2.3.5 PP2A in autoimmune diseases

SLE is an autoimmune disease, which affects the joints, kidneys, skin, brain, lungs, and blood vessels. As in other autoimmune disorders, tissue damage in SLE is caused by widespread inflammation and immune reactions (Trentin et al. 2021; Aringer and Schneider 2021; Aringer and Dörner 2018). Interestingly, as a result of SLE, T cells

are characterized by reduced IL-2 expression and enhanced catalytic PP2Ac subunit expression (Katsiari et al. 2005).

The *IL2* promoter is occupied by the TFs NF-κB, AP-1, and NFAT. An additional regulatory mechanism that controls IL-2 production in T cells involves the competitive binding of transcription activator CREB and CREM at the -180 site of IL2 the promoter (Jain et al. 1995; Rothenberg and Ward 1996). The PKA kinase phosphorylates CREB at the Ser133 residue to initiate interaction with transcription coactivators CREB and p300, and promote IL-2 production (Jain et al. 1995; Rothenberg and Ward 1996). However, T-cell activation upregulates CREM and replaces pCREB at the –180 site, and represses IL2 production (Mayr and Montminy 2001). Interestingly, the phosphatase PP2A is the primary enzyme to dephosphorylate pCREB (Mayr and Montminy 2001; Wheat et al. 1994; Choe et al. 2004; Wadzinski et al. 1993; Katsiari et al. 2005) and negatively regulates IL2 production in T cells (Wheat et al. 1994; Choe et al. 2004; Chuang et al. 2000; Falk et al. 1994; Katsiari et al. 2005).

Genetic and epigenetic mechanisms are associated with the enhanced expression of PP2A catalytic subunit PP2Ac in SLE (**Figure 4**). A CpG motif regulates the transcription of PP2Ac, and a single nucleotide polymorphism (SNP) identified during genome-wide association study on the PP2Ac proximal promoter was linked to susceptibility to SLE (Sunahori et al. 2011; Tan et al. 2011). Notably, the transcriptional repressor Ikaros recruits histone deacetylase 1 to suppress PP2A transcription. But SLE is characterized by limited Ikaros repression due to an SNP on the Ikaros binding sites and upregulation of PP2Ac transcription (Nagpal et al. 2014; Tan et al. 2011; Sunahori et al. 2011). However, significantly reduced *IKZF1* (Ikaros) mRNA expression in the peripheral blood mononuclear cells of SLE patients was also observed in a study with 60 SLE patients and 60 healthy controls (Hu et al. 2011). Therefore, multiple factors in addition to the genetic regulation of PP2Ac are likely to be associated with SLE. In SLE, hypomethylation of the CRE motif on the PP2Ac gene promoter was observed. Enhanced CREB binding due to hypomethylation of the promoter led to transcriptional upregulation of PP2Ac (Sunahori et al. 2011) regardless of the SLE disease state and was considered to be an abnormality of the disease (Katsiari et al. 2005).

Several mechanisms explain how the enhanced catalytic subunit of PP2A expression is responsible for hampered IL-2 production and SLE disease development. The enhanced PP2Ac activity in SLE causes the aberrant TCR signaling and results in reduced IL2 production. PP2A modulates the transcriptional expression of CD3ζ and FcRγ in the TCR complex (Tsokos et al. 2003). PP2A dephosphorylates TF Elf-1 (Thr-231) and results in restricted Elf-1 binding to CD3ζ and FcRγ gene promoter and former reduced expression but latter enhanced expression (Juang et al. 2008). Therefore, due to aberrant TCR CD3 signaling, there

is reduced IL-2 production in SLE T cells. However, restoring the defective CD3 signaling by CD3ζ overexpression in SLE normalizes IL2 expression in T cells (Juang et al. 2008). In addition, reduced expression and activity of PKC also promotes unopposed PP2A activity (Katsiari et al. 2005). Furthermore, PP2A mediated dephosphorylation of specificity protein-1 (SP-1; Ser59) and enhanced binding upregulate CREMα (Juang et al. 2011). CREMα suppressed IL-2 and the CD3ζ gene promoter to reduce their expression in SLE patients T cells (Juang et al. 2011).

The silencing PP2A catalytic subunit (PP2Ac) in SLE patients' T cells causes normal phosphorylation of CREB and IL2 production, thus confirming that enhanced PP2A catalytic subunit is the fundamental abnormality responsible for disease development (Katsiari et al. 2005). Therefore, enhanced PP2A catalytic subunit expression derives reduced IL-2 production in SLE immune cells and disease development but warrants further studies for therapeutic intervention.

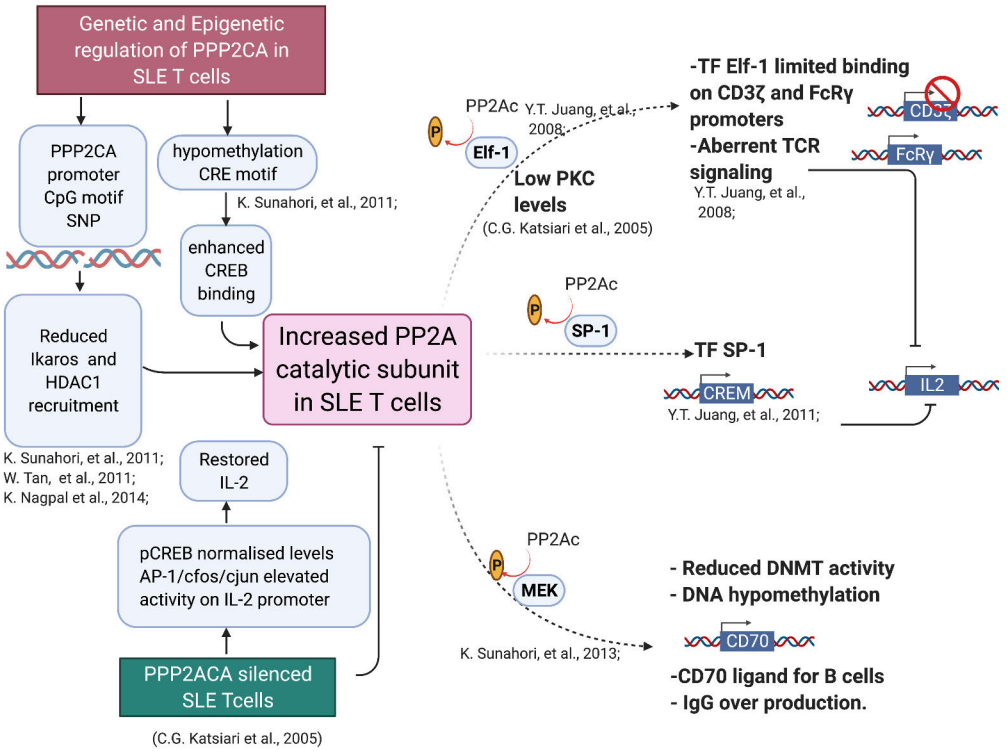

Figure 4: The mechanism of enhanced expression of PP2A catalytic C subunit and regulation of reduced IL-2 production in SLE disease development. The illustration created with BioRender.com.

2.3.6 PP2A in Th17 and Treg cells

Transgenic animals overexpressing PP2Ac have elevated IL-17 production (Apostolidis et al. 2013). In addition, PP2Ac overexpression results in the development of glomerulonephritis (Crispín et al. 2012). PP2Ac activates Rho kinase (ROCK), which phosphorylates TF IRF4. The latter promote recruitment of histone acetyltransferases (HAT) and other factors for the transcription of *IL17* (Apostolidis et al. 2013). Therefore, PP2Ac regulates IL17 expression by activation of IRF4 and enhancing histone 3 acetylation of the *IL17* locus. Specific deletion of PP2A catalytic subunit α reduces the Th17 cell differentiation and EAE disease in animals (Xu et al. 2019). PP2A regulates the phosphorylation of the SMAD2/3 and, therefore, RORγt binding on *IL17* in Th17 cells (Xu et al. 2019).

PP2A activity in Treg cells is indispensable for suppressing effector T cells (Apostolidis et al. 2016; Sharabi et al. 2018). Treg-specific deletion of PP2A in mice results in multiorgan autoimmunity. PP2A-deficient animals have a skin rash, scaly tails, and ears, wasting, dermatitis, ulcerations and a higher frequency of activated CD4+ T and CD8+ T cells in spleen and lymph nodes. *Ex vivo*-stimulated PP2A-deficient T cells produced more proinflammatory cytokines IL-17 and IL-2, IFN-γ, and TNF-α, and proliferation than cells from control animals. Gene expression profiling identified repression of *Sgms1* (SMS1; Sphingomyelin Synthase 1) gene due to Foxp3 direct binding to *its* promoter in Treg cells (Apostolidis et al. 2016). The reduced expression of SMS1 restricts ceramide and phosphatidylcholine conversion to diacylglycerol and sphingomyelin in sphingolipid metabolism and, therefore enhances ceramide levels in Treg cells (**Figure 5**). Various studies showed that the interaction of ceramide with PP2A endogenous inhibitor SET activates PP2A (Mukhopadhyay et al. 2009; Perry et al. 2012; Dobrowsky et al. 1993; Lambrecht et al. 2013; Oaks and Ogretmen 2015). Therefore, specific accumulation of ceramide inhibits TCR induced PP2A inhibitor SET, promotes PP2A activity in Treg cells. Further, PP2A direct interaction and mTOR complex-1 (mTORC1) inhibition are essential for Treg suppression of effector cells (Apostolidis et al. 2016).

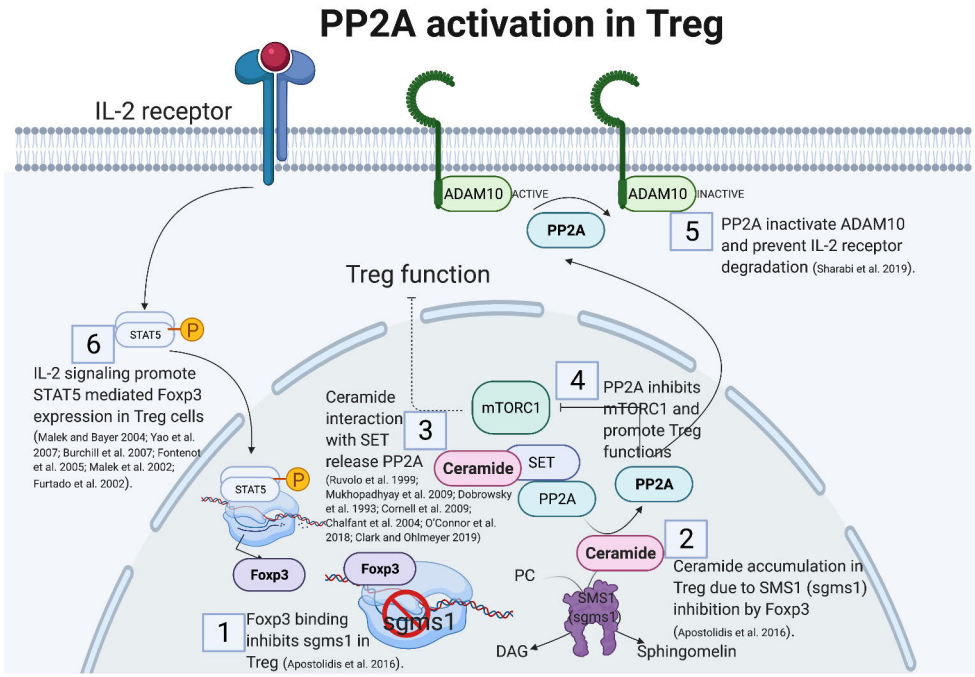

Figure 5: PP2A activation in Treg cells. Foxp3 promoter binding inhibits *sgms1* expression and promotes ceramide accumulation. The ceramide interaction with PP2A endogenous inhibitor SET activates PP2A. On the other hand, PP2A activity is important to inhibit mTORC1 and ADAM10 and promote STAT5 and Foxp3 expression in Treg cells. The illustration created with BioRender.com.

Cytokine IL-2 promotes the development of Treg cells to maintain tolerance and is also crucial for the T-cell effector and memory responses. At low concentrations, IL-2 selectively induced Treg cell responses without activation of effector cells (Hirakawa et al. 2016). The responsiveness of Treg cells towards IL-2 is due to higher expression of IL-2 receptor and enhanced activity of the phosphatase PP2A (Sharabi et al. 2019; Sharabi et al. 2018). PP2A restrains the activity of sheddase ADAM, which cleaves the IL2 receptor in T cells (**Figure 5**). Therefore, enhanced PP2A activity prevents the degradation of the IL2 receptor and thus promotes increased IL2 consumption by Treg cells, which is essential for Treg suppression of effector cells (Sharabi et al. 2019). PP2A-deficient thymic Treg cells have reduced IL2R and STAT5 tyrosine phosphorylation. The STAT5 tyrosine phosphorylation in IL-2 signaling is essential for inducing FOXP3 expression (Malek and Bayer 2004; Yao et al. 2007; Burchill et al. 2007; Fontenot et al. 2005; Malek et al. 2002; Furtado et al. 2002). Therefore, PP2A promotes IL2-STAT5 signaling and is important for Foxp3 expression (**Figure 5**) and essential for Treg cells (Sharabi et al. 2019).

2.4 Cancerous inhibitor of protein phosphatase 2A

CIP2A, also known as KIAA1524 or p90, encoded by *KIAA1524*, and located in human chromosome 3q13.13 (Soofiyani et al. 2017). CIP2A is an endogenous protein inhibitor of phosphatase PP2A (Junttila et al. 2007). Its enhanced expression in cancer cells promotes cell transformation. It directly interacts and inhibits phosphatase PP2A activity in the cancer cells. CIP2A cell-endogenous inhibition of PP2A prevents dephosphorylation and proteolytic degradation of TF and oncoprotein c-Myc S62. In addition, CIP2A regulates the dephosphorylation of several targets of PP2A, including AKT (Kauko et al. 2018; Kauko and Westermarck 2018). Structural studies have shown that CIP2A forms a homodimer and it specifically interacts with PP2A B subunits B56α and B56γ (Wang et al. 2017). The conserved N-terminal region of CIP2A binds to the B56 proteins, and CIP2A dimerization promotes this binding. Both CIP2A self-dimerization and binding to PP2A is important for CIP2A stabilization.

2.4.1 CIP2A in disease

CIP2A overexpression has been observed in several cancers and correlates with poor survival and disease progression (Khanna and Pimanda 2016; Khanna et al. 2013). Neuroblastoma is a pediatric cancer of neural crest derived embryonic peripheral nervous system. Overexpression of the TF MycN is a hallmark associated with poor prognosis and aggressive neuroblastoma disease. High expression of CIP2A is associated with MycN in regions of the neural plate and poor prognosis of neuroblastoma (Kerosuo et al. 2018).

CIP2A overexpression is associated with the poor prognosis of colon cancer (Teng et al. 2012). The direct binding of activating transcription factor 6 (ATF6) to the CIP2A promoter is associated with the latter over expression and colon cancer cell chemoresistance (Liu et al. 2018). Recently, a micro peptide named CIP2A binding peptide (CIP2A-BP) encoded by non-coding RNA LINC00665 was reported (Guo et al. 2020). CIP2A-BP interacts with CIP2A and competes for the PP2A subunit B56γ binding site. CIP2A-BP directly binds on the N-terminal of CIP2A to replace PP2A's B56γ subunit, and release PP2A activity, to decrease tumorigenesis. In cancer cells, enhanced expression of translation inhibitory protein 4E-BP1 restricts LINC00665 and CIP2A-BP expression, promote PP2A inhibition by CIP2A and metastasis (B. Guo et al. 2020). In colorectal cancer cell lines, the use of specific inhibitors or small interfering RNA (siRNA) downregulation of histone deacetylase 1 reduced CIP2A expression and restored PP2A activity (Balliu et al. 2016).

Cells maintain homeostasis by a lysosomal-mediated self-eating process called autophagy. Autophagy inhibition is commonly observed in cancer cells (Mulcahy et al. 2020). There are several mechanisms by which PP2A regulates autophagy

(Bánréti et al. 2012; Yorimitsu et al. 2009), and CIP2A was reported to inhibit autophagy (Yu et al. 2013). RNA interference (RNAi) phosphatome screening identified phosphatase PP2A and oncoprotein CIP2A among the regulators of autophagy (Puustinen et al. 2014). Immunoprecipitation studies revealed that PP2A and CIP2A are associated with kinase mTORC1 in cancer cells. The kinase mTORC1 prevents autophagy by promoting nutrient uptake for protein synthesis and cell growth. CIP2A inhibition of PP2A prevents dephosphorylation of mTORC1 targets RPS6KB1 and EIF4EBP1, known to maintain anabolic cells' state and prevents autophagy in cancer cells. Conversely, nutrient unavailability (e.g., amino acid deprivation) inactivates mTORC1 and promotes autophagic degradation of CIP2A (Puustinen et al. 2014). Thus, a positive feedback loop exists: CIP2A overexpression in cancer cells and PP2A inhibition promotes mTORC1 associated cell growth, and inhibition of autophagy prevents CIP2A degradation. CIP2A also regulates the cell cycle and mitosis by interacting with polo-like kinase 1 (Kim et al. 2013). Further, CIP2A high expression promotes a cellular energy bias towards oxidative phosphorylation, rather than glycolysis, by 5′ AMP-activated protein kinase signaling (Austin et al. 2019). Elevated CIP2A expression is observed in chronic myeloid leukemia and acute myeloid leukemia (Lucas et al. 2015; Lucas et al. 2011; 2018). Similarly, a genomic copy of CIP2A has increased in head and neck squamous cell carcinoma cancer (Routila et al. 2016; Ventelä et al. 2015).

Interestingly, as in cancer cells, CIP2A upregulation led to PP2A inhibition is associated with AD (Shentu et al. 2018; Ohlmeyer 2019). AD is a neurodegenerative and neuroinflammatory disease, characterized by aggregates of hyperphosphorylated tau protein and cognitive deficits. PP2A is the primary phosphatase in the regulation of tau phosphorylation. CIP2A overexpression in AD led to PP2A inhibition and hyperphosphorylation and aggregation of tau (Shentu et al. 2018). Thus, the pathophysiological role of CIP2A, suggesting it as a possible target for AD therapy. CIP2A also functions in the development of astrocytoma and neuroblastoma development (Yi et al. 2013; Kerosuo et al. 2018). Interestingly, CIP2A expression in the olfactory bulb and hippocampus areas in humans and mice, and its *in-vitro* role in promoting mouse neural progenitor cells was suggested (Kerosuo et al. 2010). Dandy-Walker malformation is a rare congenital malformation and developmental defect of the brain (Reith and Haussmann 2018). Recently, an activating pathogenic mutation on CIP2A via whole-exome sequencing was associated with Dandy-Walker malformation, suggesting the role of CIP2A in the disruption of neuronal development (Austin et al. 2019). Interestingly, CIP2A expression in fibroblast-like synoviocytes is associated with invasiveness and resistance to apoptosis in rheumatoid arthritis (Lee et al. 2012; 2013).

In summary, the overexpression and pathogenic function of CIP2A is well-characterized in cancer. Additional reports suggest its enhanced expression in other

diseases, such as neurodegenerative disorders, and it as a target therapeutic development. Therefore, the physiological immune-cell related functions of CIP2A deserve further attention.

2.4.2 CIP2A inhibitors

The following compounds were also found to inhibit CIP2A expression in cells:

Bortezomib. Proteasome inhibitor Bortezomib (Velcade) causes cell-cycle arrest and apoptosis. Bortezomib inhibits the 26S subunit of the proteasome (Adams and Kauffman 2004). Bortezomib and its derivatives with no proteasomal activity have antitumor activity, and they are approved by Food and Drug Administration (FDA) for multiple myeloma treatments (Niesvizky et al. 2015). In addition to antitumor effects, Bortezomib also downregulates CIP2A expression in cancers and increases PP2A activity (Yu et al. 2013; Lin et al. 2012; Hou et al. 2013; Ding et al. 2014; Chen et al. 2010). The downregulation of CIP2A occurs at the transcriptional level, but the mechanism of Bortezomib in the transcriptional regulation of CIP2A is unclear (Huang et al. 2012; Chen et al. 2010; Lin et al. 2012; Yu et al. 2013). Interestingly, Bortezomib inhibits the release of NF-κB and inflammatory cytokines from T cells. Therefore, Bortezomib has been suggested as a candidate therapy for rheumatoid arthritis, multiple sclerosis, and other inflammatory diseases treatment (Mohty et al. 2013). In adoptive transfer experiments, Bortezomib treatment to recipient animals reduces lung cancer metastases with enhanced antitumor T-cell function in the host (Mohty et al. 2013; Shanker et al. 2015).

Celastrol. Celastrol is a pentacyclic triterpenoid found in traditional Chinese medicine. It is isolated from the roots of *Tripterygium wilfordii* (Zhou 2011). Its anticancer properties are due to mitochondrial apoptotic signaling, but the effector target in mitochondrial signaling is yet to be identified (Yu et al. 2015; Yang et al. 2011; Shrivastava et al. 2015; Mou et al. 2011;Lee et al. 2012). Celastrol induces both caspase-dependent and caspase-independent apoptosis in cancer cells (Yang et al. 2011; Lee et al. 2012; Shrivastava et al. 2015). Recently, reduced levels of CIP2A upon celastrol treatments were associated with inhibition of CIP2A targets, phosphorylated AKT and c-MYC (Zi Liu et al. 2014). Celastrol directly interacts with CIP2A and promotes CIP2A proteolytic degradation by an E3 ubiquitin ligase, CHIP/STUB1, which has enhanced interaction with CIP2A in the presence of celastrol (Liu et al. 2014). Interestingly, it has anti-inflammatory properties. It prevents induction of proinflammatory cytokines IL-6 and TNF-α, and celastrol treatment in animals reduced the incidence of arthritis in an animal model for rheumatoid arthritis (Venkatesha et al. 2011; 2016; Kim et al. 2009).

Erlotinib. The epidermal growth factor receptor tyrosine kinase (EGFR) kinase inhibitor Erlotinib (Tarceva) is an FDA- approved drug for non-small cell lung

cancer treatment (Wu et al. 2015; Mendelsohn and Baselga 2006). Erlotinib binding blocks EGFR phosphorylation and downstream signaling in cancer cells. However, numerous studies identified an EGFR-independent Erlotinib mechanism of action, such as transcriptional downregulation of CIP2A (Yu et al. 2014; Chen et al. 2012; Liu et al. 2017; Wang et al. 2014). Mechanistically, Erlotinib treatment results in ERK inhibition leads to Elk-1 inactivation. Latter restricted nuclear translocation promotes CIP2A transcriptional inhibition in cancer cells (Liu et al. 2017). Interestingly, the immune response was impaired upon Erlotinib treatment, both *in vitro* and *in vivo*, including reduced T-cell proliferation (Luo et al. 2011). Down-regulation of c-Raf/ERK and other Akt signaling components is associated with Erlotinib-mediated impaired T-cell response (Luo et al. 2011). In addition, Erlotinib treatment caused an enhanced anti-tumor immune response (Im et al. 2016).

3 Aims of the Study

The Th cell activation and differentiation play central roles in providing immune protection against extracellular and intracellular pathogens. However, CD4+ T cells are also responsible for the development of several inflammatory disorders and autoimmune diseases (Hirahara and Nakayama 2016). Therefore, understanding the molecules expressed in T cells can help to develop strategies to prevent inflammatory conditions. Multiple reports about the role of the recently characterized protein CIP2A in various types of cancers and other diseases suggested it as a potential therapeutic target (Kauko et al., 2018; Khanna et al., 2016; Khanna et al. 2013). However, the role of CIP2A role in immune cell regulation has not been studied. It is important to establish the influence of CIP2A targeting on the immune response to facilitate the development of related therapies. The objective of the research presented in this thesis was to describe the role of oncoprotein CIP2A in CD4+ T-cell activation and functionally distinct Th cell lineages and particularly Th17 cells.

The presented studies were designed with the following aims:

Aim 1: To elucidate the functional role of CIP2A in T-cell activation (I).

Aim 2: To investigate the role of CIP2A in regulating Th17 cell subset differentiation (II).

Aim 3: To identify the protein Interactome of CIP2A in Th17 cells (III).

4 Materials and Methods

4.1 Human CD4+ T-cell isolation, activation, and differentiation (I, II, and III)

CD4+ T-cell isolation and Th cell differentiation were performed with umbilical cord blood. Anonymous healthy donors were used for cord blood collection at Turku University Hospital, south-west Finland, with permission from the district hospital ethical committee. Human blood from the umbilical cord was first layered on the top of Ficoll (GE Healthcare, cat# 17-1440-03) to enrich mono-nuclear cells. Further, CD4+T cells were isolated from the enriched cells with CD4+ magnetic bead kit (Invitrogen, cat # 11331D).

For Th17 *in-vitro* culture, we used serum-free X-Vivo 20 media (Lonza, Basel, Switzerland) supplemented with L-glutamine (2 mM, Sigma-Aldrich, UK), penicillin (50 U/mL), and streptomycin (50 µg/mL) antibiotics (Sigma-Aldrich). Purified naive CD4+ T cells were activated (2 million cells/well) with plate-bound anti-CD3 antibody (Beckman Coulter, cat# IM-1304, 3750 ng/well of 6-well culture plate) and 1 µg/mL of soluble anti-CD28 antibody (Beckman Coulter, cat# IM1376), and a cocktail of cytokines, including 10 ng/mL of IL6 (Roche, cat# 11138600 001), 10 ng/mL of IL1β (R&D Systems, cat#201 LB) and 10 ng/mL of TGFβ (R&D Systems, cat# 240;) in the presence of 1 µg/mL of the neutralizing antibodies anti-IFNγ (R&D Systems, cat# MAB-285) and anti-IL4 (R&D Systems cat# MAB204). In parallel, cells were cultured in X-Vivo 20 with activation and neutralizing antibodies as described above, but without cytokines, as activated nonpolarized control cells (Th0). The cells were cultured at 37°C in 5% CO_2. Successful Th17 polarization was confirmed by the following three measurements: IL17 RNA expression by TaqMan qPCR, IL17 protein secretion by Luminex or ELISA, and the surface expression of CCR6 by flow cytometry.

For human iTreg cell differentiation, we used CD4+ CD25- cells prepared by depleting CD25+ cells from isolated cord blood CD4+ cells, using LD columns and a CD25 depletion kit from Miltenyi Biotec. The cells were activated (at a density of 2 million cells/mL of serum-free X-Vivo 15 media from Lonza) with plate-bound anti-CD3 (500 ng/24-well culture plate well) and soluble anti-CD28 (500 ng/mL) in the presence of a cytokine cocktail of TGF-β (10 ng/mL), IL-2 (12 ng/mL), (both

from R&D Systems), 10 nM ATRA (all-trans retinoic acid; Sigma-Aldrich), and 10% human serum (Biowest, cat# S4190-100). Control (Th0) cells were activated similarly with plate bound anti CD3 and soluble anti-CD28 and cultured in X-Vivo 15 media without TGFβ, IL2, ATRA, and human serum. Foxp3 expression was measured at 72 h after cell activation by western blotting and flow cytometry-based intracellular staining analysis. In addition, the ability of the iTreg cells to suppress the proliferation of effector cells was used as confirmation for a successful iTreg polarization.

4.2 Cell transfection with siRNA (I, II, and II)

Human CD4+ T cells, isolated as described above, were resuspended in Opti-MEM (Gibco by Life Technologies, cat # 31985-047) at a concentration of 4 million cells in 100 µl for each transfection. The siRNA transfections were performed using the Amaxa nucleofector 2b system (Lonza). Transfected cells were rested for 48 h in RPMI medium supplemented with 10% serum and penicillin/streptomycin antibiotics.

The following siRNAs, synthesized by Sigma/Merck or Dharmacon, were used:

siNT 5′-AAUUCUCCGAACGUGUCACGU-3′ (Control siRNA)
siCIP2A-1 5′-CUGUGGUUGUGUUUGCACU-3′ (Junttila et al. 2007)
Dharmacon siGENOME individual CIP2A siRNAs
siCIP2A-2: 5′-GAACAUAAGCUAGCAAAUU-3′
siCIP2A-3: 5′-GAAACUCACACGACUAUUU-3′
siCIP2A-4: 5′-GCACGGACACUUGCUAGUA-3′
siCIP2A-5: 5′-GUACCACUCUUAUAGAACA-3′
AGK siRNAs taken from (Bektas et al. 2005)
siAGK1 5′-AACAGAUGAGGCUACCUUCAG-3′
siAGK2 5′-GAGGCUACCUUCAGUAAGA-3′
siAGK3 5′-GGAGAGACCAGUAGUUUGA-3′
siPP2A 5'-TTTTCCACTAGCTTCTTCA-3' (Junttila et al. 2007)

4.3 Mice, T-cell Isolation, and Th17 differentiation (I and II)

Mice on a C57BL/6 background in which the CIP2A gene was deleted by gene-trap technology were provided by Prof. Jukka Westermarck (Ventelä et al. 2012). The ethical committee, University of Turku, provided permission to perform experiments with the animals' cells. I have a license issued by the University of Turku to dissect the animals to obtain the tissue for isolation of T cells.

Same-gender mice of 8–12 weeks were euthanized using CO_2, and the spleens were dissected for cell isolation. A syringe plunger was used to squeeze the tissue through the 70-µm cell strainer to make a single-cell suspension. The single-cell suspension was treated with ACK RBC lysis buffer to remove RBC contamination for 10 min (cat# A10492-01, Gibco). Cells were isolated as per the manufacturer's instructions (Miltenyi Biotec, cat# 130-106-643). The purity of the cells was confirmed using the flow cytometry staining for CD4+ T cells, CD62L+ T cells, and CD25+ T cells. Cells with more than 98% CD4+CD62L+ population were considered naive cells and used for *in-vitro* culture. Cells were activated as controls (TCR control) using 1 µg/ml anti-CD3, (cat# 553238; BD Pharmingen) and 2 µg/ml anti-CD28 (cat# 557393; BD Pharmingen), both plate-bound.For Th17 cultures, naive T cells were activated in medium supplemented with cytokines: 20 ng/ml IL-6 (R&D, cat# 406-ML), 1 ng/ml TGF-β1 (R&D, cat# 240-B), 10 ng/ml IL1β (R&D, cat# 201-LB). The medium, Iscove's modified Dulbecco medium (Sigma), was supplemented with 5% (vol/vol) FCS, 0.002 M L-glutamine, non-essential amino acids (cat# 11140-035; Gibco), 100 U/ml of penicillin, 100 µg/ml of streptomycin and 50 µM β-mercaptoethanol (Gibco). For both activation and Th17 polarization, the medium was supplemented with neutralizing antibodies anti-IL4 (10 µg/ml, BD cat# 559062) and anti-IFN-γ (10 µg/ml, BD cat# 557530).

4.4 Flow cytometry (I and II)

For flow cytometry cell-surface staining, cells were first washed with phosphate-buffered saline (PBS) (two times), followed by antibody incubation in FACS I buffer (1% FBS in PBS) for 15 min, at $+4^0$C in the dark. Stained cells were then washed two times again with FACS I buffer and finally resuspended in FACS I buffer for flow cytometer acquisition or 1% formalin for next day acquisition. During intracellular protein staining, after cells were washed twice with PBS, cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature (RT). After two washes with FACS I buffer, cells were permeabilized with permeabilization buffer (10% saponin, 0.05% BSA in PBS) for 15 min on ice. Finally, cells were stained for 30 min with respective antibody incubation, followed by cell resuspension in FACS I/1% formalin. For annexin/propidium iodide (PI) staining, similarly cells (24 h post cell activation) were washed and incubated with annexin (BD, cat# 556419) and PI (BD, cat# 51-66211E) in 2X binding buffer (10 mM Hepes, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4) adjusted to a final 1X concentration in FACS I buffer. Annexin/PI staining cells were immediately acquired in a flow cytometer. For carboxyfluorescein succinimidyl ester (CFSC) cell staining, the homogenous cell suspension was incubated with 5 µM final concentration of CFSC (Invitrogen, cat# C1157) at 37^0C, followed by stopping the reaction with fetal bovine

serum. The stained cells were then cultured, and flow cytometer acquisition was performed on days 4, 5, and 6. The FlowJo (FlowJo LLC, treestar) or flowing software (Version 2.5.1) (http://flowingsoftware.btk.fi/) were used to analyze the data acquired in either BD LSR II or Fortessa/Caliber flow cytometer (BD Biosciences).

The following antibodies used for the analysis: anti-mouse CD69 (eBioscience, cat# 11-0691-82), anti-human CD69 (BD, cat# 347823), mouse IL17-PE (BD, cat# 559502), STAT3-PE (BD phosflow, cat# 557815) and pSTAT3-AlexaFlour 647 (Y705; BD phosflow, cat# 557815).

4.5 TaqMan Quantitative Real-Time Polymerase Chain Reaction (PCR) (I and II)

In TaqMan qRT-PCR analysis, cells were washed twice with PBS, and the cell pellet was resuspended in complete RLT buffer (RNeasy Mini Kit from QIAGEN, cat# 74106) with β-mercaptoethanol. The RNA was isolated, following the manufacture's protocol (RNeasy Mini Kit). The DNase (Qiagen cat# 79254) on-column treatment during the RNA isolation to remove DNA contamination. Potential residual DNA contamination was further removed by in-tube DNase I (Invitrogen™, #18068-015) digestion. The negative qRT-PCR was performed using housekeeping gene primers and probe and the isolated RNA as a template. The Nanodrop 2000 detector (Thermo Fisher Scientific) was used to measure the RNA concentration, and complementary DNA (cDNA) synthesis was performed by using either Roche Transcriptor First Strand cDNA synthesis kit (RNA amounts < 100ng; cat# 04379012001) or Invitrogen's Superscript™ II Reverse Transcriptase (RNA amounts 100 ng–1 µg; cat# 18064-014). 1:5 or 1:10 diluted cDNA was used for TaqMan qRT-PCR runs with target gene probes and primers designed by Universal Probe Library System (Roche Life Science). Quant Studio™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific) and their software were used to run the TaqMan qRT-PCR reactions and data analysis, respectively. In the data analysis, the cycle threshold (Ct) value of the gene of interest was subtracted from that of internal control (EF1alpha) to calculate delta Ct (dCT) and plotted as dCt or fold-change (2^{-dCt}).

4.6 Luminex assay (II and III)

Cytokine (IL17 or IFNγ) measurements in culture supernatants were performed using Luminex assay (Merck Millipore; HCYTOMAG-60K-01) or Duoset ELISA (enzyme-linked immunosorbent assay) kit (R&D Biosystems DY317-05, DY008), according to the manufacturer's instructions. The concentrations of cytokines were

normalized with flow cytometry-based cell counts in each culture to avoid bias due to cell death or proliferation.

4.7 Western blotting (I, II, and III)

Triton-X-100 lysis buffer (TXLB) (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton-X-100, 5% glycerol, 1% SDS) was used to resuspend the cell pellet (1–5 million T cells) and sonicated in a Bioruptor sonicator (Diagenode; 30 sec ON and 30 sec OFF) for 5–7 min, followed by high-speed spin (16,000 g) for 1–5 min. Protein concentration in the supernatant was measured by DC Protein assay (Bio-Rad, cat# 500-0111). The protein extracts (10–30 μg) were run on acrylamide gel (Bio-Rad Mini or Midi PROTEAN® TGX precast gels). For protein transfer, PVDF membranes (BioRad) were used and transfer was performed on Trans-Blot Turbo Transfer System (BioRad).

The membranes were incubated in 5% non-fat milk or BSA TBST buffer (0.1%Tween 20 in Tris-buffered saline) with primary and secondary antibodies. Western blot bands were quantified using Image J software. The antibodies used for the WB analysis were STAT3 (Cell Signaling Technology, cat# 9139), phospho-STAT3 (Y705) (Cell Signaling Technology, cat# 9131), CIP2A (Junttila et al. 2007; Côme et al. 2016), CIP2A (Cell Signaling Technology, cat# 14805), PP2A-A/B (Santa Cruz, cat# sc-15355), Trim21 (Santa Cruz, cat# sc25351), IRF4 (M-17) (Santa Cruz, cat# sc6059), PPP1A/PPP1CA (Abcam, cat# ab137512), PP1α (G-4) (Santa Cruz, cat# sc-271762), β-actin (Sigma, cat # A5441), AGK (Abcam, cat# ab137616), β-actin antibody (Sigma, cat# A5441).

4.8 Cell fractionation (III)

Twenty million Th17 cells were used to isolate the cytoplasmic and nuclear fractions for CIP2A localization studies by WB. The fractions were extracted using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, cat# 78833), following the protocol provided by the kit. The extracted proteins were analyzed by WB where α-tubulin (Santa Cruz, cat# sc-55529) and α-vimentin (Santa Cruz, cat# sc-6260) antibodies were used as control proteins for the cytoplasmic and nuclear fractions, respectively.

4.9 Immunofluorescence (II and III)

Acid-washed glass coverslips (1 M HCl and heat to 50–60 °C treatment for 4–16 hours) were used for immunofluorescence staining. Cells (Th17/Th0) were fixed with 4% PFA, RT, 10 min, and permeabilized (0.1% Triton X-100 and 30% horse

serum in PBS) 10 min at RT. The cells were washed twice with PBS, and non-specific sites were blocked for 1 h at RT with 30% horse serum in PBS and overnight primary antibody incubation at 4°C. The next day, cells were washed with PBS and Alexa fluor-conjugated (Life Technologies) secondary antibody incubation for 1 h at RT. Again, the cells were washed with PBS and mounted with Mowiol at 37°C for 30 min. The instrument Carl Zeiss LSM780 laser scanning confocal microscope equipped with 100×1.40 oil plan-Apochromat objective (Zeiss) was used for taking the images. The confocal image's corrected total cell fluorescence intensities were calculated for statistics and displayed graphically as an arbitrary unit (AU) (Burgess et al. 2010; McCloy et al. 2014).

In pSTAT3 and AGK colocalization experiments, CIP2A- and control siRNA-treated Th17 cell were stained with antibodies against both proteins, and seven views in each of three replicates was used to calculate the colocalization by image J Coloc2 plugin. The data were plotted as Pearson's correlation coefficients. The Mander's coefficients were also observed (data not shown) to avoid expression bias.

Similarly, the UBR5 Immunofluorescence in Hela cells was performed except chambered coverslip (Ibidi, cat# 80826) to culture the cells, and 10% normal goat serum (Abcam, cat# ab7481) were used. DAPI (Invitrogen, cat# D1306) was used to stain the nuclei, and anti-UBR5 (Abcam, cat# ab70311) and Alex Fluor 488 goat anti-rabbit IgG (Invitrogen, cat# A-11008) antibodies were used.

4.10 Proximity ligation assay (III)

The assay manufacture's protocol (Duolink® PLA, Sigma) was used to perform the PLA. Briefly, cells (Th17 or HeLa) were fixed, plated on a coverslip, permeabilized, and a blocking solution used to remove the background staining as described above in the immunofluorescence staining protocol. Before overnight primary antibody incubation, the cell coverslip was incubated in a humidity chamber for 30 min at 37°C. On the next day, kit buffer A was used to wash the unbound primary antibody. Next coverslips were incubated in a pre-heated humidity chamber 1 h, 30 min, and 100 min for PLA probe, ligase, and polymerase amplification reaction solution, respectively, at 37°C. The coverslips were washed with buffer B and mounted with DAPI for confocal microscopy (LSM780, Carl Zeiss)) and 3i CSU-W1 spinning disk microscope equipped with 100x1.4 O Plan-apochromat objective (Zeiss) PLA signal detection. The Cell Profiler software (Carpenter et al. 2006) was used to calculate the PLA signal out of the signal dots.

The following antibodies used in the PLA assay: anti-UBR5 (Abcam, cat# ab70311), anti-CIP2A (Santa Cruz, cat# sc-80659), anti-PPP1CA (Abcam, cat# ab137512), anti-UBR5 (Abcam, cat# ab70311), anti-TRIM21 (Santa Cruz, cat# sc-

25351), anti-GFP (mouse) (Abcam, cat# ab1218) and anti-GFP (rabbit) (Invitrogen, cat# A11122).

4.11 RNA sequencing (II)

CIP2A siRNA-silenced and control siRNA human Th17 cells were harvested at 24 h post-activation. CIP2A knock down at the indicated timepoints was confirmed by WB and TaqMan qPCR analysis before RNA sequencing for gene expression studies. RNeasy Mini Kit was used to isolate the total RNA according to manufacturer's instructions. Illumina protocol was used to prepare the libraries. An advanced Analytical Fragment Analyzer (Advanced Analytical Technologies, Heidelberg, Germany) or 2100 Bioanalyzer (Agilent) was used to study the quality of the libraries, which were quantified using Qubit Fluorometric Quantitation (Life Technologies, Thermo Fisher). The sequencing was performed at the Finnish Functional Genomics Centre (FFGC), University of Turku, using a HiSeq2500 Next-Generation Sequencing platform. The quality control of raw reads by FastQC and trimming of adapters or low-quality bases was performed by Trimmomatic (Bolger, Lohse, and Usadel 2014). The Tophat2 (D. Kim et al. 2013) was used to align the trimmed reads to the human reference genome GRCh37.75 (Ensemble release 75), and each gene's summarized read counts were calculated by HTseq-count (Anders, Pyl, and Huber 2015). Differentially expressed (DE) genes were identified with the R/Bioconductor package edgeR (M. D. Robinson, McCarthy, and Smyth 2010) applying a false discovery rate (FDR) < 0.05.

4.12 Pathway analysis (II and III)

IPA (https: www.ingenuity.com/, May 2016) with P-values < 0.01 was used to determine the significantly enriched pathways. The IPA results with positive and negative z-score were used to identify key upstream regulators and denote predicted upstream positive and negative regulators, respectively. A significance threshold for upstream regulators with p-value > 0.01 and |z-score| > 2 was applied.

4.13 Gene set enrichment analysis (GSEA) and transcription factor binding sites (TFBS) motif enrichment analysis (II)

Th17 and iTreg signature genes upregulated more than fourfold at the 24 h in the respective cell types were taken from the Th17 and iTreg time series data (Tuomela et al. 2016; Ubaid Ullah et al. 2018), and the enrichment of gene set in the CIP2A-silenced RNA-Seq data for 24 h was calculated by using a GSEA tool (Subramanian

et al. 2005). The parameters used for the analysis were as follows: permutation type: phenotype; number of permutations: 1000; gene list sorting: real; enrichment statistics: weighted; metric for ranking: Signal2Noise. TFBS analysis was performed as described in Ubaid Ullah et al. 2018. Briefly, CIP2A silenced 136 DE genes promoters (-1000 to 100 bp from the transcription start site) was analyzed by the FMatch tool of the TRANSFAC database (Release 2018.2) and TFs DE in human Th17 or iTreg cells (Ubaid Ullah et al. 2018; Tuomela et al. 2016) were used to make the matrices custom profile. Using R (version 3.4.3), the enrichment was calculated by the binomial test and the Benjamini & Hochberg method was used to correct the p-value, an FDR < 0.05.in was used.

4.14 Immunoprecipitation (II and III)

Specific antibodies were used to immunoprecipitate STAT3 and CIP2A along with their respective Immunoglobulin G (IgG) control IPs. In addition, CIP2A interactors were compared by using two CIP2A-specific antibodies that recognize distinct regions of CIP2A protein. For IP, we used Pierce™ MS-Compatible Magnetic IP Kit (Thermo Fischer, cat# 90409), following manufacturer's instructions. Briefly, cells were harvested on ice and washed twice with PBS to remove the culture media. The ice-cold IP-mass spectroscopy (MS) cell lysis (IP-MS) buffer supplied in the kit was used to resuspend the cells for 10 min with periodic mixing for cell-lysis. The cell lysate was centrifuged at full speed to remove membranes and other debris. The supernatant was transferred to new tube, and protein measurements were performed using the NanoDrop™ spectrophotometer (Thermo Scientific). 500–1000 µg of protein lysate in IP-MS cell lysis buffer was incubated with antibody (1:50 dilution) at +4ºC overnight. The immune complex was incubated with protein A/G magnetic beads for 1 h at RT on second day. The target protein and their respective interactors were later eluted after washing the complex and the magnetic beads with the washing buffer supplied in the kit. The eluate was later transferred to a new low binding Eppendorf sample tube. The elution buffer was removed by vacuum dry centrifugation for SDS PAGE and WB analysis.

4.15 Mass spectrometry (II and III)

The respective biological IP samples were denatured with 8 M urea and dithiothreitol, reduced alkylated with iodoacetamide, digested with trypsin (37^0C, 16 h). The samples were desalted using Empore C18 disks (3M) (Fisher scientific, cat# 2215). Based on Nanodrop-1000 UV spectrometer detection, proteomics analysis of equivalent aliquots of the digested peptides were made in triplicates by liquid chromatography-tandem MS (LC-MS/MS) using a Q-Exactive HF quadrupole–

Orbitrap mass spectrometer (Thermo Fisher Scientific) with EASY-nLC 1200 UPLC system. The i.d. pre-column 20 x 0.1 mm and i.d. analytical column 150 mm x 75 µm packed in conjunction with Reprosil 5-µm C18-bonded silica (Dr Maisch GmbH) were used with a gradient of 8–45% B for 78 min at a flow rate of 300 nl/min for separation and, water with 0.1% formic acid (A) and 80% acetonitrile 0.1% formic acid (B) compositions for the mobile phase.

4.16 Selected reaction monitoring mass spectrometry (III)

The MS detected protein targets of interest (CIP2A and AGK for publication II and 20 for publication III) were validated by SRM MS. The synthetic peptide analogs isotopically labelled (lysine $^{13}C_6$ $^{15}N_2$ and arginine $^{13}C_6$ $^{15}N_4$) were selected based on their consistency in discovery data, LC-MS/MS using a Q Exactive HF quadrupole–Orbitrap mass spectrometer (Thermo Scientific). The integrity of the signals from the transitions measured for the heavy labelled and native peptide MS/MS spectra was evaluated by the Skyline software (MacLean et al. 2010).

To validate the MS-detected interactome by SRM-MS, the CIP2A IP samples were prepared as for the discovery measurements and spiked with synthetic heavy-labeled peptide analogs of the targets and retention time standard (MSRT1, Sigma). The TSQ Vantage Triple Quadrupole Mass Spectrometer (Thermo Scientific) coupled to Easy-nLC 1000 liquid chromatograph (Thermo Scientific) was used for the LC-MS/MS analyses. A faster gradient of 8–43% B in 27 min, then to 100% B in 3 min, at a flow rate of 300 nl/min was used to separate the peptide, although the column configuration was same as in the discovery measurements with the Q-Exactive. Similarly, the compositions of the mobile phases were water with 0.1 % formic acid (A) and 80% acetonitrile 0.1% formic acid (B). PASSEL (Farrah et al. 2012) with dataset identifier PASS01186 was used to deposit SRM data.

4.17 MS data analysis (II and III)

A MaxQuant with the inbuilt Andromeda search engine (Jürgen Cox and Mann 2008; Jürgen Cox et al. 2011) was used to search their data using a UniProt human protein sequence database (version June 2016, 20,237 entries). The database search parameters used were methionine oxidation as a variable modification, cysteine carbamidomethylation as a fixed modification, and two missed cleavages. An FDR of 0.01 at the level of peptide and protein was applied. To calculate the relative protein intensity profiles across the samples, MaxQuant's label-free quantification (LFQ) algorithm was used with the "match between runs" function enabled. The Perseus statistics and informatics platform was used to process the values of LFQ

protein intensity (MaxQuant output). The output was filtered to remove the proteins detected by less than two peptides (Jürgen Cox et al. 2014; Tyanova et al. 2016). The Perseus permutation-based t-test was used to evaluate the relative abundance of the proteins detected in the pull-downs. The Resource for Evaluation of Protein Interaction Networks interface was used to analyze the filtered LFQ protein intensities (Mellacheruvu, et al. 2013). Briefly, the average LFQ of the triplicate were calculated for each biological replicate of the IPs and the mock baits. The relative protein intensities were analyzed using the Significance Analysis of INTeractome (SAINT) algorithm, and only proteins were included with a SAINT score $\geq$ 0.7 (Choi et al. 2011) were included. In addition, a catalog of immunoprecipitation experiments (CRAPome) was used to filter and remove common protein contaminants from the interaction list (Mellacheruvu, et al. 2013). The cut-off of 60% of the proteins present in the contaminant repository database was excluded as there was no direct match between the samples and the IP conditions.

4.18 Network and enrichment analysis (II and III)

The functional protein association network database STRING version 11 (Szklarczyk et al. 2017) was used to map and download the known factors (STAT3 and CIP2A), from which the high confidence interactions, (interaction score $\geq$ 0.7) networks were visualized with Cytoscape version 3.7.1 (Shannon et al. 2003). The Cytoscape plug-in cluster Maker v2 algorithm MCL Cluster (Morris et al. 2011) with the granularity parameter (inflation value) set to 1 (STAT3 interactome) and 1.8 (CIP2A interactome) were used to identify the clusters using the Markov clustering algorithm in Cytoscape (Brohée and van Helden 2006). The SAINT probability scores (SP) were used to define combined SP to define the strength of the protein interactions with STAT3 in CIP2A silenced and control conditions. The combined SP was calculated as SP siCIP2A - SP siNT and range from -1-0-1, which designate interactions only in control, equal strength of interactions and only in the CIP2A silenced condition, respectively, with a coded continuous color gradient from blue (-1) to white (0) to red (1). The CIP2A interactome immuno-precipitates and the controls IgG MS intensity differences were calculated as the normalized log2-transformed intensities and coded as continuous gradient from the white to grey node inner color.

The Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 (Huang et al., 2009a; Huang et al., 2009b) and the Protein Annotation Through Evolutionary Relationship (PANTHER) classification system version 13.0 (Mi et al. 2013; 2017) were used to identify STAT3/CIP2A interactome associated enriched Gene Ontology (GO) biological processes. The analysis was performed

using the STAT3/CIP2A interactome as the input and the Th17 proteome as background reference (Tripathi et al. 2019). The DAVID GO FAT terms were considered to filter the non-specific terms (Huang et al., 2009b). The GO FAT enriched biological process was defined for any cluster with more than four members. To summarize, the enriched biological process in the STAT3/CIP2A interactome and the GO SLIM terms from PANTHER were used to identify the broadest terms and filter the more specific ones. The enriched biological process was defined at FDR ≤ 0.05. The IPA analysis was used to summarize the cellular location and classification of the identified proteins.

4.19 Data availability (II and III)

The RNA-Seq data in publication II is deposited under the accession number GSE118094 at National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database. The ProteomeXchange Consortium via the PRIDE (Vizcaíno et al. 2016) partner repository with identifiers PXD010612 and PXD008983 were used to deposit the STAT3 and CIP2A interactome, respectively. The CIP2A interactome validation SRM MS data was submitted with identifier PASS01186 to Peptide Atlas (Desiere et al. 2006), and the raw reads are available through PASSEL (Farrah et al. 2012).

5 Results

5.1 Defective T-cell activation and proliferation upon CIP2A depletion (PART I and II)

RNA-Seq gene expression profiling of human and mouse T cells showed that CIP2A (KIAA1524) expression is upregulated in TCR-activated CD4+ T cells (Tuomela et al. 2016). In our further investigations, both WB and TaqMan qPCR analysis were used to demonstrate this difference. In contrast, CIP2A expression was undetectable in naive T cells (I; Figure 4; II, Figure 1 and S1). WB analysis additionally confirmed CIP2A expression in activated CD8+T cells (I; Figure 4).

The effects of CIP2A depletion in CD4+ T cells were observed to identify the significance of CIP2A in T-cell activation. Five siRNAs (named as siCIP2A1-5) targeting different regions of CIP2A were tested and the best three (siCIP2A1, 4, and 5) were selected (II; Figure 2) to exclude CIP2A depletion off target effects. Flow cytometry analysis of CD69 and CD25 expression was used to compare differences in the activation of human cord blood CD4+ T cells transfected by CIP2A-targeting and control siRNA, and CIP2A deficient/sufficient mouse CD4+CD62L+ T cells isolated from the spleen. These measurements revealed that expression of CD69 and CD25 was reduced in activated CIP2A-depleted human and mouse T cells (I, Figure 4; II, Figure S2). In addition, the effects of CIP2A ablation in human and mice cells on T-cell proliferation were monitored by CFSC dilution. The absence of CIP2A in T cells resulted in the delayed proliferation of both human and mouse Th cells (II; Figure S2). Using the annexin and PI flow cytometry assay, analysis of cell viability demonstrated no significant cell death upon CIP2A depletion in T cells (II; Figure S2).

Following these observations, we sought to investigate whether CIP2A expression is induced by TCR triggering or autocrine/paracrine IL-2 signaling. TaqMan PCR analysis of T-cell activation using TCR triggering or IL2 alone indicated CIP2A expression was induced upon TCR activation but not with IL-2 stimulation (II; Figure 1). The results demonstrated that T-cell activation upregulates CIP2A expression, and its absence results in defective T-cell activation and proliferation. While PP2A and CIP2A regulate cell proliferation in cancer cells (Ventelä et al. 2012; Yang et al. 2016; Wang et al. 2017; Kauko et al. 2018; Kerosuo

et al. 2018; De et al. 2014), this study was the first to report the influence of CIP2A on T-cell activation. Therefore, CIP2A is a TCR- but not an IL-2-induced protein and is essential for T-cell activation and proliferation. Moreover, CARMA1 is an adapter protein that functions as a molecular scaffold to recruit signaling mediators essential for T-cell activation (Eitelhuber et al. 2011). Interestingly, PP2A plays a significant role in limiting T-cell activation by direct interaction and dephosphorylation of scaffolding proteins CARMA1 (Eitelhuber et al. 2011). This suggests that hampered T-cell activation due to the absence of CIP2A might be due to enhanced PP2A activity. Further studies are required to investigate whether CIP2A prevents CARMA1 dephosphorylation from PP2A and promotes T-cell activation.

5.2 CIP2A negatively regulates IL17 expression in human Th17 cells (PART II)

We first studied IL-17 expression between CIP2A-targeting and non-targeting siRNA-treated human CD4+ T cells at 72 hours in Th17 polarizing conditions. The enhanced expression of IL-17 and CCR6 in CIP2A-silenced human Th17 cells was observed (II; Figure 2). Similarly, upregulation of IL-17 expression was also seen in mouse CIP2A-deficient cells under Th17 polarizing conditions for 5 days (II; Figure 2). The detection of *IL17* expression was performed at the mRNA level by TaqMan PCR and the protein level by intracellular flow cytometry staining or IL-17 protein secretion. Similar to our observations in activated cells, CIP2A silencing reduced Th17 cell proliferation (II; Figure S2). Thus, the upregulation of *IL17* expression upon CIP2A depletion in Th17 cells was not due to enhanced cell proliferation.

5.2.1 Gene expression analysis of CIP2A-silenced Th17 cells (PART II)

To gain insight into the underlying mechanism of the enhanced *IL17A* expression observed with CIP2A depletion, genome-wide (RNA Seq.) gene expression analysis was performed for 24 h polarized human Th17 cells. With CIP2A knockdown, 136 DE genes were detected with an FDR cutoff of <0.05 and $\log_2$ fold-change ($\log_2$FC) increase in expression of 1.0 (II; Figure 3; table 1). Among these were several well-known Th17 genes that were upregulated upon CIP2A depletion, including the transcription factors *RORC*, *RORA* and *MAF*, further supporting hypothesis for negative regulation of IL-17 signaling by CIP2A. In contrast, *IRF8* and *IFNG*, which repress Th17 differentiation, were among the downregulated genes in CIP2A-silenced Th17 cells. Among the other genes of interest downregulated upon CIP2A silencing were regulatory associated protein of MTOR complex 1 (*RPTOR*), and

colony stimulating factor 2 (*CSF2*). RPTOR is a component of signaling in response to nutrient availability that controls cell growth and autophagy. RPTOR modulates the activity of the rapamycin complex 1 (mTORC1) by acting as the scaffold for recruiting mTORC1 substrates. Intriguingly, a positive loop exists between CIP2A and mTORC1 in the inhibition of autophagy (Puustinen et al. 2014). CIP2A inhibits PP2A to prevents dephosphorylation and degradation of mTORC1, and the latter prevents autophagic degradation of CIP2A (Puustinen et al. 2014). Thus, our results support the concept of transcriptional regulation of the mTORC1 subunit RPTOR by CIP2A, in addition to the previously reported CIP2A mTORC1 regulation. CSF2 (colony stimulating factor 2) is a cytokine essential for EAE disease development in animals (Gaffen et al. 2014; Stockinger and Omenetti 2017). Accordingly, it will be important to assess CIP2A depletion in an autoimmune animal disease model.

GSEA (Subramanian et al. 2005) confirmed the global upregulation of Th17 signature genes upon CIP2A ablation in Th17 cells. However, iTreg gene signature was not enriched. Th17 and iTreg signature genes were defined based on their upregulation in respective cell types at 24 h, FDR <0.05, log2[FC] > 2) (Tuomela et al. 2016;Ullah et al. 2018). CIP2A-silenced DE genes in Th17 cells were used for IPA to identify the enriched pathway and classification of DE genes in CIP2A-silenced Th17 cells. The IPA analysis revealed that enzyme was the most abundant class of DE genes and IL-17 regulated pathways in epithelia, macrophages, and Th cell, were the most enriched, further supporting IL17 signaling inhibition by CIP2A in Th17 cells (II; Figure 3).

5.2.2 CIP2A controls STAT3 signaling to constrain Th17 differentiation (PART II)

IPA analysis was performed to predict the upstream regulators of the genes DE upon CIP2A silencing in Th17 cells. In addition, the promoters of the DE genes were analyzed for TFBS enrichment. Among the key upstream regulators |Z score <-2 or > 2|, STAT3 enrichment was found in IPA analysis (II; Figure 4). The direct binding of STAT3 is known to regulate *IL17* expression during Th17 differentiation, and its deficiency in animals prevents EAE disease development (Liu et al. 2008). Notably, we found increased and sustained STAT3 phosphorylation (Y705 and S727) levels in CIP2A-silenced cells (II; Figure 4). To further investigate this association, protein interactions with pSTAT3 (Y705) in CIP2A sufficient and deficient Th17 cells were determined by MS (II; Figure 5). LC-MS/MS proteomics analysis was made for immunoprecipitants of pSTAT3 (Y705) in CIP2A-silenced and control Th17 cells. Confirming the successful pulldown, STAT3 was the most enriched protein (II; Figure 5; Table S4). After statistical analysis and filtering on the basis of cut-offs (P < 0.05, and SP >0.7) (Emani et al. 2015), 217 protein interactions were common

between the conditions, but 69 and 49 interactions were exclusive to pSTAT3 proteins in CIP2A-sufficient and -deficient Th17 cells, respectively (II; Figure 5). GO and network analysis of this pSTAT3 interactome revealed that immune response was among the most enriched biological processes related to the STAT3 interactors (II; Figure 5). The pSTAT3 interactome identified in this study provides a resource for future studies

Interaction between kinase AGK and ubiquitin ligase TRIM21 with pSTAT3 were demonstrated in CIP2A-sufficient and -deficient Th17 cells, respectively (II; Figure 5-6). Furthermore, TRIM21 and AGK interaction was also observed in the interactome of CIP2A in Th17 cells (Khan et al. 2020b). TRIM21 is known to regulate proinflammatory IL-17 producing cells. *Trim21* depletion in animals results in enhanced production of proinflammatory cytokines (including IL-17), and autoimmunity (Espinosa et al. 2009; Ahn et al. 2017). In addition, TRIM21 is known for IRF8 proteasomal degradation. The *IRF8* downregulation was also observed by RNA-Seq analysis of *CIP2A*-silenced Th17 cells. However, since *CIP2A* silencing resulted in increased pSTAT3 without influencing the total STAT3 levels, this suggests that the interaction of TRIM21 with pSTAT3 in CIP2A-sufficient conditions would not explain the upregulation of pSTAT3 observed with CIP2A depletion. AGK regulates the JH2 autoinhibitory domain of JAK2 and STAT3 phosphorylation in cancer cells (Chen et al. 2013). *AGK* silencing downregulates pSTAT3 phosphorylation, but overexpression results in enhanced pSTAT3 in cancer cells (Chen et al. 2013).

Therefore, we hypothesize CIP2A interaction with AGK could be the mechanism for the enhanced STAT3 phosphorylation in CIP2A-silenced Th17 cells. The stronger interaction between pSTAT3 and AGK in *CIP2A*-silenced Th17 cells was validated by pSTAT3 IP, followed by WB analysis (II; Figure 6). The confocal microscopy as an additional validation confirmed the enhanced interaction between AGK and pSTAT3 in *CIP2A*-silenced Th17 cells (II; Figure 6). Notably, we demonstrated downregulation of pSTAT3 levels in *AGK* RNAi-silenced Th17 cells, but total STAT3 levels was not affected upon *AGK* silencing which is comparable to earlier report in cancer cells (X. Chen et al. 2013). Furthermore, the simultaneous depletion of both *AGK* and *CIP2A* can rescue enhanced pSTAT3 and IL-17 levels (II; Figure 6). Thus, these measurements demonstrated that the CIP2A interaction with AGK regulates pSTAT3 and IL-17 expression levels in Th17 cells.

5.2.3 PP2A independent CIP2A functions in Th17 cells (PART II)

Initially, CIP2A inhibition of PP2A activity towards c-Myc and AKT was identified from studies of CIP2A overexpression in cancer cells (Junttila et al. 2007; O'Connor

et al. 2018; Kauko and Westermarck 2018). The reported interaction between CIP2A and PP2A in cancer cells was further demonstrated in Th17 cells (Junttila et al. 2007;Wang et al. 2017; Khan, et al. 2020b). Other reports have shown PP2A positively regulates IL-17 expression and PP2A gene knock out animals display impaired Th17 cell differentiation (Xu et al. 2019; Apostolidis et al. 2013). Therefore, we hypothesized that the enhanced IL-17 expression during inhibition of CIP2A in Th17 cells could be due to an increase in PP2A activity. To test this hypothesis, PP2A was inhibited by two different concentrations of siRNA specific to the PP2A-A subunit. The PP2A-A subunit was selected since it is a scaffolding subunit, and its inhibition could disperse the holoenzyme composition. In addition, a chemical inhibitor of PP2A, okadaic acid, was used in Th17 cells. Okadaic acid has broad activity as a serine threonine phosphatases inhibitor, but at low concentration, okadaic acid is more specific to PP2A than to other serine threonine phosphatases (Kauko et al. 2018; Kauko and Westermarck 2018). Therefore, we used the least tolerated okadaic acid doses for experiments and included the siRNA to compare the results. We also used FTY720, an immune suppressive compound and a PP2A activator drug (O'Connor et al. 2018; Kauko and Westermarck 2018; Chun and Hartung 2010). FTY720 activates PP2A by disrupting its interaction with PP2A endogenous inhibitor SET/I_2^{PP2A}. In disease conditions, FTY720 suppresses inflammatory cytokines, including IL-17. Interestingly, PP2A inhibition by either RNAi or OA in Th17 cells resulted in upregulation of IL-17 expression, and PP2A activator FTY720 treatment led to reduced IL-17 expression in Th17 cells (II; Figure 7).

Previous studies targeting the same molecule in human and mouse cells resulted in the development of opposite phenotypes (Ciofani et al. 2012; Tripathi et al. 2019). For example, silencing of the chromatin regulator *SATB1* in human Th17 cells upregulates IL-17 expression, but its deficiency in mice reduces IL-17 in Th17 cells (Ciofani et al. 2012; Tripathi et al. 2019). Similarly, the effects of PP2A inhibition in mice and human Th17 cells are not same. In line with this, only a small degree of overlap of transcriptome and proteome between human and mice Th17 cells was reported (Tripathi et al. 2019; Tuomela et al. 2016). Therefore, phenotypic characterization of a gene should be first carried out in human cells before using the *in-vivo* animal mouse model for detailed characterization (Stockinger and Omenetti 2017). Furthermore, CIP2A participates in a specific interaction with the PP2A-B56 subunit that is indispensable for the stability of CIP2A (J. Wang et al. 2017). Also, the composition of PP2A depends on the cellular expression of the subunit isoforms, as this can dictate the functions of the PP2A holoenzyme (Janssens et al. 2001; Janssens et al. 2008; O'Connor et al. 2018; Kauko et al. 2018). In summary, the upregulation of IL-17 expression both by inhibition of CIP2A and PP2A could be due to the substrate specificity of CIP2A towards the PP2A-B56 subunit.

Subsequently, it will be important to establish the significance of PP2A subunit specific immune responses in future studies.

A range of studies indicated the importance of CIP2A as a target for drug therapies for cancer treatment (O'Connor et al. 2018; Kauko and Westermarck 2018). In the work presented in this thesis, increased expression of the proinflammatory cytokine IL-17 was observed upon CIP2A depletion (Khan, et al. 2020a). Nevertheless, an increase in IL-17 expression in the absence of CIP2A does not necessarily mean that there will be a twist towards an inflammatory phenotype upon targeting CIP2A. This is because IL-17 also provides protection against fungal and bacterial pathogens (Stockinger and Omenetti 2017). Similar to CIP2A, a deficiency of Tbet resulted in the upregulation of IL-17 expression (Lazarevic et al. 2010). However, *Tbet* deficiency in animals ameliorated EAE disease (Wang et al. 2014). Interaction of Tbet and Runx1 inhibits the binding of Runx1 to the *Il17* promoter, and therefore, absence of Tbet removes this inhibition and increased IL-17 expression (Lazarevic et al. 2010). However, Tbet and Runx1 are indispensable for the ontogeny of EAE in animals (Wang et al. 2014). Interestingly, CIP2A also interacts with the Runx1 (Khan et al. 2020a). Therefore, future studies are needed to further clarify the role of CIP2A in T cells and related immune diseases.

5.3 CIP2A protein interactome in Th17 cells (PART III)

To further understand the molecular mechanisms of CIP2A functions in T cells, we analyzed CIP2A protein interactions in human Th17 cells. The choice of cell type was motivated by the observed role of CIP2A in Th17 cell differentiation (Khan et al. 2020a). To avoid detection of non-specific interactions and to improve reproducibility, two CIP2A specific antibodies (Ab1 and Ab2) targeting different regions of CIP2A were used together with their respective control IgG (IgG1 and IgG2) antibodies (III; Figure 1; S1). Ab1 is monoclonal and specific to the N-terminal region (residues surrounding Val 342) and Ab2 polyclonal, targeting the C-terminus (Hoo, Zhang, and Chan 2002) (III; Figure 1; S1). The use of two specifically validated antibodies made it possible to carry out IP of CIP2A in primary human T cells, without the need for CIP2A overexpression. For these measurements, CIP2A IP was performed in 72-h polarized human Th17 cells.

After trypsin digestion of the IP samples for proteomics analysis, 680 CIP2A proteins were detected by LC-MS/MS. An acceptance criterion of two or more unique peptides of confidence threshold 99% or more was applied. The list of proteins was further filtered to reduce the contribution of non-specific interactions and common contaminants. Using a software package for scoring protein-protein interactions (SAINT), proteins with probability scores of greater than or equal to

0.95 were retained, and proteins frequently detected (>60%) in a database aggregated from negative controls (CRAPome) were removed (Mellacheruvu et al. 2013). In total, 335 putative CIP2A protein interactions and over 80% of interacting proteins were identified with both antibodies. The top 50 were represented (III; Figure 1). CIP2A(KIAA1524) protein was among the most enriched proteins detected with both antibodies.

Included in the top CIP2A interactions were beta-spectrin (SPTBN1), TRIM21, phosphatase PP1, adaptor protein DOCK8, and LIMA. In addition, among the most significant interactions identified were phosphatase PP1 regulatory (PPP1R18 and PPP1R12A) and catalytic (PPP1CA) subunit. Previously, the lead inhibitory role of CIP2A has been linked to phosphatase PP2A. However, in this study, we showed for the first time the interaction of CIP2A with PP1. Further studies are needed to establish whether the inhibitory functions of CIP2A are common to other phosphatases. Additionally, increased phosphatase PP1 activity has been observed in rheumatoid arthritis. The PP1 dephosphorylate (Ser418) and degrade FOXP3 and thus, impaired Treg response and inflamed synovium (Nie et al. 2013). The known associations of CIP2A (Lee et al. 2012; 2013) and PP1(Nie et al. 2013) with RA and their interaction in human Th17 cells (Khan et al. 2020b) merits further investigations.

The protein SPTBN1 regulates the shape of cells and organelles and has not been studied in the context of CIP2A (Machnicka et al. 2012). Interestingly, both CIP2A and SPTBN1 are overexpressed in neurodegenerative AD pathology, and SPTBN1 has been considered as an AD biomarker (Sihag et al. 1996; Yan et al. 2012). In AD, CIP2A overexpression is associated with PP2A inhibition, and hyperphosphorylation, and mis-localization of tau (Y. P. Shentu et al. 2018). In addition, CIP2A overexpression in animals induced AD-like cognitive deficits (Shentu et al. 2018). Accordingly, it will be essential to determine whether the interaction of CIP2A with SPTBN1 plays a role in the development of AD.

The E3 ubiquitin ligase TRIM21 is involved in proteasomal regulation of the interferon regulatory factor (IRF) family, and its gene or genetic polymorphism has been linked to the autoimmune disease SLE. TRIM21 provides protection in inflammatory bowel diseases by inhibiting Th1 and Th17 cells in the intestinal mucosa (Espinosa et al. 2009; Yoshimi et al. 2012; Zhou et al. 2017). In addition, *Trim21*- deficient animals have reduced ubiquitination of protein IRF5 and enhanced B-cell differentiation, and its dysfunction has been suggested for SLE disease pathogenesis. Enhanced PP2A expression and activity leads to IL2 repression and represents a central component of the immunopathology of SLE disease (Katsiari et al. 2005). Therefore, further studies can identify the significance of CIP2A interactions with TRIM21 and PP2A in SLE disease.

Another notable CIP2A-interacting protein is the adaptor protein DOCK8. Mutations in the *DOCK8* gene have been associated with combined immunodeficiency in humans in a very similar manner as has been observed with STAT3 mutations (Su 2010; Q. Zhang et al. 2009). In addition, both STAT3 and DOCK8 are components of the same signaling pathway. The latter acts as an adaptor protein for the recruitment of kinases, regulating STAT3 phosphorylation in B cells (Jabara et al. 2012). It would be interesting to characterize the relevance of enhanced STAT3 phosphorylation, and its interaction with DOCK8 in CIP2A silenced cells in future studies.

5.3.1 CIP2A interactome–cellular distribution and the associated processes (PART III)

Previous studies with epithelial cancer cells indicated that CIP2A is mainly localized in the cytoplasmic subcellular region and has some presence in the nuclear region (Myant et al. 2015; Junttila et al. 2007). By confocal microscopy and WB analysis, we further confirmed CIP2A localization in the cytoplasmic and nuclear regions in Th17 cells (III; Figure 2; S2)**.** Phalloidin and DAPI were used to differentiate the cytoplasmic and nuclear regions in confocal microscopy. In addition, vimentin and tubulin were used as control proteins for WB analysis of the nuclear and cytoplasmic fractions, respectively. Annotation of the cellular locations of the proteins interacting with CIP2A was performed by IPA (Qiagen). Consistently, the CIP2A-interacting proteins were distributed in both of these sub-cellular compartments (III; Figure 2). Overall, this dataset provides a comprehensive view of CIP2A interacting proteins, reflecting localization of CIP2A in both the nuclear and cytoplasmic cellular compartments regions.

Further, GO and bioinformatics interaction tools performed network analysis for the biological processes associated with the CIP2A interactome. The CIP2A interactome network determined from the STRING database was visualized with Cytoscape (Shannon et al. 2003; Szklarczyk et al. 2017). The process most frequently linked with the interactome was “RNA metabolism or splicing”. (III; Figure 3). This is consistent with a previous phospho-proteome study linking CIP2A with RNA splicing (Kauko et al. 2020). However, further studies are required to dissect the role of CIP2A in the regulation of RNA biogenesis. Based on the intensities of protein interactions with both CIP2A antibodies, the top proteins interacting with CIP2A are represented in the form of a heatmap (III; Figure S3**).** The functional enrichment analysis by bioinformatics tools DAVID and PANTHER similarly revealed CIP2A-interacting proteins involved in RNA-related processes, including splicing among the most important biological processes (Huang et al. 2009b; Mi et al. 2017; 2013). IPA analysis revealed that the molecular function of

the majority of CIP2A interacting proteins was enzymes (III; Figure 3). Also, in addition to these, putative interactions with a diverse class of proteins, some not well known, were shown.

In summary, the interactome bioinformatics analysis revealed that the majority of the CIP2A-associated proteins are involved in RNA processes, including splicing. In cancer cells, splicing of apoptotic proteins produces variant proteins that promote anti-apoptotic pathways (David and Manley 2010; Schwerk and Schulze-Osthoff 2005). It will be interesting to study further the splicing-associated role of CIP2A in cancer cell transformation. Splicing of Treg lineage TF FOXP3 plays a role in Th17 cell inhibition (Mailer et al. 2015). Future studies could establish whether there are splicing-related functions of CIP2A in T-cell differentiation and cancer development.

5.3.2 CIP2A protein interactome validation (PART III)

SRM-MS, PLA, confocal microscopy, and WB were used to validate CIP2A protein interactions. SRM-MS analysis of three independent biological replicates confirmed more than 20 CIP2A interaction targets (III; Figure 4; Supplementary Table 4-5). Interactions between the phosphatase PP1 and CIP2A were identified and validated, in addition to the known CIP2A interaction with PP2A (III; Figure 4-5). Potentially, the identified CIP2A interaction with the catalytic subunit of both PP1 and PP2A phosphatase. The WB and PLA also confirmed CIP2A interactions with PPP1CA, TRIM21, STAT1, IRF4, UBR5, and PP2A. (III; Figure 5). Additionally, siRNA ablation of CIP2A in Th17 cells identified the functional significance of the CIP2A interaction with NF-κB2 by phosphorylation upregulation of NF-κB2 in CIP2A-silenced Th17 cells (III; Figure 5).

Previously, the interaction between CIP2A and the PP2A-A and PP2A-B (B56) subunits was identified in cancer cells (Junttila et al. 2007; Wang et al. 2017). In this study, we verified these CIP2A-PP2A interaction in Th17 cells. In addition, we showed, for the first time, the interaction of CIP2A with the PP2A-C (PPPCA and PPPCB) catalytic subunits. Since the phosphatase PP1 catalytic subunit was also identified among the CIP2A interactors and the catalytic subunit of both phosphatases is highly conserved in evolution (Shi 2009), the interaction could be because of their structural similarity. Further, the SNP on the catalytic subunit of PP2A has been associated with SLE, and its overexpression is linked with the pathology of the disease (Tan et al. 2011; Katsiari et al. 2005). Accordingly, it will be important to study if CIP2A has a role in SLE. In addition, high PP2A activity was demonstrated indispensable for the suppressive functions of Treg cells and IL17 expression in mouse T cells (Apostolidis et al. 2013; Xu et al. 2019; Apostolidis et al. 2016). In the work presented in this thesis, we have also shown PP2A regulation

of IL17 expression in human Th17 cells (II; (Yang et al. 2016)). In human Th17 cells, PP2A siRNA depletion or inhibition by okadaic acid negatively regulates *IL17* expression. In line, the use of PP2A activator FTY720 downregulates IL-17 in human Th17 cells. PP2A activation for treatment of cancer and other diseases seems to be promising. Thus, our results further supporting PP2A activation could be beneficial in human inflammatory diseases. However, PP2A promotes IL-17 expression in mice T cells and ameliorated EAE in PP2A gene-deficient animals. The differences could be due to species specific differences in PP2A functions.

The validated CIP2A interactions by SRM-MS and WB analysis with T-cell-associated TFs are RUNX1, IRF4, STAT1, and DDX5. IRF4 initiates Th17 differentiation, and PP2A regulation of IRF4 has been studied (Apostolidis et al. 2013; Ciofani et al. 2012). In addition, DDX5 is also important for Th17 differentiation (W. Huang et al. 2015). Further studies are needed to understand the importance of these interactions for Th17 cell differentiation.

6 Summary

The activation and differentiation of CD4+T cells into the functionally distinct effector and regulatory T helper cell lineages enable balanced immune responses, protecting against pathogens. In addition to the hallmark transcription factors and cytokines, numerous molecules affect these processes, and a detailed understanding of T helper cells signaling pathways and regulatory mechanisms is vital for the treatment and prevention of immune-mediated diseases. In the work presented in this thesis, the role of CIP2A in T helper cell activation, differentiation and functions of Th17 cells functions was studied. To gain further insight into the targets and interactions of CIP2A in Th17 cells, its protein interactome and the influence of silencing, and the associated changes in the pSTAT3 interactome were determined.

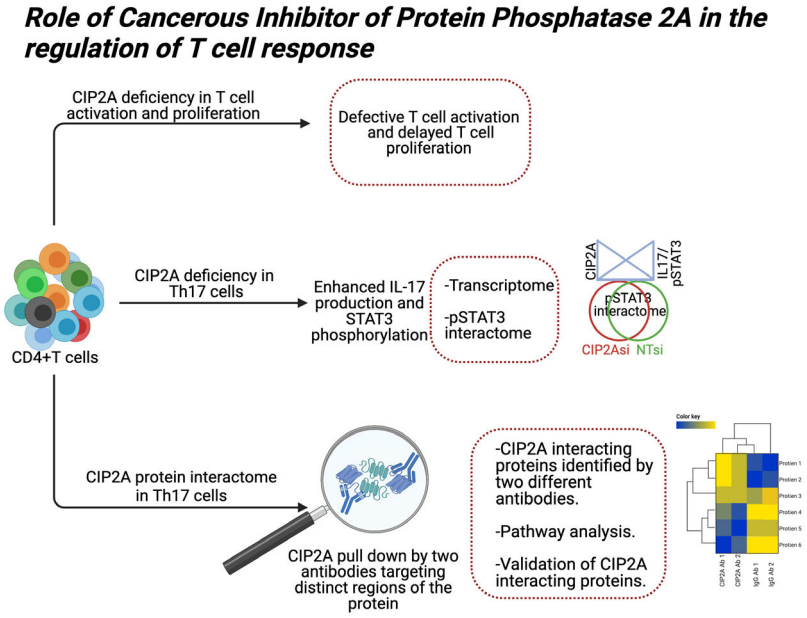

Figure 6. Summary of thesis research study results in the form of Illustration. The upper panel showing CIP2A depletion hampered T-cell activation. Middle panel, upregulation of IL-17 and phosphorylation of STAT3 was further studied by transcriptome analysis and pSTAT3 interactome in CIP2A-silenced cells. The last arrow shows the CIP2A protein interactome in Th17 cells using two antibodies targeting different regions of CIP2A.

Among the T helper subsets, Th17 cells are central in the eradication of fungal and bacterial infections. However, aberrant Th17 cell response can impair this protection or lead to inflammation and autoimmune diseases. Accordingly, strategies to prevent excessive Th17 response have been actively developed for the treatment of inflammatory disorders. CIP2A inhibits PP2A and drives cancer cell transformation and is considered a potential target for cancer therapies. In addition, CIP2A has been associated with the development of AD (Kerosuo et al. 2018; Kauko et al. 2018; C. Y. Liu et al. 2017; De et al. 2014; Khanna et al. 2013). PP2A regulates immune responses and autoimmunity (Apostolidis et al. 2016; Eitelhuber et al. 2011). Prior to the current work, the role of CIP2A in immune cells had not been studied.

We found that expression of CIP2A was induced by T-cell activation (Côme et al. 2016; **Figure 6**). Further, CIP2A-deficient mice and siRNA-mediated CIP2A-silenced human primary CD4+ T cells, resulted in reduced T-cell activation and proliferation. CIP2A-deficient T cells in mice and humans showed increased Th17 differentiation and enhanced and prolonged phosphorylation of the key Th17 transcription factor, STAT3 (Y705) (Khan, et al. 2020a; **Figure 6**). Proteomics analysis of the pSTAT3 interactome in CIP2A silenced and control Th17 cells revealed that CIP2A regulates the interaction between the transcription factor STAT3 and kinase acylglycerol kinase (AGK). In CIP2A-deficient cells, enhanced interaction of AGK resulted in prolonged STAT3 phosphorylation, facilitated Th17 differentiation, and upregulation of IL-17 in Th17 cells.

Comprehensive identification of protein interactome landscapes can uncover the mechanistic basis of their cellular functions by providing information about key regulators and target molecules. The CIP2A interactome in Th17 cells was determined by proteomics using MS analyses of immunoprecipitates with two antibodies targeting different regions of CIP2A (Khan, et al. 2020b; **Figure 6**). A panel of the interactors were validated using targeted MS, WB, and proximity ligation assay. Among these, in addition to the known interactions with the subunits of phosphatase PP2A (i.e., PP2A-A and PP2A-B) interaction with the catalytic subunit PP2A-C was demonstrated. Moreover, the interaction of CIP2A with the phosphatase PP1 was shown for the first time. The CIP2A interaction network provides insight into the contribution of CIP2A in known and novel pathological and physiological functions to be further studied.

In summary, the results of these studies provided new knowledge on the role of CIP2A in T-cell functions. The data measured for the CIP2A interactome have revealed unreported functions that should be further studied, particularly when developing therapies targeting CIP2A.

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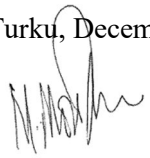

Mohd Moin Khan

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