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T CELL SIGNATURES AND MODULATION BY REGULATORY T CELLS

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T cell signatures and modulation by regulatory T cells

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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The public defense is scheduled to take place at L8:00 Lecture hall, Centre for Molecular Medicine, Karolinska University Hospital, Solna, Sweden on April 12, 2019

Dedicated to the anonymous blood donors who will probably never know about their contributions…

"Be water, my friend."
Bruce Lee

ABSTRACT

CD4 T cells are crucial coordinators of protective immune responses against microbes and tumors. However, immune responses misdirected towards self-antigens are the hallmarks of autoimmune diseases. Regulatory T cells (Tregs) maintain peripheral immune tolerance, mainly by suppressing effector T cells. Unfortunately, immunosuppression by Tregs can also favor cancer. Regulating the susceptibility of T cells towards Treg-meditated immunosuppression presents an exciting approach to reconcile between immune activation and tolerance in a disease-specific way. However, less is known about the regulation and mechanism of Treg-mediated suppression in target T cells. The work presented in this thesis is dedicated to unraveling the modulation of signaling cascades in T cells upon T cell receptor (TCR) stimulation and suppression by Tregs.

By using unbiased phosphoproteomics, we have mapped the global phosphoproteome of T cells upon TCR stimulation and suppression by Tregs. Our data indicate that Tregs suppress T cells mainly by opposing activation-induced phosphorylation. We discovered that Tregs revert activation-induced phosphorylation of DEF6 at specific sites (T 595 / S 597). Utilizing phospho-mutants, we discovered novel functions of these phosphorylations in disrupting the interaction of DEF6 with the IP_3R and regulating T cell signaling *via* modulation of NFAT activation and transcriptional regulation of T cell cytokines. Upon further exploration of the phosphoproteomic candidate list, we observed similarly altered phospho-regulation of protein phosphatase 1, regulatory subunit 11 (PPP1R11) by Tregs. We revealed that silencing of PPP1R11 renders T cells resistant to Treg-mediation suppression. Our data indicates PPP1R11 to be a novel regulator of T cell activation and Treg-mediated suppression. In light of several reports on resistant T cells in multiple diseases, the phosphoproteomic mapping of suppressed T cells and the insights into novel roles of DEF6 and PPP1R11 from our study may aid in regulating the sensitivity of T cells towards immunosuppression by Tregs in cancer and autoimmune diseases.

Besides phosphorylation of proteins, the subcellular localization of proteins is also an important regulatory mechanism of protein activity. Protein localization and activity is modulated by TCR stimulation and was shown for well-known TCR signaling proteins such as NFAT to be affected by Tregs as well. However, these mechanisms are not understood on a global level. Since there is no global data set available on subcellular protein localization and TCR stimulation-induced translocation in primary human T cells, as the first milestone in this field, we have performed a comprehensive mapping of the spatial proteome of T cells and TCR-induced subcellular protein translocation.

Besides exploring the target T cell side, in this work we also studied novel methods to induce Tregs, which represents the “other side” of Treg-based immune therapies. We present a novel method of inducing iTregs by using supernatants from M2 macrophages. Our data demonstrate that M2 macrophages induce iTregs by binding and re-releasing TGF-β, which may be explored for Treg induction *in situ* in the future.

Together, by presenting a global picture of T cell protein signaling yet with fine resolution, our work provides new mechanisms and data sets to revisit the role of T cells in therapy, especially in the context of T cell suppression by Tregs.

LIST OF SCIENTIFIC PAPERS

I. **Joshi, R.N.**, Binai, N.A., Marabita, F., Sui, Z., Altman, A., Heck, A.J.R., Tegnér, J., and Schmidt, A. (2017). Phosphoproteomics reveals regulatory T cell-mediated DEF6 dephosphorylation that affects cytokine expression in human conventional T cells. Front. Immunol. 8.

II. **Joshi R.N.**, Fernandes S.J., Shang M.M., Kiani N.A., Gomez-Cabrero D., Tegnér J., Schmidt A. Phosphatase inhibitor PPP1R11 modulates resistance of human T cells towards Treg-mediated suppression of cytokine expression. (Accepted for publication in Journal of Leukocyte Biology)

III. Schmidt, A., Zhang, X.-M., **Joshi, R.N.**, Iqbal, S., Wahlund, C., Gabrielsson, S., Harris, R.A., and Tegnér, J. (2016). Human macrophages induce CD4 + Foxp3 + regulatory T cells via binding and re-release of TGF-β. Immunol. Cell Biol. 94.

IV. **Joshi R.N.**, Vesterlund M., Stadler C., Tegnér J., Schmidt A., Lehtio J. TcellSubC: an atlas of the subcellular proteome of human T cells. (Manuscript)

ADDITIONAL PUBLICATION

Publications during doctoral studies that are not included in this thesis

1. Schmidt, A., Éliás, S*., Joshi, R.N.*, and Tegnér, J. (2016a). In vitro differentiation of human CD4+FOXP3+induced regulatory T cells (iTregs) from naïve CD4+T cells using a TGF-β-containing protocol. J. Vis. Exp. 2016.*shared second authors

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LIST OF KEY ABBREVIATIONS

Treg	Regulatory T cell
Tcon	Conventional T cell
Th	T helper
Ag	Antigen
TCR	T cell receptor
Ca^{2+}	Calcium
IL	Interleukin
IFN	Interferon
MHC	Major histocompatibility complex
HLA	Human leukocyte antigen
APC	Antigen presenting cell
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
AP-1	Activator protein 1
HiRIEF	High-resolution isoelectric focusing
IMAC	Immobilized metal affinity chromatography
MS	Mass spectrometer
CD	Cluster of differentiation
SOCE	Store-operated calcium entry
IP_3	Inositol 1,4,5-trisphosphate
DAG	Diacylglycerol
IBD	Inflammatory bowel diseases
PPP1R11	Protein phosphatase 1, regulatory subunit 11
DEF6	Differentially expressed in FDCP 6 homolog
SLAT	SWAP-70-like adaptor protein of T Cells
STAT	Signal transducer and activator of transcription
FOXP3	Forkhead box P3
TGF	Tumor growth factor

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

A competent immune system is essentially a coordinated interplay between activatory and tolerogenic waves to ensure adequate responsiveness and protection against harmful antigens and tumors while also maintaining non-responsiveness to self and innocuous antigens. Controlling these accelerations and brakes of the immune system serve as the basis of devising immunotherapeutic interventions against malignancies. Broadly speaking, immunological defense in vertebrates comprises of 2 main legions: innate (natural) immunity conducting the early reactions and adaptive immunity conducting the later steps which are based on cues acquired from previous exposure to antigens (Ags). The immunological battle is fought on several fronts; B and T lymphocytes (T cells) are crucial mediators of humoral and cell mediated-adaptive immune responses, respectively. Depending on the expression of the coreceptor, T cells are aptly divided into helper CD4+ T cells and cytotoxic CD8+ T cells. We have come far since J. Miller being publicly reminded that B and T were the first and last letters of "bullshit" when he proposed the existence of these two subsets of lymphocytes in a scientific conference in 1968 (Miller, 1999) till present day when CD4 and CD8 T cell counts are routinely used even in basic clinical tests. With several chemo- and immunotherapeutic treatment against cancers, infections, autoimmune diseases, allergies and transplant rejections targeting T cells, both in pipelines and clinics along with the recently approved CAR T cell therapies, it is truly an exciting time to work with T cells.

1.2 CD4 T CELLS: DEVELOPMENT AND TOLERANCE

T cells, like other blood cells, originate from the hematopoietic progenitor cells in bone marrow. Unlike other hematopoietic cells, T cells mature in the thymus (Shortman and Wu, 1996). Immature thymocytes are CD4-CD8-, and are hence termed double negative (DN). DN thymocytes proliferate and mature in thymus and eventually acquire a double positive state (CD4+CD8+) (DP) (von Boehmer et al., 1988). During the process of maturation, they acquire mature T cell receptor (TCR) proteins (αβ or γδ) determining their specificity and selectivity to detect the vast array of Ags even before they have been encountered. DP thymocytes which recognize the self Ag:MHC complex expressed by the cortical epithelial cell with low affinity undergo positive selection and mature into functional CD4 or CD8 T cells depending on their association with MHC class II or MHC class I respectively (Mizuochi et al., 1992). Simultaneously, over 90% of DP thymocytes with none or too low affinity to the Ag:MHC complex undergo apoptosis in lack of survival cues by the process aptly named "death by neglect.". Random combination of TCR gene segments to engineer over 10^9 unique TCRs (Lythe et al., 2016) using a limited number of TCR genes is a genetic masterstroke but also gives rise to TCRs with strong affinity to self-Ags (Feeney et al., 1994). The generation of these potentially autoimmune T cells is averted by the process of "central tolerance". These DP T cells with too high affinity to self Ag:MHC complex on medullary thymic epithelial cells are negatively selected for "clonal deletion" (Shortman and Wu, 1996).

Further, immature T cells which exhibit affinity stronger than the positively selected population described above, but weaker than the clonally deleted population are rescued and skewed to differentiate as regulatory T cells (Tregs) by the process of “clonal diversion” (Klein et al., 2019).

Although about 98 % of cells do not survive central tolerance mechanisms in the thymus, some autoreactive T cells still evade to systemic circulation which are then to be controlled by various mechanisms of peripheral tolerance, as described here. TCR stimulated T cells lacking co-stimulation undergo functional inactivation called “anergy”. Repeated activation of T cells with persistent Ags leads to apoptosis via the process termed “activation-induced cell death” (Green et al., 2003). Autoreactive T cells are further controlled by regulatory T cells (Tregs) in the periphery.

1.3 CD4 T HELPER CELLS AND SUBSETS

CD4 T cells are the most abundant lymphocytes in peripheral blood which initiate and sustain diverse immunological responses mainly by regulating other immune cells, hence aptly named helper T cells. Depending on the immune micro milieu and the type of antigenic stimulation, CD4 T cells have tendencies to polarize into several distinct subsets. These subsets have specialized immunological functions which are mainly mediated by the expression of signature cytokines and have unique genetic signatures mainly guided by their lineage-defining “master” transcription factors. Further, preferred transcription factors of the signal transducer and activator of transcription (STAT) family are also activated upon cytokine receptor stimulation of the T cells, which then also regulates the cytokine profile, upstream and in addition to the respective master transcription factors. Figure 1 depicts a simplified and unidirectional map of CD4 T cell differentiation and functions. While it is well understood that plasticity exists between the subtypes and functions (O’Shea and Paul, 2010; Oestreich and Weinmann, 2012), CD4 T cells can be divided into two broad functional categories: immunogenic effector T cells (Th1, Th2, Th17, and Tfh) and immunosuppressive Tregs as depicted in Figure 1 and described further.

1.3.1 Th1 and Th2 cells

Th1 and Th2 cells were the first subtypes of effector CD4 T cells that were discovered. The polarization of Th1 cells is mainly mediated by IL-12 and IFN-γ secreted by innate immune cells, NK cells, and T cells. Signaling mediated by STAT4 and the master transcription factor T-bet dictate the immunological function of Th1 cells via secretion of IL-2 and IFN-γ as their hallmark cytokines. Th1 cells activate and recruit macrophages and cytotoxic CD8 T cells to clear out cells infected with intracellular pathogens like bacteria and viruses. Similarly, Th2 cells are generated in the presence of IL-2 and IL-4 which drives the STAT6 and subsequently GATA3-mediated secretion of Th2 cytokine profile consisting of IL-4, IL-5, and IL-13. Th2 cells contribute to immunity against extracellular pathogens like parasitic helminths and stimulate repair of tissue damage (Romagnani, 1991, 2014; Walker and

McKenzie, 2018). Anomalies in Th1 and Th2 cells contribute to autoimmunity and allergies respectively.

1.3.2 Th17 and Tfh cells

Th17 and Tfh are more recent additions to the CD4 T cell subsets, and further subsets have been proposed (Th3, Th9, and Th22) that are not discussed here. The polarization of Th17 cells is regulated majorly by IL-6 and TGF-β which drive the STAT3 and subsequently RORγ-mediated secretion of IL-17A, IL-17F, and IL-22 (Romagnani, 2014), although other cytokine combinations have also been described to induce Th17 cells. Th17 cells are involved in clearing extracellular microbes like fungi and bacteria at the mucosal surfaces. Tfh cells are specialized T cell subsets, mainly present in the follicles of lymph nodes where they contribute to B cell maturation and activation. Human Tfh cells can be generated in the presence of IL-6, IL-21 and TGF-β which guides the STAT3 and BCL-6 mediated expression of IL-21 while they also produce other Th cytokines (Crotty, 2014). Generally, the differentiation factors and cytokines driving the above-described subsets are here noted in a simplified way, since depending on the stimulation conditions and other factors in the medium, different cytokines may mediate the effects. Further, species differences exist, most notably also between human T cells and commonly used murine T cells.

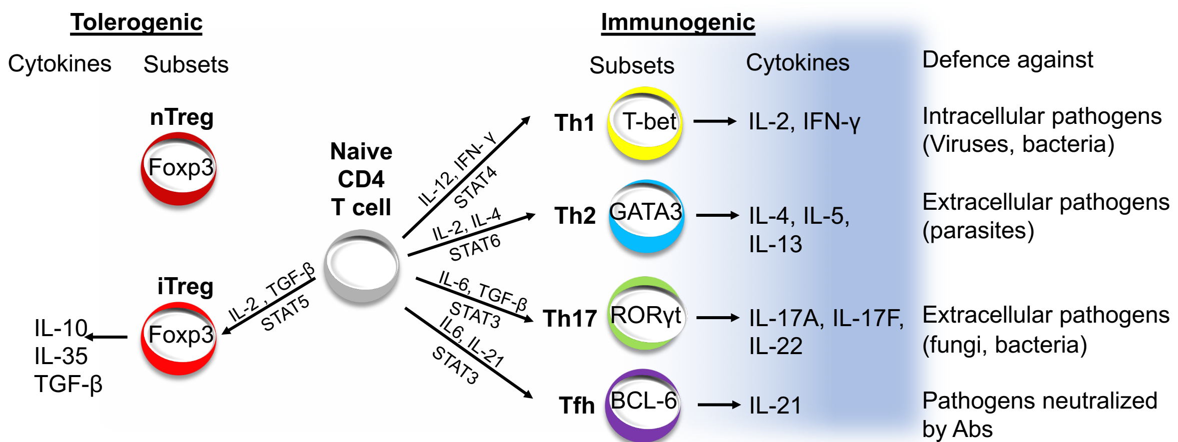


Figure 1: **CD4 T helper cell subsets**. Naïve CD4 T cells (grey) can differentiate into immunogenic effector T cells (Th1, Th2, Th17, and Tfh) (right side) and immunosuppressive Tregs (left side) (different colors) in the presence of TCR stimulation and CD28 co-stimulation. Cytokines that can drive the polarization along with master transcription factors and STAT molecules which are expressed by individual subsets are also mentioned. Additionally, major cytokines produced upon differentiation and primary immune targets of each subtype are also depicted.

1.4 REGULATORY T CELLS

Tregs are tolerogenic subsets of CD4+ T cells which mediate peripheral self-tolerance by suppression of effector T cells and other immune cells.

1.4.1 Tregs, a historical perspective

The history of Tregs dates back to the early 1970s after the discovery of thymus-derived cells exhibiting suppressive effects on other immune cells (Gershon et al., 1972) and preventing thymectomy-induced autoimmunity on adoptive transfer to the recipient mice (Kojima et al., 1976). However, the next two decades witnessed a dramatic decline in research interest in the field for these "suppressor cells", mainly because of lack of characteristic molecular markers and partly because of findings negating the proposed mechanism of suppression being mediated by soluble factors (Shevach, 2011). The whole field showed some signs of rejuvenation after the discovery of CD25 (IL-2 receptor α chain) as a surface marker for Tregs in mice (Sakaguchi et al., 1995) and finally in humans (Baecher-Allan et al., 2001; Stephens et al., 2001). It was after the discovery of Foxp3 as a lineage-defining transcription factor for Tregs (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003) that the field of Tregs started its "renaissance" and started to attract tremendous interest from the immunological community. Now, Tregs are established as the primary mediator of peripheral tolerance crucial for the aversion of autoimmune disease, allergies, transplant rejection and graft *versus* host disease (GvHD). Several therapies involving Tregs are in experimental phase and clinical trials in Type 1 Diabetes, Inflammatory bowel diseases (IBD) and organ transplantation (Duggleby et al., 2018).

1.4.2 Phenotype of Tregs

Phenotypically, Tregs are broadly defined as CD4+, CD25++, Foxp3+ suppressor cells (Sakaguchi, 2011). Although the expression of CD25 (IL-2 receptor α chain) is limited to Tregs in naïve mice, activated T cells are also known to transiently express medium levels of CD25 (Baecher-Allan et al., 2001), hence Tregs in humans may rather be characterized by high expression of CD25. Moreover, Foxp3 is widely accepted as the lineage-defining "master" transcription factor for Tregs. In fact, it is the mutation in Foxp3 which mediates systemic autoimmune disease both in mice (*scurfy*) and humans (IPEX syndrome) and hence primarily established the importance of Tregs in autoimmunity. In contrast to murine T cells, human conventional T cells have been reported to express medium levels of Foxp3 upon activation (Pillai et al., 2007). Despite being the most characteristic marker for Tregs to date, expression of Foxp3 cannot be an absolutely specific marker for Tregs and neither can CD25. In light of such ambiguity, the methylation pattern in the Treg-specific demethylated region (TSDR) in the Foxp3 locus and lack of or low expression of CD127 (IL-7Rα) can be used to distinguish Tregs from activated T cells (Huehn et al., 2009; Liu et al., 2006).

Naturally occurring Tregs (nTregs) consist of thymus-derived Tregs (tTregs) and peripherally-derived Tregs (pTregs), whereas *in vitro* generated Tregs are termed as iTregs (Rudensky et al., 2013). For all further discussions, the term "Treg" will indicate nTregs. Apart from the formal location-based classification of Tregs, they may also be classified according to the expression of FOXP3 / CD25 and CD45RA (a marker of naïve T cells) into CD45RA+FOXP3 low resting Tregs and CD45RA-FOXP3+ activated Tregs (Miyara et al., 2009). Furthermore, specific suppressors of Tfh; follicular Tregs expressing low or no CD25

and IL-10 producing Type 1 regulatory T cells which have transient or no FOXP3 expression have also been widely considered as functional classes of Tregs (Groux et al., 1997; Wing et al., 2018). Such classifications reflect uncovered knowledge about Treg biology.

1.4.3 Generation of Tregs

As mentioned earlier, thymic Tregs are differentiated from immature thymocytes in the thymus by the process of "clonal diversion". Strong TCR signal by self Ag:MHC along with CD28 co-stimulation drive the expression of CD25 which enables the cellular response towards IL-2 signaling and subsequent expression of FOXP3 mediated by STAT5 and other factors (Burchill et al., 2008).

Although the majority of Tregs are estimated to be constituted of tTregs (70-80% in both mice and men) as defined by the expression of *IKZF2* (Helios) and reviewed in (Shevach and Thornton, 2014), some portion of nTregs also consists of extrathymically derived pTregs. While Helios is used to differentiate tTregs and pTregs, it is not an absolute marker, and instead, Neuropilin-1 is recommended to be a more suitable marker of tTregs, at least in mice (Yadav et al., 2012).

pTregs are either differentiated from naïve T cells or may be generated by conversion from other subsets of T helper cells in the periphery. The generation of pTregs is primarily shaped by IL-2 and TGF-β. Furthermore, vitamin A metabolite all-trans retinoic acid (ATRA) and commensal microbiota-derived short chain-fatty acids, mainly butyrate have also shown to favor the induction of pTregs in the gut region. The mediators of pTreg generation have not been fully understood and are also reported to be tissue-dependent and shaped by other immune cells like macrophages and DCs (Arpaia et al., 2013; Schmitt and Williams, 2013).

In vitro generation of iTregs from naïve T cells has been an excellent platform to understand the development of pTregs. Protocols to generate iTregs have mainly been based on mimicking the *in vivo* regulators of pTreg generation like IL-2 and TGF-β, and recent works have also suggested the suitability of using molecules such as ATRA, butyrate and the mTOR inhibitor Rapamycin (Lu et al., 2014; Schmidt et al., 2016; Schmitt and Williams, 2013).

1.5 TREG-MEDIATED SUPPRESSION OF T CELLS

CD4+CD25- conventional T cells (here called T cells or Tcons), being the most abundant cell type among the lymphocytes, are crucial targets for immunosuppression by Tregs to maintain peripheral tolerance. Tregs employ myriads of suppression mechanisms presumably depending on the cytokine micro milieu and site of immune reaction as well as type and activation status of target cells and Tregs themselves to control the effector cytokine production and proliferation of T cells, as illustrated in Figure 2 (Schmidt et al., 2012). Understanding the individual mechanisms and their relevance in specific disease settings may be helpful to devise effective and suitable Treg-mediated therapies. The major mechanisms of suppression are described below.

1.5.1 Indirect suppression by modulation of APC function

Tregs have been well documented to downregulate the stimulatory capacity of antigen presenting cells (APCs) hence contributing to the suppression of T cells indirectly. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) has been shown to capture its ligands CD80 / 86 by trans endocytosis (Qureshi et al., 2011; Wing et al., 2008). Murine Tregs deficient in CTLA-4 have been shown to be less suppressive mainly due to reduced potency to downregulate CD80 / 86 on APCs. Strikingly, Treg-specific deletion of CTLA-4 resulted in severe lymphoproliferative diseases (Wing et al., 2008). Competitive inhibition of co-stimulatory signals to CD28 in T cells has also been partly attributed to downregulation of CD80 / 86 in murine dendritic cells (DCs) by Lymphocyte function-associated antigen 1 (LFA-1) from human Tregs (Tran et al., 2009). Tregs have also been reported to stimulate expression of tryptophan metabolizing enzyme, indoleamine 2,3-dioxygenase (IDO) in APCs via CTLA-4-dependent signaling to promote starvation and direct cell cycle arrest (Fallarino et al., 2006). Additionally, Tregs also recruit surface molecules like cluster of differentiation 40 (CD40), neuropilin-1, Lymphocyte-activation gene 3 (LAG-3) and the antigen-presentation attenuator A20 to downregulate the antigen (Ag) stimulatory capacity of DCs (Schmidt et al., 2012). Further, one recent study has also shown Tregs to hamper antigen presentation by capturing the MHC complex from APCs (Akkaya et al., 2019).

1.5.2 Suppression by secretion of soluble molecules

Tregs have been shown to produce various immunosuppressive cytokines like IL-10, TGF-β, and IL-35 for suppression of T cells. The importance and redundancy of these cytokines for Treg-mediated suppression of T cells seem to differ depending on the disease model, species and *in vitro* setup (Schmidt et al., 2012).

Murine TGF-β has been shown to confer Treg-mediated suppression *in vitro* (Nakamura et al., 2004) and has also been shown to be crucial for preventing colitis (Read et al., 2000). Conversely, other groups had contradictory findings with reports of TGF-β knock out (KO) Tregs still being suppressive *in vitro* (Read et al., 2000). Furthermore, TGF-β plays a positive role in the generation of iTregs (Marie et al., 2005). Similarly, the importance of IL-10 has also been shown in several disease models like the experimental autoimmune encephalomyelitis and most prominently colitis (Asseman et al., 1999; McGeachy and Anderton, 2005).

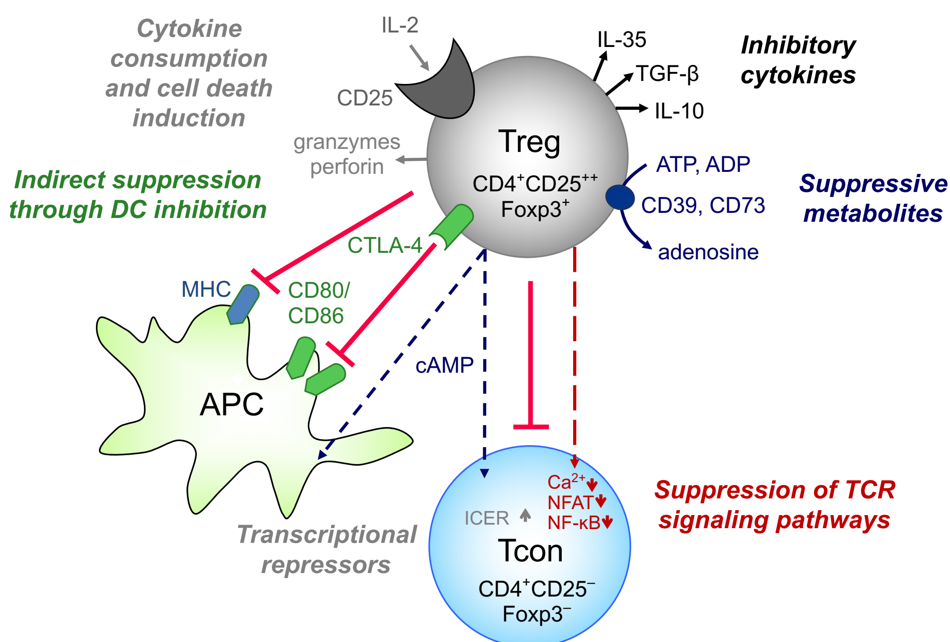
Studies have shown IL-35 to be important in Treg-mediated suppression of T cells in murine models; however human Tregs do not express IL-35 (Bardel et al., 2008).

Tregs produce other repressive molecules like cyclic adenosine monophosphate (cAMP) (Bopp et al., 2007) and adenosine for direct or APC-mediated suppression of T cells (Borsellino et al., 2007). Particularly, cAMP seems to be more important for direct Treg-mediated suppression of T cells. Treg-mediated suppression of IL-2 expression in T cells was initially suggested to act by the induction of inducible cAMP early repressor (ICER), which is a transcriptional repressor (Bodor et al., 2007). While ICER has a role in cytokine gene

expression, recent studies found ICER to be dispensable, and instead showed cAMP to act by promoting exchange protein directly activated by cAMP (EPAC) (Vang et al., 2013). Further Tregs have also been reported to utilize granzyme / perforin-mediated apoptosis induction in T cells (Grossman et al., 2004).

1.5.3 Suppression by IL-2 consumption

Tregs have been shown to induce IL-2 deprivation for T cells by consumption of IL-2 (an important growth factor for T cell proliferation) via IL-2R containing CD25 (IL-2R α chain) due to its high affinity for IL-2. Although shown to be important in an IBD model (Pandiyan et al., 2007), the resultant net effects on target T cells have been unclear. Tregs have also been shown to outcompete naïve T cells for IL-2 and utilize it for the induction of IL-10 production (Barthlott et al., 2005).



Adapted from Schmidt *et al.* 2012 Front. Immunol.

Figure 2: **Mechanisms of Treg-mediated suppression of T cells**. Tregs regulate T cells by several immunosuppressive mechanisms illustrated in separate colors. Tregs can suppress T cells indirectly by modulating APCs. Tregs also directly suppress T cells by secretion of inhibitory cytokines and suppressive molecules. Furthermore, Tregs also suppress T cells by competing for IL-2 and apoptosis induction. Additionally, Tregs can rapidly suppress TCR-induced calcium-mediated signaling pathways in a contact-dependent manner.

1.5.4 Contact-dependent suppression: Does it exist?

While Treg-mediated suppression of T cells is mainly visualized within the boundaries of mechanisms mentioned above (acting via APCs and secretion of soluble factors), the relevance of direct suppression of T cells by Tregs is not widely accepted. This is in part because of lack of detailed knowledge of molecular pathways mediating this contact-dependent suppression and evidences proving its *in vivo* relevance and occurrence. However, multiple *in vitro* studies have conclusively shown that Tregs can directly suppress

proliferation and / or activation of T cells in a contact-dependent manner without mediation by APCs or soluble factors in mice and humans (Ermann et al., 2001; Hagness et al., 2012; Huang et al., 2012; Oberle et al., 2007a; Thornton and Shevach, 1998). While earlier microscopic studies in intact explanted or intravital lymph nodes concluded that stable contact does not occur *in vivo* between Treg and T cells (Mempel et al., 2006; Tang and Krummel, 2006), a recent breakthrough study reported that Tregs stably contact T cells at the site of inflammation in murine non-lymphoid target tissues (Miska et al., 2014). In this study of murine pancreatic islet graft transplantation model, the stable contact between Tregs and T cells occurred both with and without mediation by APCs with only a minor role for CTLA-4 (Miska et al., 2014). Furthermore, another imaging study has reconfirmed the direct interaction between endogenous Tregs and adoptively transplanted T cells in murine lymph nodes in a CTLA-4-dependent manner (Matheu et al., 2015). Further works are required to understand the *in vivo* significance and mechanism of direct suppression of T cells by Tregs.

1.6 T CELL RECEPTOR SIGNALING AND ROLE OF PHOSPHORYLATIONS

TCR signaling is essential for proliferation and cytokine secretion of T cells (Guy et al., 2013). T cell activation requires recognition of Ag:MHC complex by TCR (Signal I), co-stimulation via binding of CD80 / 86 to CD28 (Signal II) and finally signaling via cytokines (Signal III) (Corthay, 2006). TCR signaling induces the activation of three core transcription factors in T cells, which control cytokine transcription and decide cell fate. The central transcription factors include nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), nuclear factor of activated T cells (NFAT), and activator protein 1 (AP-1).

Ligation of the TCR complex with Ag:MHC complex initiates TCR signaling to result in activation of two Src kinases, CD3-associated Fyn kinase (via dephosphorylation by Tyrosine (Tyr) (Y) phosphatase CD45) and CD4-associated Lck kinase. Fyn-mediated phosphorylation of ITAMs in the cytoplasmic ζ chain of the CD3 receptor induces subcellular translocation of inactive cytosolic zeta-chain-associated protein kinase 70 (ZAP-70) to membrane-bound immunoreceptor tyrosine-based activation motif (ITAMs) of CD3, where ZAP-70 is further activated upon phosphorylation by Lck. ZAP-70, in turn, phosphorylates and activates linker for activation of T cells (LAT), which serves as an important branching point for further downstream signaling. Phosphorylation of Y132 on LAT recruits phospholipase Cγ1 (PLCγ1) to induce calcium-dependent NFAT and NF-κB or RAS-MAPK pathways, which is primarily considered calcium independent. Phosphorylation of Y171, Y191, and Y226 on LAT activate guanine nucleotide exchange factor, SOS and SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), which in turn regulate essential GTPases like Ras, Rac, and Rho and subsequently activate mitogen-activated protein (MAP) kinases like p38, JNK, and ERK1/2. The action of these kinases activates the AP-1 pathway by dimerization of c-Jun and Fos (to form the AP-1 transcription factor) upon translocation into the nucleus. Membrane recruitment and activation of PLCγ1 by LAT is mediated by activation of adaptor protein SLP-76 and IL2 inducible T cell kinase (Itk). PLCγ1 enzymatically cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) generating the second messengers,

diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 is crucial for mediating calcium influx into the cytoplasm mainly by regulating Ca^{2+} gates of the endoplasmic reticulum (ER) by binding to IP_3 receptor (IP_3R). The resulting decrease in the ER Ca^{2+} levels activates stromal interaction molecules 1 and 2 (STIM 1 and 2) which then regulate the opening of Ca^{2+} channel in the plasma membrane composed of Calcium Release-Activated Calcium Modulator 1 and 2 (Orai1&2), resulting in store-operated Ca^{2+} entry (SOCE). Signaling induced by DAG and IP_3 consequently activates NF-κB and NFAT respectively by unmasking nuclear-localizing sequences in the targets which mediates nuclear translocation of the activated NFAT and NF-κB from the cytosol (Prole and Taylor, 2019; Smith-Garvin and Koretzky, 2009; Vaeth and Feske, 2018; Zhang and Dong, 2005).

1.7 TREG-MEDIATED SUPPRESSION OF PROLIFERATION VERSUS CYTOKINE EXPRESSION

Broadly speaking, suppression of proliferation is considered a necessary condition for Treg-mediated suppression of T cells, while suppression of activation-induced cytokines is usually considered as an intermediate step to install suppression of proliferation. However, it has been shown that cytokine expression can be independent of proliferation, since distinct TCR signaling pathways drive proliferation and cytokine secretion (Guy et al., 2013). Further, T cells have been shown to proliferate even when major components of TCR signaling and subsequently cytokine expression are disturbed (Oh-Hora et al., 2008). Even Tregs have been reported to suppress the expression of certain cytokines without affecting proliferation in CD4 T cells (Sojka and Fowell, 2011) and similarly, suppressing effector activity without affecting proliferation in CD8 CTLs (Mempel et al., 2006). Work by Schmidt *et al.* has also indicated that depending on the time point after the onset of suppression, Tregs can suppress cytokine secretion and proliferation independently (Schmidt et al., 2011). While suppression of cytokine expression and proliferation have been mostly dependent for the APC-mediated effect of Treg on T cell priming, suppressing the production of inflammatory cytokines might be more crucial in the inflamed tissues, at least in the early phase.

1.8 RAPID SUPPRESSION OF TCR SIGNALING IN T CELLS BY TREGS

Deciphering the details of rapid and early suppression by Tregs could enable the discovery of upstream mediators of suppression. It might be feasible to manipulate the machinery of Treg-mediated suppression more effectively and specifically by targeting these upstream molecules.

Initial studies in mice have characterized the dynamics for Treg-mediated suppression of IL-2 expression in T cells, ranging from 6 to 15 hours (Sojka et al., 2005; Thornton and Shevach, 1998). Oberle *et al.* demonstrated that human Tregs suppress cytokine transcription even more rapidly, within 1 to 3 hours in T cells independently of IL-2 consumption or secretion of soluble molecules (Oberle et al., 2007a). Using stronger TCR activation to enable an earlier robust read-out of cytokine expression, Schmidt *et al.* showed that the suppression of cytokines occurred as early at 30 minutes when pre-activated Tregs were used. The study

showed that Tregs rapidly suppress calcium and calcium-dependent NF-κB and NFAT pathways while the AP-1 pathway was primarily unaffected in T cells as depicted in Figure 2. Surprisingly, suppression was not mediated by known modification of proximal events of TCR signaling like ZAP-70, PLC-γ1, IP_3 levels, or PKCθ phosphorylation. Additionally, this contact-dependent suppression was shown to occur both in the presence and absence of APCs and was independent of CTLA-4. More importantly, the hallmark of this study was the finding that Tregs suppress T cells by inhibition of TCR-induced depletion of the intracellular calcium stores and hence preventing the activation of NF-κB and NFAT1, which could be visualized as early as 5 minutes after activation (Schmidt et al., 2011).

In a study on multiple sclerosis patients, Schwarz and colleagues have also demonstrated that Tregs fail to suppress calcium signaling but do not disrupt NFAT2 nuclear translocation in T cells (Schwarz et al., 2013). Further, Huang and Fowell claimed that Tregs selectively attenuate specific TCR signaling pathways to induce suppression. They have demonstrated that murine Tregs attenuate NF-κB signaling in T cells after 6 hours of activation in a setting independent of APC-activity. However, they observed sustained expression of NFAT1 and NFAT2 in suppressed cells (Huang et al., 2012). While studies mentioned above present NFAT as an activatory molecule for T cells especially within early time points, several studies claim NFAT1 to exert suppressive effects on T cells at later time points (Bopp et al., 2005; Chellappa et al., 2015; Shin et al., 2014). This would be well in accordance with the dual role of NFAT mediating activation, but also the state of unresponsiveness (anergy) in T cells in the absence of costimulation (Macian et al., 2002). A recent work by Aandahl and colleagues suggested that human Tregs pre-activated for a prolonged time (48 hours) were able to suppress TCR-proximal signaling molecules as well as NF-κB and AP-1 upon 48 hours of coculture and activation (Chellappa et al., 2015). Taken together, these studies hint at the possibility of multiple mechanisms mediating rapid suppression of T cells. While the NF-κB pathway appears to be consistently attenuated by Tregs across several studies, the role of NFAT in suppressed T cells may be dependent on activation time points, Treg pre-activation, NFAT isoforms, and experimental setups. Additionally, a growing amount of recent works have reported impaired signaling in TCR pathways mainly involving Akt-MAPK-AP-1 pathway to give rise to T cells resistant towards suppression by Tregs. Hence the importance of understanding the details of Treg-mediated signaling in T cells is even more evident.

1.9 RESISTANCE AGAINST TREGS (VIVA LA REVOLUCIÓN)

Presently, most of the research into Treg-mediated therapies either in experimental phase or clinical trials are mainly focused on regulating or strengthening the suppressive capacity of effector Tregs. Since the efficacy of treatment ultimately depends on the effect these Tregs have on the target cells, there can be untapped potential in modulating the susceptibility of the target T cells as well. Further, an increasing number of evidence in multiple autoimmune diseases shows that Tregs fail to suppress T cells partly because of resistance-induction in T cells (Buckner, 2010; Mercadante and Lorenz, 2016; Walker, 2009). These resistant T cells

serve as “red flags” and potential hurdles for therapies targeting T cells and Tregs. Less is known about the exact mechanisms of resistance. Studies report that T helper cell subsets vary in their susceptibility to Tregs. Th17 and Th2 cells along the diseases modulated by respective Th subsets have been shown to be more resistant to Tregs than Th1 cells (Cosmi et al., 2004; Stummvoll et al., 2008; Taams et al., 2007). T cell resistance has been associated with several cell-extrinsic factors, mainly impaired cytokine micro milieu involving the expression of TNF-α (Valencia et al., 2006; Wehrens et al., 2013), IL-4 (Pace et al., 2005; Pillemer et al., 2009), IL-6 (Schneider et al., 2013) and IL-15 (Ben Ahmed et al., 2009) in both experimental and clinical settings. The knowledge is even sparser regarding the cell-intrinsic causes of resistance in T cells partly because the understanding of signaling events in the T cell upon suppression by Tregs is severely limited to canonical molecules and yet incomplete. Deregulation of SMAD7 (Fantini et al., 2009), CD28 (Thewissen et al., 2007), TRAF6 (King et al., 2006) and phosphatase SHP1 signaling (Mercadante and Lorenz, 2017), ultimately affecting the Akt-MAPK pathway, have been linked as some cell-intrinsic causes of resistance so far. There is a pronounced need for unbiased global studies to elucidate the molecular events initiated in T cells upon Treg-mediated suppression to fill the knowledge gap and resolve the potential issues arising from T cell resistance.

1.10 PHOSPHOPROTEOMIC STUDIES IN T CELLS (TRADING THE TORCH FOR THE FLOODLIGHT)

TCR signaling is largely mediated by phosphorylations, which is apparent by the involvement of multiple kinases and phosphatases, as described earlier. It is feasible that Tregs suppress T cells by regulating these already known or even unknown phosphorylations as shown for Treg-mediated regulation of some key phosphorylations of TCR signaling molecules (Chellappa et al., 2015; Huang et al., 2012; Schmidt et al., 2011). Hence the global mapping of the phosphoproteome of T cells upon suppression by Tregs might elucidate comprehensive mechanisms of Treg-mediated suppression of T cells. Since post translational modifications (PTMs) like phosphorylations can occur within seconds, mapping the phosphoproteome is even more suitable to study the mechanism of rapid suppression by Tregs which can occur as soon as 30 minutes of contact between Tregs and T cells and is then acting immediately after TCR stimulation, as soon as TCR signaling such as calcium influx becomes detectable (Schmidt et al., 2011).

In eukaryotes, protein phosphorylations mainly occur on serine (Ser; S), threonine (Thr; T) and tyrosine (Tyr; Y) residues and mediate multiple signal transduction pathways. Besides these well-known substrates, phosphorylation can also occur on histidine, lysine, and arginine residues (Hunter, 2012). Although these additional phosphorylations might have some biological importance, their abundance is still debated and they are currently understudied (Fuhs and Hunter, 2017). Phosphorylation is mediated by the action of kinases and phosphatases which add and remove phosphate group to and from target proteins, respectively. 518 protein kinases and 189 protein phosphatases are identified in humans till date (Chen et al., 2017; Manning et al., 2002). Their importance in regulating biological

pathways are reflected by the observations that over 30% of proteins encoded by the human genome occur in a phosphorylated state (Cohen, 2002) and drugs targeting kinases are among the fastest growing drug family in recent years (Santos et al., 2016). Understanding the potential roles of kinases and phosphatases in mediating the phosphorylation-dependent effect of Tregs on TCR signaling in T cells could have significant potential for basic research as well as clinical application.

Despite recent advancements in mass spectrometry (MS), limited attempts have been successful in mapping the global phosphoproteome and subcellular proteome of CD4 T cells in resting state and upon TCR activation. Few attempts at global profiling of TCR-induced T cell proteome are based on Jurkat T cell lines, not primary cells (Chylek et al., 2014; Nguyen et al., 2015a). The recently updated Lymphos 2.0 database provides comprehensive coverage of activation-induced changes in the phosphoproteome of human primary CD3+ T cells (Nguyen et al., 2015b). Further publications have also surfaced in profiling the phosphoproteome of murine CD8+ T cells (Navarro et al., 2011). In light of the central contribution of CD4+ T cells in immunity, in particular autoimmune diseases and several T cell-based immunotherapeutic approaches, there is a need of unbiased global profiling of TCR-induced changes in human CD4+ T cells. Impressive works have been accomplished in mapping the phosphoproteome of murine CD4+ T cells (Tan et al., 2017) and murine Tregs (van Ham et al., 2017) in recent years.

Lacking any global study on the direct effect of Tregs on T cells, most knowledge is based on targeted studies of selected canonical molecules. Although these targeted studies have installed a backbone for understanding the signaling in suppressed cells, they are limited to the discovery of known and canonical molecules (Chellappa et al., 2015; Schmidt et al., 2011; Shin et al., 2014). Hence there is a need to span beyond probing known TCR signaling molecules for their role in Treg-mediated suppression by performing a global and unbiased mapping of the dynamics in the phosphoproteome in T cells upon Treg-mediated suppression.

1.11 MS-BASED SUBCELLULAR PROTEOMICS IN T CELLS (BUILDING A PROTEOME-WIDE "GPS")

Subcellular localization is an important determinant of protein function, since specific intracellular compartments can provide favorable niches for specific reactions, protein interactions, modifications and stability (Lundberg and Borner, 2019). Deregulation of subcellular localization has been implicated in several disorders like cancer, neurodegeneration, obesity, genetical and protein misfolding diseases (Hunter, 2012; Kau et al., 2004; Luheshi et al., 2008; Wang et al., 2016). Hence the determination of precise subcellular location/s can be invaluable in understanding a protein's biological function and devising therapeutic interventions. While the determination of subcellular location by tagged proteins (Huh et al., 2003) and antibody-based detection have been successful for targeted approaches (Thul et al., 2017), MS-based proteomics methods can provide high coverage and generate unbiased proteome-wide subcellular location data. For example, recent

advancements in MS techniques have led to the curation of high-resolution maps of the subcellular proteome in human cell lines (Itzhak et al., 2016; Orre et al., 2019), human fibroblasts (Jean Beltran et al., 2016), murine pluripotent stems cells (Christoforou et al., 2016), rat tissues (Jadot et al., 2017) and yeast (Nightingale et al., 2019). Although these studies have immensely improved the general classification of the subcellular proteome, there is a lack of context-specific classification of the subcellular proteome for primary human immune cells like lymphocytes. Present efforts to classify the subcellular proteome of CD4+ T cells are mainly limited to subtractive approaches of profiling only a particular fraction of interest (Filén et al., 2005; Graessel et al., 2015; Moltu, 2013; Moulder et al., 2010; Procaccini et al., 2016) or have rather low coverage of the global proteome (Solstad et al., 2011). High-resolution, global mapping of the subcellular location of the T cell proteome would decrease the dependence on subcellular data from unrelated cellular sources and hence, could guide a more specific and targeted validation for the spatial information of the protein of interest for T cell-specific works.

2 AIMS

1. To elucidate the molecular mechanisms behind rapid suppression of T cells by Tregs (Paper I & II)
    a. To map the global effect on the phosphoproteome of T cells upon suppression by Tregs (Paper I)
    b. To study the role of phosphatase inhibitor PPP1R11 in modulation of the resistance of T cells against Treg-mediated suppression (Paper II)
2. To elucidate the mechanism and role of macrophages in the generation of iTregs (Paper III)
3. To map the subcellular proteome and proteome-wide stimulation-induced subcellular translocation in T cells (Paper IV)

3 METHODS SUMMARY

To achieve the aims described above, several different methods were employed. Below follows a summary of the main methods. Of note, for a more detailed description please refer to the individual articles.

3.1 T CELL:TREG COCULTURE SYSTEM AND STIMULATION

In order to facilitate the study of rapid signaling upon short term T cell:Treg coculture, we used a previously optimized allo-disparate coculture system using cells magnetically isolated from human peripheral blood (Schmidt et al., 2011). In short, freshly isolated and HLA-A2 disparate T cells (CD4+CD25-) were cocultured with pre-activated Tregs (CD4+CD25++; O.N. anti-CD3 stimulation) or control T cells for 85 mins and then stimulated for 5 mins with cross-linked anti-CD3/anti-CD28 antibodies (referred to as TCR stimulation) for phosphoproteomic and phospho-Western Blot studies and 3-5 hours for transcriptomic studies such as cytokine mRNA analysis (Paper I). Following quick magnetic separation based on HLA-A2 expression, the target T cells were processed for further downstream analysis. Alternatively, for long-term stimulation, responder T cells were labeled with CFSE and stimulated alone or with Tregs for 3-5 days and proliferation was measured by flow cytometry (Paper II). For iTreg generation, naïve CD4+CD25-CD45RA+ T cells were cultured with supernatants of macrophages such as M2 macrophages, or TGF-β1 + IL-2 as a control (Paper III). Specific details regarding HLA-A2 disparity, pre-activation for Tregs, nature and time point of stimulations and cocultures are specifically given in each publication (Paper I – IV).

3.2 MS-BASED PHOSPHOPROTEOMICS

Apart from the technological limitations, the main constraint of performing phosphoproteomics is the required amount of starting material. Utilizing comparatively low amount of starting materials (57-196 µg protein per sample) we have been able to perform global phosphoproteomics. In brief, trypsin-digested peptides from suppressed T cells, stimulated T cells and resting T cells isolated from T cell-Treg, T cell-T cell cocultures and untreated T cells (N = 3 donors) respectively were individually labeled with stable isotope dimethyl labeling (Boersema et al., 2009) and mixed to enable multiplexing. Phosphopeptide enrichment was carried on Titanium ion immobilized metal affinity chromatography (IMAC) (Zhou et al., 2013) before processing in the LC-MS. The analyses were based on relative quantification of the dimethyl ratios (Paper I).

3.3 NUCLEOFECTION OF UNSTIMULATED T CELLS

Nucleofection of plasmids and siRNAs (Paper I & II) were conducted by Neon transfection system and Amaxa technology respectively, with several optimizations. The possibility of optimizing transfection conditions and voltages enables Neon transfection to be suitable for transfection of plasmids (1 kbp) with considerably good viability while transfection with Amaxa technology yielded higher efficiency of transfection albeit with compromised cellular

viability. Although these methods provide a relatively economical and possibly less labor-intensive (once optimized) alternatives to study the biological effect of genetic perturbations in cellular contexts, they come with their limitations. Of note is that we observed electroporation in general affects cellular physiology with elevation in calcium signaling and other activation-associated signaling molecules including cellular responses to stimulation. Although these effects can be normalized with the use of empty vector or control siRNA, caution needs to be administered while resolving differences which are small in magnitude. DEF6 plasmids (phospho-mutants, wild type or empty vectors) were transfected in primary human T cells. The cells were rested for 8 hours and then stimulated with 5 minutes or 3 hours of TCR stimulation for Western Blot and mRNA studies (Paper I). For siRNA studies, silencing with PPP1R11 and control siRNAs were performed by incubation at 37°C for 4.5 days (Paper II). In general, cells were stimulated with crosslinked TCR stimulation for 3-6 hours (RNA studies) or 4.5 days with plate-bound anti-CD3 and soluble anti-CD28 stimulation (protein studies), or as described for specific experiments.

3.4 SUBCELLULAR FRACTIONATION AND MS-BASED PROTEOMICS

The subcellular fractionation strategy was mainly aimed at enabling robust isolation of basic cellular meta-components from limited starting materials (20 Million T cells; at least 48 µg protein per fraction) suitable for clinical and biological samples of a rare nature. Qproteome Cell Compartment Kit was used to isolate cytosolic, nuclear and membrane fractions from T cells upon steady state and following 15 or 60 minutes of TCR stimulation. It needs to be mentioned that the membrane fraction consists of the plasma membrane as well as the membranous organelles. Enzymatically digested peptides were labeled with 10 plex Tandem mass tag (TMT) labeling. The multiplex mixtures were pre-fractionated by immobilized pH gradient (IPG)-isoelectric focusing on pH 3-10 IPG strips using the high-resolution isoelectric focusing (HiRIEF) method (Branca et al., 2014). Peptides were extracted from the gel strips into 72 separate fractions which were analyzed by LC-MS/MS. Downstream analyses were based on the relative ratios of the individual TMT intensities (Paper IV).

4 RESULTS AND DISCUSSION

4.1 MOLECULAR MECHANISMS OF RAPID SUPPRESSION OF T CELLS BY TREGS (PAPER I)

A series of work by Oberle *et al.* and Schmidt *et al.* (Oberle et al., 2007b; Schmidt et al., 2011) have contributed to the process of understanding the mechanism and signaling in target T cells during Treg-mediated direct and rapid suppression of T cells by Tregs. The most upstream effect of Tregs was shown to be the inhibition of depletion of intracellular calcium stores and suppression of downstream pathways like NFAT and NF-κB in T cells. Despite "kick-starting" the discovery of the involved molecules, targeted studies including and similar to these studies (Chellappa et al., 2015; Huang et al., 2012; Shin et al., 2014) paint an ultra-canonical and yet incomplete view of this process. To understand the global effect of Treg-mediated suppression on T cells, we decided to pursue an unbiased strategy by comprehensibly mapping the phosphoproteome of T cells upon suppression. Owing to the rapid nature of suppression (starting as soon as after 30 mins of T cell:Treg contact and detectable with 5 mins of TCR stimulation) and the heavy involvement of phosphorylations in regulating TCR signaling we postulated that Tregs suppress T cells by regulating the phosphoproteome of T cells.

4.1.1 Tregs revert the activation-induced phosphorylation in T cells

5 minutes of TCR stimulation with or without the presence of Tregs produced a massive alteration in the T cell phosphoproteome (42% and 19% of total detected phosphopeptides changing over 25% upon stimulation and suppression respectively). TCR stimulation mainly induced elevation of phosphorylation (397 out of 431 regulated phosphopeptides) and strikingly Tregs seemed to reverse the activation-induced phosphorylated state with the majority of phosphopeptides exhibiting reduced phosphorylation compared to stimulation without Tregs (158 out of 198 regulated phosphopeptides). Further analysis with a statistical cutoff of $P < 0.05$ confirmed that most of these phosphopeptides with reduced phosphorylation upon suppression exhibited increased phosphorylation upon activation. This is the first indication that Tregs suppress T cells by globally altering the phosphoproteome of the target T cells.

4.1.2 Tregs suppress DEF6 phosphorylation in T cells

Analyses of phosphoproteins regulated by TCR stimulation and / or by Tregs have unmasked several novel candidates, the majority of which are not previously known to be involved in TCR signaling, supporting the strength of our unbiased approach. Hence these phosphorylations hold potential to unravel novel events in Treg-mediated suppression of T cells which are poorly understood so far. These candidates exhibit significant enrichment in cytoskeletal remodeling, which is one of the proximal events mediating TCR signaling and possibly early signaling events upon T cell and Treg contact. Interestingly, a recent study involving phosphoproteomic characterization of murine Tregs and T cells reports a Treg specific activation of cytoskeletal regulators (van Ham et al., 2017), although suppressed

target T cells were not studied here. Taken together, early signaling events upon T cell and Treg contact may be mediated by molecules involved in cytoskeletal remodeling .

Among the molecules that were differentially phosphorylated by Tregs in our study, a lymphocyte-specific guanine nucleotide exchange factor (DEF6 / SLAT) appeared. DEF6 has been shown to regulate TCR signaling by IP_3R-mediated regulation of Ca^{2+} / NFAT signaling and activation of cytoskeleton-regulating Rho-GTPases (Bécart et al., 2008; Gupta et al., 2003). Our discovery that Tregs prevent the activation-induced phosphorylation of DEF6 T 595 and S 597 holds more relevance since these phospho-sites are located in the DH domain of DEF6 which is crucial for the activity of DEF6 (Bécart et al., 2008; Cote et al., 2015; Fos et al., 2014). Hence, we hypothesized that DEF6 T 595 and S 597 may be involved in mediating the functionality of DEF6 in T cell activation and suppression.

4.1.3 DEF6 T 595 and S 597 phosphorylation contribute to T cell activation

The initial experiments involved generating mutants of the above-discovered DEF6 phospho-sites, and transfection of phospho-silent mutants of DEF6 T 595 and S 597 in HEK 293 cells. This revealed that these DEF6 phospho-sites mediated the interaction of DEF6 with the IP_3R which is crucial for the regulation of intracellular calcium levels. Next, we studied the functional role of these phosphorylations in shaping the stimulation-induced signaling events in primary T cells. Transfection of the phospho-silent mutants of DEF6 led to reduced activation of NFAT upon TCR stimulation as compared to wild type DEF6. Similarly, T cells overexpressing the phospho-silent mutant DEF6 protein also exhibited lower expression of T cell activation-induced cytokines like *IL2* and *IFNG* mRNA; notably, the hallmark cytokines to be rapidly suppressed by Tregs. On the other hand, overexpression of the phospho-mimic mutants led to enhanced expression of these cytokines as compared to the phospho-silent mutants. A similar trend of regulation of other NFAT target genes like *IL2RA* and *FASLG* was also observed with both the phospho-mutants. Taken together these results suggest that these DEF6 phosphorylations positively regulate T cell activation.

Regulation of T cell activation via interaction with IP_3R and NFAT activation by DEF6 may be mediated by several domains of DEF6. Membrane translocation of DEF6, mediated by domains other than the DH domain (containing T 595 and S 597) seems to be vital for the influence of DEF6 on regulation of T cell activation (Bécart et al., 2008; Fos et al., 2014). Expression of the DH domain alone has been shown to be insufficient to regulate DEF6:IP_3R binding unlike other subunits (Fos et al., 2014). However, upon forced membrane translocation, the DH domain, alone can completely restore NFAT activation and cytokine regulation in murine DEF6 -/- T cells. DEF6:IP_3R interaction was not checked in this case (Bécart et al., 2008). Taking these observations together with our results, it is highly likely that upon proper membrane localization, phosphorylation of DEF6 T 595 and S 597 (in the DH domain) regulate the DEF6:IP_3R binding and hence stimulation of Ca^{2+}/NFAT signaling in T cells. It is feasible that these phospho-sites may distort the overall DEF6 structure to hinder the DEF6:IP_3R binding, which remains to be further determined.

In conclusion, we propose DEF6 T 595 and S 597 phosphorylations to be novel mechanisms to regulate T cell activation and Treg-mediated suppression via mediating DEF6:IP_3R interaction and Ca^{2+} / NFAT signaling, as depicted in Figure 3.

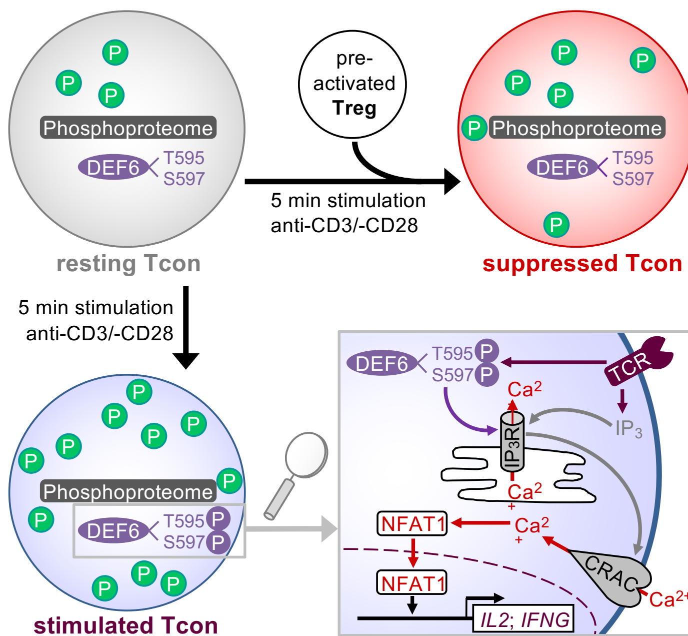


Figure 3. Proposed model of phosphoproteomic regulation of T cell activation and suppression. Tregs suppress T cells (red) by reverting the activation-induced phosphorylation (blue). The phosphoproteome of suppressed T cells partially resembles resting T cells (grey). Tregs suppress T cells by inhibiting activation-induced phosphorylation of DEF6 T 595 and S 597, possibly abrogating DEF6:IP_3R interaction and downstream NFAT signaling and cytokine expression.

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4.2 PPP1R11: A REGULATOR OF T CELL RESISTANCE TO TREGS (PAPER II)

In Paper I, we found that the phosphoproteins regulated by Tregs are functionally enriched in cytoskeletal remodeling and we subsequently discovered novel DEF6 phosphorylations which regulated T cell activation. When we analyzed the phosphoproteins-regulated by Tregs instead for overrepresented protein classes, we observed enrichment of phosphatases, kinases and transcription factors. As discussed earlier, TCR signaling is heavily regulated by phosphorylations, plus kinases and phosphatases are viable drug targets for clinical intervention. Hence, we investigated the phosphoproteomic data for phosphatases and their regulators. We observed that Tregs reverted the activation-induced phosphorylation of PPP1R11 S 73, S 74, T 75 and S 77 ($P = 0.057$). PPP1R11 is an inhibitory (regulatory) subunit of PP1 phosphatase (Zhang et al., 1998). These 4 phospho-sites constitute most of the reported phospho-sites (4 out of 5) in the 12 amino acids long motif of PPP111. Further, this motif which houses these phospho-sites, is crucial in maintaining the suppressive effect of PPP1R11 on PP1 (Zhang et al., 2008). Since PP1 is the most common of the eukaryotic phosphatases, and regulation of PP1 activity is highly dependent on regulatory subunits (Bollen et al., 2010), we strived to investigate the relevance of PPP1R11 in shaping the response of T cells towards Tregs. We performed siRNA-mediated silencing of PPP1R11 in T cells and asked: “Does PPP1R11 affect Treg-mediated suppression?”

4.2.1 PPP1R11 modulates resistance to Tregs in T cells

We utilized an allogeneic T cell:Treg coculture setting with T cells treated with PPP1R11 siRNA and control siRNA respectively, and subsequently measured the resulting IL-2 and IFN-γ levels (mRNA and protein) to analyze the effect of PPP1R11 silencing in shaping the T cell response towards activation and Treg-mediated suppression. We observed that Treg-mediated suppression of stimulation-induced IL-2 and IFN-γ was compromised upon PPP1R11 silencing. Further, the extent of abrogation of cytokine suppression was proportional and correlated to the efficiency of PPP1R11 silencing in the respective T cells. These results show that PPP1R11 regulates resistance of T cells towards Treg-mediated suppression.

4.2.2 PPP1R11 regulates TCR stimulation-induced cytokine expression, and PPP1R11 knockdown imparts an activated phenotype to T cells

While PPP1R11 has been shown to be involved in cell cycle regulation and apoptosis mainly by suppression of PP1 in non-immunological settings, the role of PPP1R11 in human immunology is not widely studied. We further dissected the direct effect of PPP1R11 on T cells in experiments similar to those introduced in the previous paragraph. PPP1R11 silencing upregulated the expression of T cell stimulation-induced cytokines like IL-2 and IFN-γ upon TCR stimulation (mRNA and protein). Hence, it is plausible that these overactivated T cells cannot be sufficiently suppressed by Tregs anymore, as described above. Additionally, PPP1R11 silencing also upregulated the TCR-induced expression of *CD69*, a marker of early T cell activation while late activation markers like *IL2RA* and *CTLA4* were not significantly affected. Furthermore, PPP1R11 silencing also downregulated PTPN22 (mRNA and protein), a phosphatase. Since PTPN22 is reported to negatively regulate the proximal TCR signaling (Bottini and Peterson, 2014), affecting PTPN22 might be an additional mechanism how PPP1R11 regulates TCR activation, besides its primary target PP1. Some works that have already indicated a role of PP1 in TCR stimulation (Thomas Mock, 2012; Wabnitz et al., 2018) prompted us to follow up on these results, especially in the context of PPP1R11 as a novel regulator of T cell activation. It is noteworthy that we observed neither an effect of PPP1R11 silencing on PP1A mRNA or protein expression. Our observation is in line with earlier works (Bollen et al., 2010; Ceulemans and Bollen, 2004) where it is suggested that regulatory subunits instead influence substrate specificity and activity of PP1 by altering its subcellular localization and interacting with PP1 substrates. To understand the role of PP1 itself in T cells and draw comparisons with the effect of PPP1R11 on T cells, we performed chemical inhibition of PP1 by tautomycetin (Mitsuhashi et al., 2001), an antifungal agent under investigation for usage as an immunosuppressive agent following organ transplantation (Wee et al., 2010). PP1 inhibition by tautomycetin suppressed the expression of IL-2 and IFN-γ (mRNA and protein). The seemingly reciprocal nature of regulation of the TCR activation-associated cytokines between chemical silencing of PP1 and siRNA-mediated silencing of PPP1R11 correlatively suggest that PPP1R11 regulates T cell activation via repressing its target PP1. In line with our conclusion, PP1A has been indicated as a positive

regulator of TCR-induced IL-2 and IFN-γ expression by regulating NF-κB by so far unknown mechanism (Thomas Mock, 2012).

To gain additional understanding of PPP1R11 effects on T cells besides well-known targets and TCR signaling regulators, we next performed RNAseq on PPP1R11-silenced T cells in the resting stage and upon 6 hours of TCR stimulation to study the potential mechanism and global effect of PPP1R11 silencing on the T cell transcriptome. We observed that PPP1R11 differentially regulated the stimulation-induced expression of several genes which were highly enriched in pathways associated with T cell activation. Among this subset of genes were surface and proximal mediators of T cell signaling and most of the genes were associated with phosphatidylinositol and AKT / MAPK pathways. Furthermore, we also observed downstream products of the NF-κB pathway. Our observation indicates that the PPP1R11-mediated effect on T cell activation might involve alterations in the MAPK, AKT, AP-1 and NF-κB pathways, all of which are reported to be involved in the induction of resistance in T cells towards Treg-mediated suppression (Mercadante and Lorenz, 2016; Wohlfert and Clark, 2007). However, targeted Western Blot analysis did not exhibit a significant difference in the phosphorylated or the total levels of exemplary canonical molecules in the MAPK-AP1, NFAT and NF-κB pathways upon PPP1R11 silencing. It needs to be considered that regulation may occur via phospho-sites other than the ones we have inspected or even by PTMs other than phosphorylation. Further, modulation may also be dependent on the time point of activation, all of which were not feasible to be tested by targeted studies.

Taken together, we propose PPP1R11 as a novel negative regulator of T cell activation-induced cytokine expression and regulator of susceptibility of T cells towards Tregs, as depicted in Figure 4.

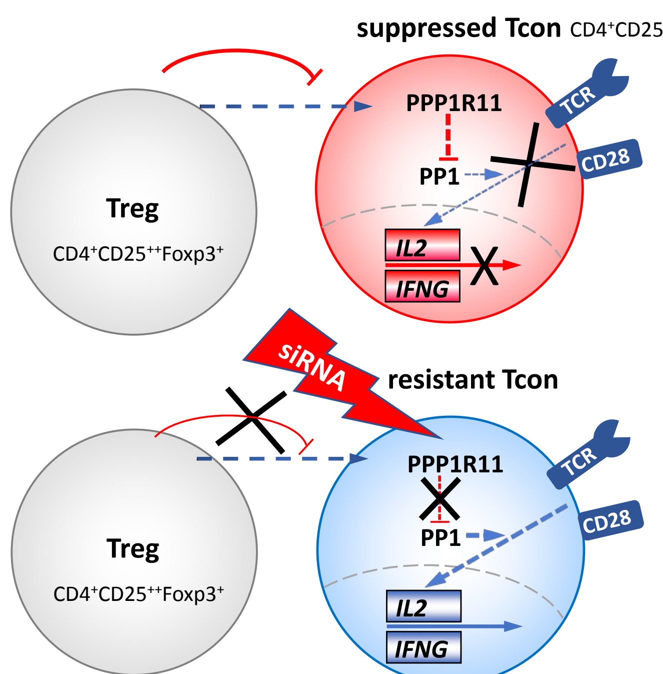

Figure 4: Novel role of PPP1R11 in the induction of resistance in T cells towards immunosuppression by Tregs. We propose PPP1R11 to modulate Treg-mediated suppression of cytokine expression in T cells possibly via repression of PP1, which itself augments cytokine expression in T cells.

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4.3 UNRAVELING THE EFFECT OF MACROPHAGES IN THE GENERATION OF ITREGS (PAPER III)

In Paper I and II we studied the effect of Tregs on the target T cells, but the differentiation, suppressive capacity, and stability of the Tregs themselves are influenced by other immune cells (Sakaguchi et al., 2008). Further, the suppressive mechanisms employed by iTregs are basically unstudied. *In vitro* differentiation of iTregs from naïve T cells, mainly by protocols involving IL-2 and TGF-β and / or other stimulants have been an excellent option to elucidate Treg biology and have also been proposed to be a possible alternative to *ex vivo* isolated Tregs (Lan et al., 2012). However, none of the iTreg-inducing protocols so far has been successful in specifically generating TSDR demethylation in the *FOXP3* locus, which is one of the distinct Treg signatures. Hence the stability and integrity of iTregs are the biggest concerns for their clinical application. Conversely, *in vivo* generated pTregs acquire TSDR demethylation in several mouse models (Ohkura et al., 2012; Schmitt and Williams, 2013); this suggests that it may be feasible to generate TSDR demethylation and stable iTregs if the *in vitro* induction protocols are optimized to recreate the *in vivo* generation of pTregs. Furthermore, macrophages have been reported to be involved in the generation of Tregs *in vivo*. Recent studies involving the adoptive transfer of tolerogenic macrophages or similar cell types in experimental models of autoimmunity have resulted in positive prognosis, possibly aided by the generation of iTregs (Haribhai et al., 2016; Weber et al., 2007). Hence in Paper III, we studied the feasibility of using supernatants from anti-inflammatory M2 macrophages generated by using a novel stimulatory protocol (IL-4 / TGF-β / IL-10) (Mia et al., 2014; Parsa et al., 2012) in generating human iTregs from naïve T cells. We observed that the M2 supernatants could induce iTregs with expression levels of FOXP3 as high as in nTregs. These iTregs also expressed high levels of other Treg signature molecules like CD25 and CTLA-4 with low expression of inflammatory cytokines like IFN-γ. Further, M2-iTregs possessed superior suppressive potential regarding *in vitro* proliferation of responder T cells. However, suppression assays with iTregs come with their own complications compared to assays with nTregs, one being that the iTreg cells are highly activated and expanded (i.e. not anergic) *in vitro*, hence being able to overgrow responder T cells and suppress unspecifically, for example by IL-2 consumption. Therefore, correct controls (like activated T cells generated without TGF-β hence not expressing FOXP3) are crucial in such assays, and often unspecific yet dose-dependent suppression by non-Tregs is indeed observed (Schmidt et al., 2016). Studying the differences and similarities in Treg suppression mechanisms between iTregs and nTregs is therefore intricate. Although it would be interesting to study whether iTregs suppress by similar mechanisms like nTregs regarding DEF6 and PPP1R11, preliminary experiments on the suppression of *IL2* and *IFNG* mRNA in Tcons by iTregs were inconclusive, perhaps due to the problems associated with the highly activated state of iTregs and control T cells as mentioned above (Angelika Schmidt, unpublished data). Therefore, we could not further follow up on the mechanistic aspects of iTreg-mediated suppression.

FOXP3 induction in the iTregs was found to be mediated by TGF-β, initially used to generate M2 macrophages. Despite extreme washing and complete removal of soluble TGF-β, macrophages captured and rereleased active TGF-β which largely mediated the iTreg differentiation. Interestingly, knocking down *TGFB* expression in macrophages and hence blocking new TGF-β expression showed no significant effect on the secreted levels of active TGF-β or FOXP3 levels in the iTregs, confirming that initially added TGF-β conferred the effects. Notably, none of the iTreg populations we tested acquired TSDR demethylation as displayed by nTregs, and consequently, FOXP3 expression was not stable upon restimulation. However, M2-induced macrophages exhibited somewhat more stable FOXP3 expression than iTregs which were generated with IL-2 and TGF-β directly, despite the importance of TGF-β in the generation of iTregs by M2 supernatants. In addition, M2-induced iTregs exhibited superior IFN-γ repression compared to TGF-β-induced iTregs. These observations highlight the importance of additional unknown factors besides TGF-β in the M2 macrophage-driven iTreg generation. Taken together, instead of *in vitro* generation of iTregs that come with concerns, M2 macrophage transfer may be a more viable option to induce Tregs for therapeutic purposes *in vivo*, which should be further explored in the future.

4.4 MAPPING THE SUBCELLULAR PROTEOME AND SUBCELLULAR PROTEIN TRANSLOCATION IN T CELLS (PAPER IV)

Paper I and II helped us to appreciate how “looking beyond” the well-known molecules can give us novel perspectives in biological pathways like T cell activation and Treg-mediated suppression. Since phosphorylation and other PTMs are reported to regulate subcellular localization of proteins, we were excited by the idea of mapping the spatial proteome of T cells. As discussed earlier, shuttling of several critical elements of TCR signaling across subcellular compartments play key roles in T cell activation, so we decided to map the proteome-wide subcellular translocation upon TCR activation.

4.4.1 TcellSubC: an atlas of the subcellular proteome of T cells

T cells from three different donors in steady state or stimulated states (15 minutes or 1 hour of TCR stimulation) were fractionated into three subcellular components, namely cytosol, membranes (including organelles), and nuclei. Using high-resolution fractionation and MS-based peptide detection, our study identified and allocated subcellular localization for proteins corresponding to more than 8,000 genes with more than 7,000 shared between the three donors. The three clusters acquired by k means clustering corresponded strongly with the gene ontology (GO) classification of cytoplasm, membrane, and nucleus and additionally with the comparable subcellular classification generated by SubCellBarCode, a recently published high-resolution mapping of the subcellular proteome of 5 different cell lines (Orre et al., 2019).

4.4.2 Stimulation-induced subcellular protein translocation

By considering the proteins that were simultaneously changing in the opposite direction in 2 or more compartments with $|\log_2 FC|>0.201$ upon 60 minutes of TCR stimulation, we were

able to identify 210 potentially translocating proteins. The majority of these translocating proteins involved the membrane fraction (which also includes organelles). Since GO analysis on the translocating proteins exhibited components of oxidative phosphorylation and mitochondrial complexes this might hint at the importance of protein shuttling during the early metabolic changes initiated upon TCR stimulation. Besides, we also observed several important transcription factors that were translocating upon activation like NFKB2, NFATC1, NFATC3, STAT3, STAT5A, which are already known to translocate upon TCR activation (Busino et al., 2012; Meier et al., 2017; Okamura et al., 2000).

Since PTMs like phosphorylations are known to regulate the subcellular localization of proteins, we integrated our translocating proteins with curated sets of activation-induced phosphoproteins in T cells in Paper I and other published data sets (Joshi et al., 2017; Nguyen et al., 2015b) including PTMs experimentally verified to regulate subcellular localizations (Hornbeck et al., 2019). We identified 21 translocating molecules with associated phosphorylations or ubiquitinations, which have strong biological relevance to be studied as regulators of subcellular localization in T cells.

Further, orthogonal verification of subcellular translocation was performed with an image-based approach which produced reproducible results involving stimulation-induced nuclear translocation of complement component 3 while the well-studied nuclear translocation of NFATC2 was used as a control. This indicates the robust nature of our findings regarding subcellular location and stimulation-induced protein shuttling.

5 CONCLUDING REMARKS AND PERSPECTIVES

(Paper I) We have curated a novel resource describing the phosphoproteomic characterization of human T cells under resting, stimulated and suppressed states. Our data indicate that Tregs suppress T cells mainly by negating the activation induced-phosphorylation of T cells. We believe that these phosphoproteins have paved the way for the discovery of novel regulators and mechanisms of T cell stimulation and suppression of T cells by Tregs. For instance, we have discovered novel roles of DEF6 phosphorylations in regulating the interaction of DEF6 with IP_3R as well as in promoting T cell stimulation via activation of NFAT and transcription of activation-induced genes. Our data indicate that Tregs target these DEF6 phosphorylations to rapidly suppress T cell activation. However, the specific molecule on the surface of Treg and T cells that interact with each other and orchestrate suppression along with the exact kinase or phosphatase that regulate DEF6 phosphorylation is still unknown. Further studies focusing on the immune synapse formed upon Treg-T cell contact and interaction of several phosphorylations in our study are required for a comprehensive understanding of the direct suppression pathway. Further, it would be of importance to generate DEF6 phospho-mutants in a DEF6 knockout background and study the effects on T cell activation and Treg suppression, also in disease models, to further understand the importance of DEF6 phosphorylation *in vivo*.

It is to be considered that the phospho-regulation of contact-dependent rapid suppression is one among various mechanisms of suppression which vary depending on the immune micro milieu and possibly the subsets of both T cells and Tregs. The direct regulation of T cells by Tregs is more likely to be relevant in the inflamed tissue to control the inflammation and probably serve as an additional mechanism to implement peripheral tolerance when the suppression of classical DC-mediated priming of T cells in the lymph nodes fail or is insufficient.

(Paper II) By following up on candidates from our phosphoproteomic screen, we have identified the role of phosphatase inhibitor PPP1R11 in inducing resistance towards Tregs in T cells, and as a novel negative regulator of TCR activation-induced cytokine expression. The underlying molecular mechanisms mediating the effect of PPP1R11 in T cells is still unclear. However, our data indicate that PPP1R11 affect T cell signaling by inhibiting PP1 phosphatase possibly via regulating the substrate specificity, activity of PP1 or competing with PP1 substrate rather than direct transcriptional or translational regulation of PP1. Our data also point at the possible involvement of AP-1 and NF-κB pathways while the identification of the exact molecule/s and mode of regulation still remains elusive. Since the efficiency of siRNA-based transient silencing of PPP1R11 dilutes with each cell division, future follow-up studies with stable PPP1R11 knockout as well as phospho-mutants in cellular and animal models are required to elucidate the exact molecular mechanism and *in vivo* relevance.

Discovery of PPP1R11 as a regulator of T cell resistance and a potential role of PTPN22 phosphatase in mediating part of the effects of PPP1R11 from our study and reports of SHP-1

phosphatase being involved in T cell resistance from another study (Mercadante and Lorenz, 2017) support the possibility of using phosphatase-modulating drugs in the therapeutic intervention of T cell resistance. Although the PP1 inhibiting drug Tautomycetin is presently available, targeting a ubiquitous and multi-functional phosphatase, which may even have opposing functions in different cell types (Gu et al., 2014; Thomas Mock, 2012), needs to be done in a cautious and cell type-specific manner. Instead of targeting the catalytic core of PP1 as done for kinases, it might be more specific to target regulatory subunits like PPP1R11 which have been reported to control the substrate specificity and activity of the phosphatase and even its cell-type specific function (Bollen et al., 2010).

The biological significance of PPP1R11, DEF6, and our phosphoproteomic database provide novel targets and avenues to revisit the role of T cells in immunotherapy, especially to modulate the sensitivity of T cells towards suppression of T cells by Tregs. Interesting clinically relevant and open questions remain to be explored:

1. Can the candidate molecules from our phosphoproteomic study be used to predict disease prognosis or therapy outcome for ongoing clinical trials involving Tregs?
2. Can the candidate molecules be targeted to regulate the T cell susceptibility to Tregs in disease situations?

(Paper III) By shifting our focus to the generation of iTregs, we discovered the ability of M2 macrophages in generating human iTregs mainly by capturing and re-releasing TGF-β, primarily used in the differentiation of M2 macrophages themselves. The superior stability and suppressive capabilities of M2-iTregs over TGF-β-iTregs may be accredited to additional factors produced by M2 macrophages which are yet to be identified. We provide a novel protocol for *in vitro* generation of iTreg using M2 macrophages induced by TGF-β-containing optimized cytokine cocktail. In contrast to the systemic delivery of TGF-β, adoptive transfer of M2 macrophages might be a more specific and effective alternative for targeted delivery of TGF-β and restoration of immune suppression possibly via Treg induction. Whether this scenario could be exploited *in vivo*, and which molecular mechanisms of suppression are employed by iTregs, remain to be investigated.

(Paper IV) We have curated a high-resolution subcellular proteomic map of primary human T cells, divided into cytosolic, nuclear and membrane (including organelles) fractions under steady-state conditions and upon 15 minutes and 1 hour of T cell receptor (TCR) stimulation respectively. The subcellular classification is presently based on clustering analysis and can certainly be improved by applying machine learning aided subcellular classification in the future as done in other studies (Christoforou et al., 2016; Orre et al., 2019). Our database will particularly support functional studies of the novel molecules identified from several global omics and prediction studies which are getting more and more common with the advent of high throughput technologies. The subcellular location from our study can be readily used as a basis for hypothesis generation for T cell-specific cellular function of proteins, as well as for studies exploring the importance of these localizations in Treg-mediated suppression of T cells.

Mapping the spatial proteome by targeted experiments, tagged proteins and antibody-based imaging approaches like Human Cell Atlas (Huh et al., 2003; Thul et al., 2017) and global MS-based studies (Christoforou et al., 2016; Itzhak et al., 2016; Jadot et al., 2017; Jean Beltran et al., 2016; Nightingale et al., 2019; Orre et al., 2019) including our present study have contributed an immense amount of data involving subcellular localization of proteins in different cells and contexts. The next challenge of the field is to integrate the data for meta-analysis of subcellular proteome acquired from several of these approaches and make it more accessible via a user-friendly interface. Ultimately, it would be of great interest to study subcellular protein translocations globally also in T cells upon suppression by Tregs. However, the amount of starting material required despite highly sensitive proteomics methods employed so far, precluded us from performing these studies with Treg-suppressed T cells as well.

In conclusion, with these novel phosphoproteomic and subcellular proteomic data in T cells and transcriptomic data on resistant T cells, we set the stage for further studies employing targeted analysis of the relevance of these novel findings in TCR activation and Treg-mediated suppression. Our data contribute to understanding and revisiting the role of T cells in basic biology and disease, and ultimately, to develop better therapeutic strategies for autoimmune diseases, allergies and cancer.

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