The role of Ten Eleven Translocation proteins on regulatory T cell differentiation and function

MS Thesis submitted by

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Certificate

This is to certify that this dissertation entitled "The role of Ten Eleven Translocation proteins on regulatory T cell differentiation and function" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Sameer Kumar Jagirdar at the Turku Centre for Biotechnology, Turku, Finland under the supervision of Prof. Riitta Lahesmaa, Academy Professor, Turku Centre for Biotechnology, Turku, Finland during the academic year 2017-2018

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Supervisor

Declaration

I hereby declare that the matter embodied in the report entitled "The role of Ten Eleven Translocation proteins on regulatory T cell differentiation and function" are the results of the work carried out by me at the Turku Centre for Biotechnology, Turku, Finland, under the supervision of Prof. Riitta Lahesmaa and the same has not been submitted elsewhere for any other degree.

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Abstract

Regulatory T cells (Tregs) are an important part of the adaptive immune system. They function as negative regulators of the immune response, thereby preventing inflammatory diseases. They also maintain immunologic tolerance to self and commensal antigens. FOXP3, a transcription factor characterised as the master regulator of Tregs, can be modified in a variety of ways including epigenetic mechanisms. The FOXP3 locus contains a Treg specific demethylated region (TSDR) which is actively demethylated by Ten eleven translocation (TET) enzymes. This is important for the stability of FOXP3 expression and Treg development. Therefore, it is necessary to study them further to understand the underlying mechanisms. We sought to do this by using a TET1/TET2/TET3 triple knockdown approach to see the effect on development and functionality of in vitro induced Treg cells (iTregs) and further study their downstream targets. Firstly, we used a siRNA mediated approach which did not generate consistent results. Therefore we changed strategy and used a CRISPR/Cas9-based approach to stably silence the TET genes. We were successful in obtaining one functional CRIPSR crRNA for each TET which could efficiently knockdown the targeted gene for up to two weeks. Further, the procedure will have to be standardised for a triple knockdown.

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I would like to thank my mentors, Andrabi Syed Bilal Ahmad and Dr. Omid Rasool for guiding me throughout the course of my project and giving me great inputs along the way. It has been very enjoyable working with them. Not once did I feel that work was tiresome. They always kept the excitement going.

I would like to thank Dr. Subhash Tripathi for showing me the Th17 way of life and letting me work on a very interesting part of his project.

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Introduction

The plague of Athens (430-426 BC) at the outbreak of the Peloponnesian War was a significant contributor to the decline and fall of classical Greece. It caused the death of the great politician, Pericles and obliterated the population. But it was also a great point in history for science. It marked the earliest known reference to immunity as we know it. Thucydides, a survivor of the great epidemic, noticed that a person who had survived and recovered from a previous case of the disease could help treat the sick without contracting it again (Retief and Cilliers, 1998). The 18th and 19th centuries saw great and rapid advancements in the field of immunology through scientists like Pierre-Louis Moreau de Maupertuis, Louis Pasteur, Paul Ehrlich and Elie Metchnikoff (Ostoya, 1954; Plotkin, 2005).

The immune system is a collection of cells, tissues and organs working together towards achieving a common goal; to preserve the integrity of the body, by protecting it from environmental agents such as microbes and chemicals. In a more fundamental sense, the immune system has the ability to recognise and distinguish between self and non-self cells. As a whole, it provides a multi-layered defence system of increasing specificities. First, there is the physical barrier (skin, mucous), followed by the innate and adaptive immune systems. Hematopoietic stem cells (HSCs) in the bone marrow give rise to two different cell types: the common myeloid and lymphoid progenitors. The common myeloid progenitor gives rise to the cells involved in the innate immune response including neutrophils, eosinophils, basophils and macrophages. The lymphoid progenitor gives rise to B and T cells which are involved in adaptive immunity. The innate immune response is triggered when pathogen-associated molecular patterns (PAMPs) on the invading microbes are recognised by pattern recognition receptors (PRRs). It is usually the first response to an invading pathogen and is non-specific and short-lived. It further activates the adaptive immune response. On the other hand, the adaptive immune response takes longer to activate but is ‘antigen-specific’, long lasting and is associated with immunological memory. It is capable of giving an enhanced response against a pathogen that it has already encountered. This is the basis of vaccination.

The B and T cells of the adaptive immune system play a role in humoral and cell-mediated immunity respectively. B cells, when activated, differentiate into plasma

cells and secrete antibodies which bind to a specific antigen and help in its clearance. This is the humoral aspect of the adaptive immune system. Cell-mediated immunity is brought about by two types of T cells: CD8+ cytotoxic T cells (Tc) and CD4+ helper T cells (Th). Tc cells otherwise called killer T cells, induce apoptosis of cells that are infected with pathogens or are otherwise damaged or dysfunctional. T helper cells play a very crucial role in the immune system, in that, they orchestrate the full panoply of the immune system. To name a few processes, they help in recruitment of different cell types to the site of infection by producing certain chemokines and cytokines, enhance or suppress the activity of certain cells and also help B cells produce pathogen specific antibodies.

T cell development occurs in the thymus. It contains cortical and medullary areas surrounded by a capsule. Lymphoid progenitors which have developed from HSCs in the bone marrow, migrate to the thymus for further development and selection. They first enter the sub-capsular cortical area where they encounter the thymic stroma and endure a term of proliferation. They move from the cortex to the medulla as they differentiate from CD4-CD8- double negative cells into CD4+CD8+ double positive cells and further into single positive naïve CD4+ (Th) or CD8+ (Tc) cells which are then released from the thymus into the periphery (Overgaard et al., 2015; Schwarz and Bhandoola, 2006).

In the periphery, a T cell is activated by three signals. The first is the binding of the T cell receptor (TCR) to its cognate peptide on the major histocompatibility complex (MHC) on an antigen presenting cell (APC). This triggers initial activation of the T cell. The second signal comes from co-stimulation. In the case of Th cells, initial co-stimulation is provided by binding of the co-stimulatory receptor CD28 on the T cell to the B7 protein on the APC. This induces T cell proliferation. Without the second signal, the T cell becomes anergic. The third and final signal that decides which type of responder the T cell will become, is in the form of cytokines (Smith-Garvin and Koretzky, 2009). Table 1 shows the different Th cell subtypes, their stimulating cytokines and the associated master regulator (transcription factor) where known (Luckheeram et al., 2012). Each effector subtype has different functions. Th1 cells help in the elimination of intracellular pathogens while also being associated with organ specific autoimmunity. Th2 cells drive the immune response against extracellular parasites and play a major role in the induction and persistence of many

allergic diseases (Prete, 1992). Th17 cells also drive the immune response against extracellular parasites, specifically, bacteria and fungi and are also involved in the generation of autoimmune diseases (Annunziato et al., 2007). Regulatory T cells (Treg) play the role of negative regulation of an immune response, thus protecting against immunopathology. They are very important as they maintain immune homeostasis and immunologic tolerance to self and foreign antigen (Sakaguchi et al., 2008).

CD4+ Th cell subtype	Stimulating cytokine(s)	Master regulator
Th1	IL12, IFNγ	T bet
Th2	IL4, IL2	GATA3
Th17	IL6, IL21, IL23, TGFβ	RORγt
Tfh	IL6, IL21	
Treg	IL2, TGFβ	FOXP3
Th9	IL4, TGFβ	

Table 1: Th cell subtypes, their stimulating cytokines and associated master regulators

As described in table 1, the transcription factor Forkhead box P3 (FOXP3) has been characterised as the master regulator of Tregs. In humans, CD4+FoxP3+ Tregs are generated in the thymus (tTregs) or from naïve CD4+ T cells in the periphery upon antigen stimulation in the presence of IL-2 and transforming growth factor β (TGFβ) (pTregs). Tregs generated by the latter method *in vitro* are called induced Treg (iTregs). tTregs are important to prevent autoimmunity as they express a TCR repertoire with a bias for self-antigens while pTregs are mostly found in the gut and maternal placenta and consequently are thought to be important in establishing tolerance against commensal bacteria, allergens and the foetus in the pregnant mother (Beissert et al., 2006; Fontenot et al., 2003). Any kind of disruption in the functioning or development of Tregs may lead to inflammatory or auto-immune diseases.

Mutations or deletions in the FOXP3 gene in humans lead to fatal autoimmune and/or inflammatory diseases due to the inability of CD4+ T cells to develop into Tregs. In humans, IPEX (Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) is a fatal genetic disorder caused by a mutation in the FOXP3 locus. A similar phenotype is seen in the mutant mouse strain scurfy. Experiments on these mice confirmed that FOXP3 was indispensable for the thymic development of

CD4+CD25+ Tregs. Moreover, it was shown that ectopic expression of FoxP3 in conventional CD4+CD25- T cells can induce the suppressive phenotype. Therefore, stable FOXP3 expression is particularly necessary for the development and proper functioning of Tregs (Bennett et al., 2001; Fontenot et al., 2003). FOXP3 is essential for the suppressive ability of Tregs (Gavin et al., 2007).

Several signalling pathways have been implicated for the transcriptional regulation of Foxp3. For example, signalling pathways initiated by TCR, RA, IL-2R/STAT pathway, TGFβ/SMAD pathway, PI3K/Akt/mTOR axis and Notch signal pathway (Belkaid et al., 2011; Harris and Pierpoint, 2012; Pyzik and Piccirillo, 2007; Rudensky, 2012). Additionally, epigenetic mechanisms have been proposed for the regulation of Foxp3 (Floess et al., 2007). It was found that a conserved CpG island in the conserved non-coding sequence 2 (CNS2) region of the FOXP3 locus is hypomethylated in natural Tregs as against conventional CD4+ T cells. The methylation status of this region, referred to as the Treg-specific demethylated region determines the expression level of FOXP3 and Treg stability (Baron et al., 2007; Polansky et al., 2008).

TSDR demethylation is achieved by an active mechanism that involves the successive oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) in an Fe(II) and 2-oxoglutarate dependent mechanism by enzymes of the ten-eleven-translocation (TET) family which is then removed and replaced by an unmodified cytosine by the thymine DNA glycosylase (TDG)/Base excision repair (BER) pathway (Nair et al., 2016; Pastor et al., 2013; Rasmussen and Helin, 2016; Toker et al., 2013). The TET family includes three mammalian proteins, namely, TET1, TET2 and TET3. Yang et al. reported that TET1 and TET2 deletion led to hypermethylation of the FOXP3 locus and impaired Treg cell differentiation and function (Yang et al., 2015). Nakatsukasa et al. showed that TET2 and TET3 are redundantly involved in TSDR demethylation and are important for the stability and homeostasis of Tregs (Nakatsukasa and Yoshimura, 2017). Yue et al. showed using TET2/TET3 double knockout mice that TET2/3 proteins mediate the demethylation of CNS1 and CNS2 of the FOXP3 gene and that FOXP3 expression is markedly compromised in these mice. They also report that Vitamin C potentiates TET activity (Yue et al., 2016). These findings show that the TET proteins are important for the maintenance of the demethylated region on the

FOXP3 locus and for the development and stability of Tregs. Therefore, there is a need to elucidate the role(s) they play in Treg differentiation and function and the mechanisms thereof.

Objectives

1. To study the role of TET proteins in the differentiation and functioning of Tregs
    a. The proposed method of the study is as follows:
        i. Standardize TET1/TET2/TET3 triple knockdown using siRNA-mediated gene silencing approach
            I. Study the effect of TET knockdown on Treg suppressive ability and FOXP3 expression
            II. If knockdown and its effect is successful and consistent, prepare samples for RNA-seq to find the downstream targets
    b. To study the difference in the expression of TETs at different time-points in Tregs differentiated from CD4+CD25- cells isolated from peripheral blood mononuclear cells (PBMCs) as opposed to those isolated from human umbilical cord blood.
    c. To study the effect of media on the polarisation of Tregs (X-Vivo 15 that is normally used vs X-Vivo 20)

Materials and Methods

CD4+ cell isolation: Blood (human umbilical cord blood or buffy coat) is diluted 1:1 with PBS and mononuclear cells extracted using Ficoll Paque Plus (GE Life Sciences) density gradient centrifugation. CD4+ cells were isolated from the mononuclear cells using the DynabeadsTM CD4 positive isolation kit from ThermoFisher.

CD25 depletion: CD4+ cells were incubated with CD25 Microbeads II (Miltenyi Biotec) in MACS buffer and run through a MACS separation column LD 25 (Miltenyi Biotec). The flow-through containing CD4+CD25- cells is collected. (MACS buffer: PBS with 0.5% BSA, 2mM EDTA pH 7.2 and filter sterilised)

Nucleofection: 4x106 cells in 100µl Optimum (Gibco) were mixed with 6µg siRNA in a nucleofection cuvette and nucleofected with TET targeting or non-targeting siRNAs

(Table 2) using Amaxa Nucleofector II. Cells were immediately collected using pre-warmed RPMI (Lonza) (supplemented with penicillin/streptomycin, L-glutamine and 10% FBS) and transferred to a 6 well plate. Cells were rested for 48 hours in the incubator at 37°C.

siRNA target	siRNA sequence (5'-3')
TET1	ACGAUUAGCUCCAAUUUAU[dT][dT]
TET2	CAAGGGCAGUCCCAAGGUA[dT][dT]
TET3	UGGAGUCACCUCUUAAGUA[dT][dT]

Table 2: TET siRNA sequences

Culturing Tregs: 48h post nucleofection, cells were collected and washed with PBS and then suspended in X-Vivo 15 (Lonza) (supplemented with penicillin/streptomycin and L-glutamine). They were cultured in α-CD3 coated plates under Th0 (X-Vivo 15 + α-CD28) and Treg (X-Vivo 15 + α-CD28 + IL-2 + TGF-β + RA + Serum) conditions.

Intracellular cytokine (IC) staining: Cells were washed with FACS-I buffer (2% FBS, 0.1% Sodium azide in PBS) and then fixed in a 1:3 solution of fixation/permeabilization concentrate:diluent (eBioscience™) for 30 minutes at 4°C in the dark. After fixing, they were permeabilised in permeabilization buffer (eBioscience™) and incubated at 4°C with a fluorochrome-conjugated antibody for 30 minutes in the dark. They were washed again with FACS-I buffer and stored in 1% formalin. Cells were analysed using a BD LSRII flow cytometer.

Surface staining: Cells were washed two times with FACS-I buffer (2% FBS, 0.1% Sodium azide in PBS) and then incubated with a fluorochrome conjugated antibody for 30 minutes at 4°C in the dark. They were washed again with FACS-I buffer and stored in 1% formalin. Cells were analysed using a BD LSRII flow cytometer.

iTreg suppression assay: Responder cells (CD4+ CD25- Tconv cells) were stained with CellTraceTM Violet and then plated along with iTregs in a 96-well U-bottom plate coated with α-CD3 in 3 different ratios (Responder: iTreg = 1:1, 1:0.5, 1:0.025). The plate was kept in the incubator at 37°C for 4 days. Then the cells were washed with FACS-I buffer and stored in 1% formalin. Cell counting was done on BD LSRII flow cytometer.

Cas9/gRNA Ribonucleoprotein editing: gRNA was prepared by mixing crRNA (TET1, 2 or 3- specific Edit-R predesigned crRNAs) and tracrRNA (both from Dharmacon) in a 1:1 ratio and incubated at 37°C for 30 minutes to hybridize the RNAs. Ultramer oligo was added to the above solution. The RNP mixture was assembled by a 2:1 ratio mixing of the gRNA and Cas9 protein and incubated for 10 minutes at 37°C. 1.2 million cells were added to this mixture and nuclefected with the 96-well nucleofector shuttle system (Amaxa) using the EH115 nucleofection program. Cells were washed with pre-warmed media and transferred to a 96-well culturing plate. 24 hours post nucleofection, the cells were activated. Adapted from (Simeonov et al., 2016).

PCR amplification and sequencing: Primers against each of the TETs were designed such that 400bp surrounding the target of TET1 crRNA1, TET2 crRNA3 and TET3 crRNA1 would be amplified. KAPA HiFi Hot start kit was used to PCR amplify DNA

isolated from TET1 crRNA1, TET2 crRNA3 and TET3 crRNA1 KD samples with the following primers:

TET1_crRNA1_F-primer: 5´-GATCTCCCGTTCAACCA-3´

TET1_crRNA1_R-primer: 5´-TGTGACTTTGTGGATGCTTGG-3´

TET2_crRNA3_F-primer: AGCAGTGGAGAGCTACAGGA

TET2_crRNA3_R-primer: CCTTGGGACTGCCCTTGATT

TET3_crRNA1_F-primer: 5´-AACCAGCAAACATGTCTCCCA-3´

TET3_crRNA1_R-primer: 5´- GTGTGGCAGTTGGCACAAATA-3´

The program used was as follows:

95°C 5min

98°C 20sec / 65°C 20sec (-0.5°C /cycle) / 72°C 2min (14X)

98°C 20sec / 58°C 20sec / 72°C 2min (35X)

72°C 10min

4°C

The PCR reactions were run on a 1.4% agarose gel and products (400bp) eluted using the gel extraction kit from Machery Nagel. The extracted PCR products were sent for sequencing to the sequencing unit of Institute for Molecular Medicine Finland, Helsinki.

Results and Discussion

Role of TET proteins in the differentiation and function of iTregs

Stable FOXP3 expression is a signature of Tregs. This depends on the DNA demethylation status at the TSDRregion within the FOXP3 locus. Recent evidence suggested that active demethylation at the TSDR is regulated by the TET proteins, primarily by TET2 and TET3. Earlier experiments from Lahesmaa lab studying TET protein kinetic expression in cells isolated from cord blood showed that all the three TETs were differentially expressed between Th0 and iTreg at early time-points (12h, 24h). In these experiments, FOXP3 was specifically upregulated in iTregs at 72h time-point post cell activation (Fig. 1). Encouraged by these results, we decided to study the effect of TET knockdown on FOXP3 expression and iTreg suppressive ability.

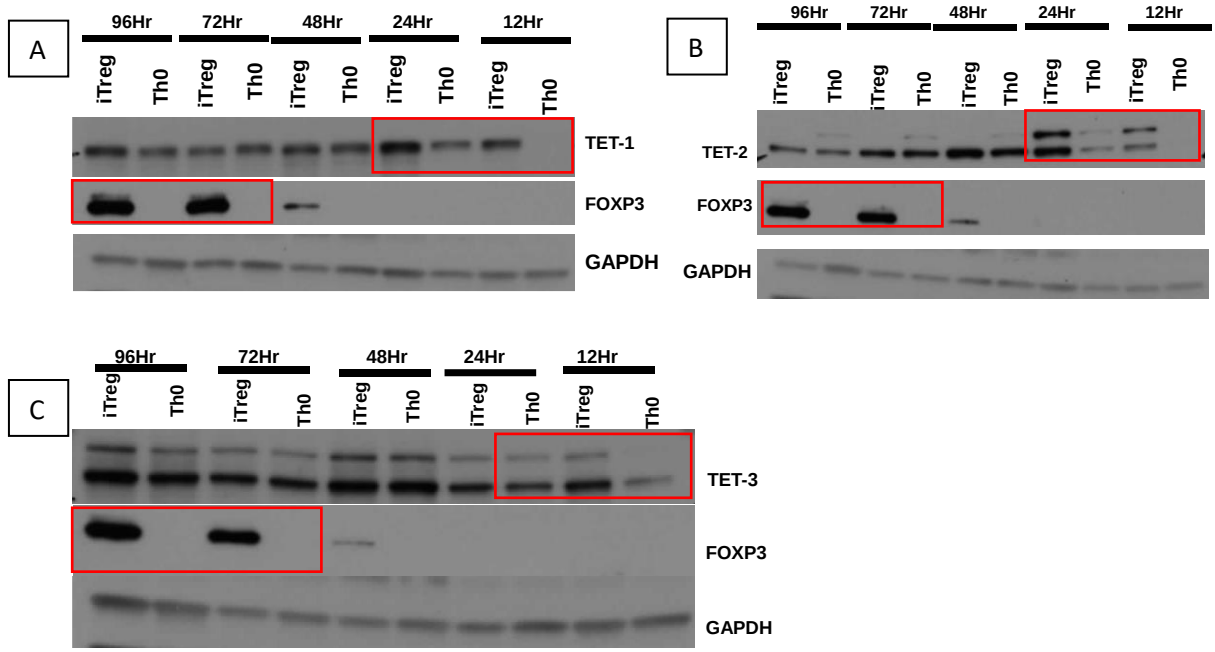


Figure 1. Western blots showing expression of A) TET1 B) TET2 C) TET3 along with FOXP3 at the time points: 12h, 24h, 48h, 72h and 96h. (Picture courtesy: Andrabi Syed Bilal Ahmad)

As can be seen in figure 1, TET1 and TET2 are very highly and clearly differentially expressed between iTreg and Th0 at the 12h and 24h time-points while TET3 is differentially expressed at the 12h time-point. Previous RNAseq results from the lab showed a higher expression of TET1 and TET2 and not so much for TET3 in

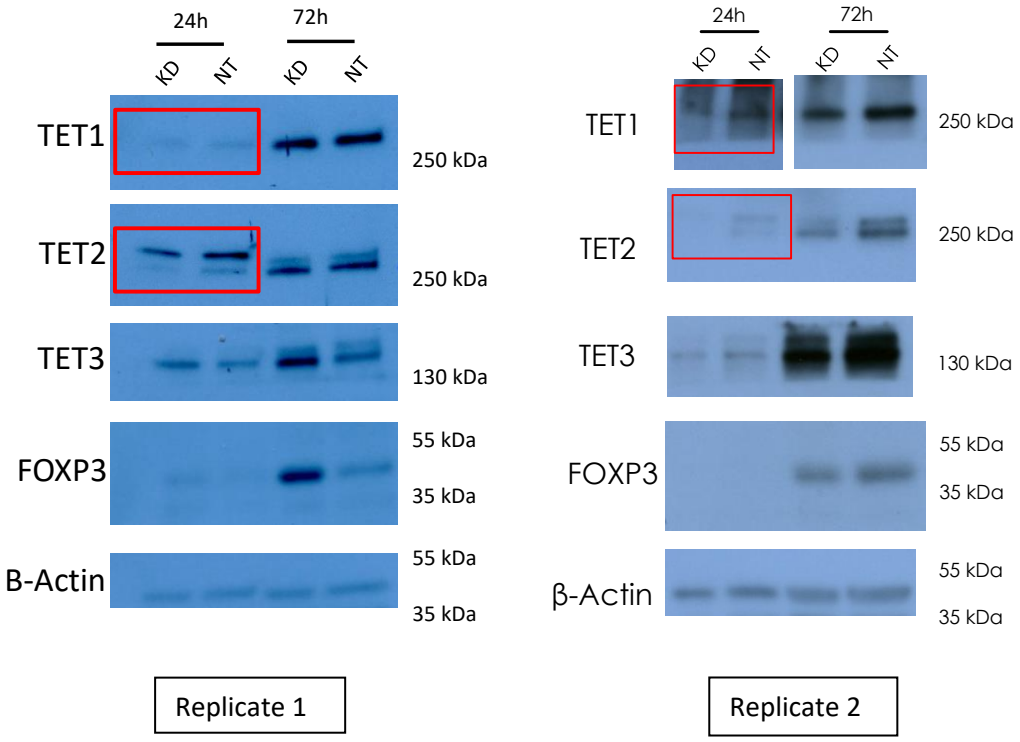
iTregs as compared to Th0. Taking this into consideration, we decided to start with TET1/TET2 double knockdown experiment and look at its effect on the iTreg cells.

Previously, different siRNAs against each TET had been validated in the lab for consistent knockdown effect and out of them the best 3 (one for each TET) were selected for TET double and/or triple knockdown experiments.

CD4+ CD25- cells were isolated from cord blood and nucleofected with a master mix of the TET1 and TET2 siRNAs or non-targeting siRNA as a control. Transfected cells were rested for 48h and then activated under Treg culturing condition for different time-points. Cells were harvested at 24h and 72h time-points post cell activation.

Figure 2 shows a representative comparison between two replicates (out of three) of the TET1/TET2 double knockdown experiment. The western blots show evident knockdown of TET1 and TET2 (Fig. 2 A) while the effect on FOXP3 expression and iTreg suppressive ability were not consistent (Fig. 2 B and C).

A



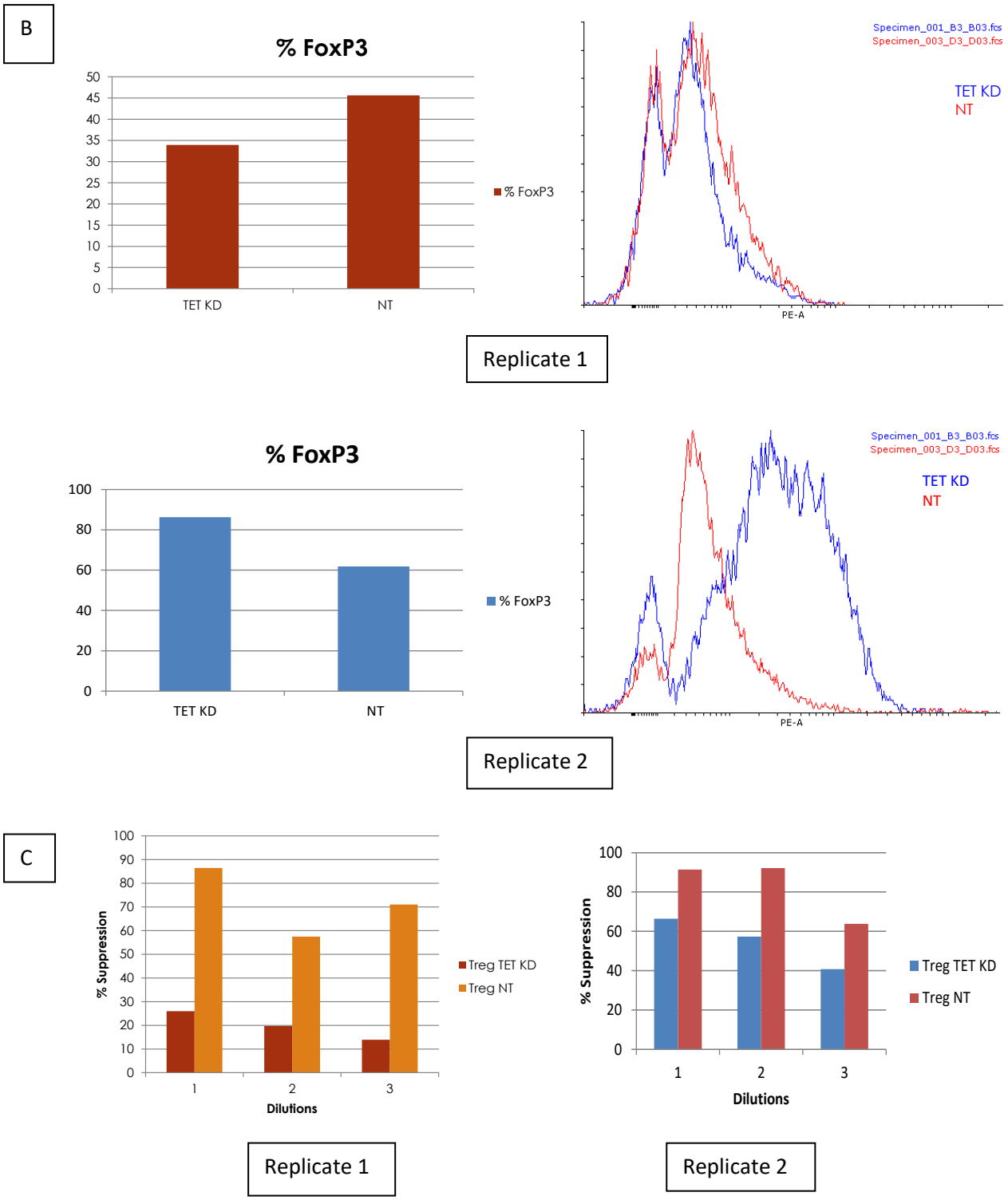
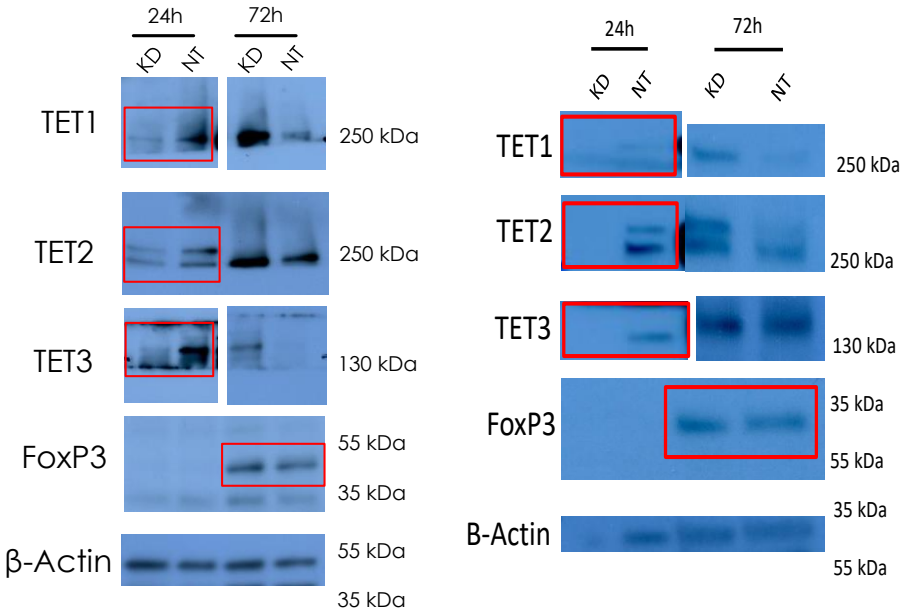


Figure 2: A) Western blot showing knockdown of TET at the protein level. B) Effect of knockdown on FOXP3 depicted by a bar graph showing percentage of FOXP3 and an overlay histogram. C) Effect of Knockdown on suppressive ability by bar graphs showing percentage suppression of responder cells). TET KD – TET knockdown; NT – Non-targeting; Dilutions – Treg:Responder – 1 (1:1), 2 (0.5:1), 3 (0.025:1)

Even though there was knockdown in all the three replicates, the effect on FOXP3 expression and iTreg suppression ability was not consistent among them. This

led us to go ahead with our plan to do a TET1/TET2/TET3 triple knockdown using the selected siRNAs. Figure 3 shows a comparison between two replicates of the TET1/TET2/TET3 triple knockdown experiment. Similar to the double knockdown experiment, knockdown was evident (Fig 3. A) but the effect on FOXP3 expression was not clear (Fig 3. B).

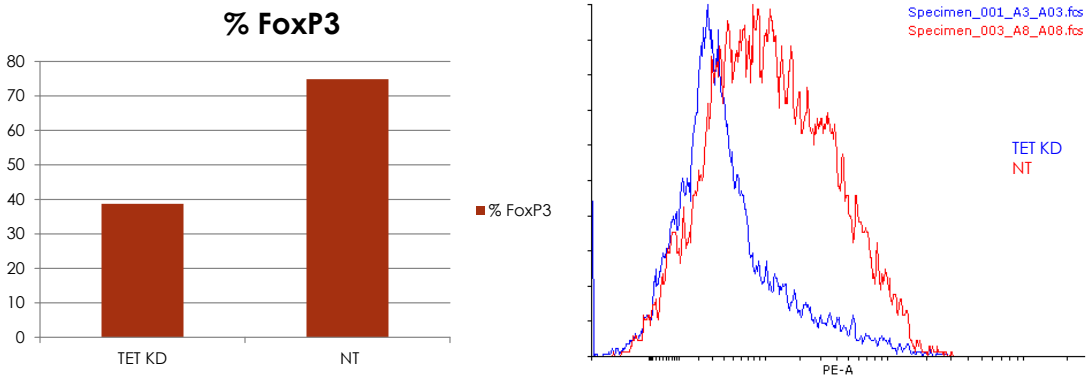
A



Replicate 1

Replicate 2

B



Replicate 1

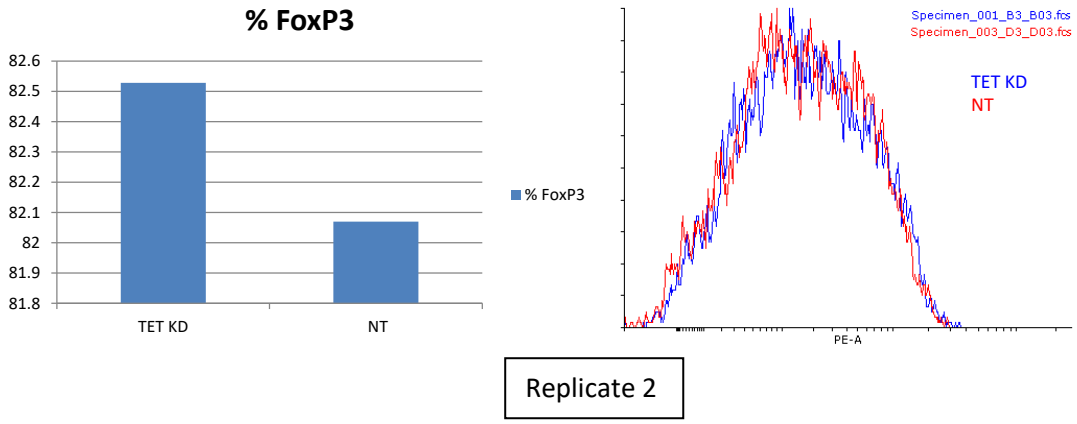


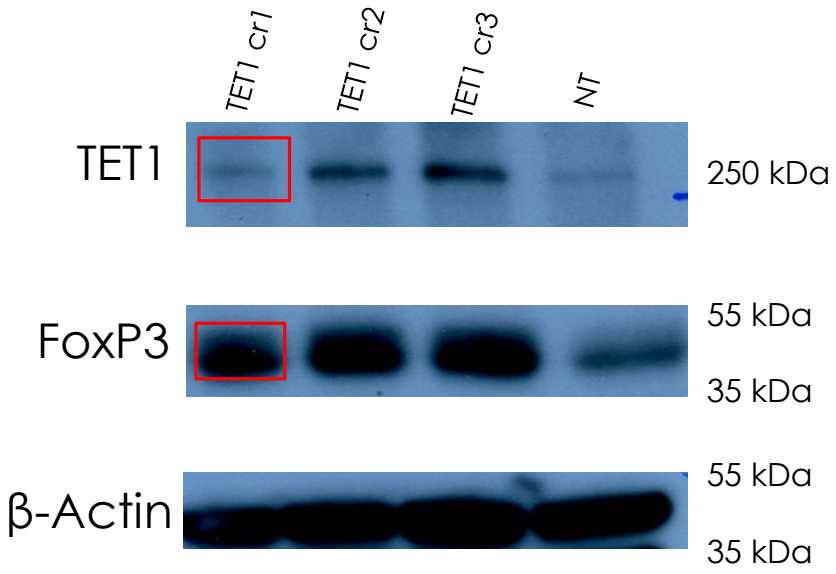
Figure 3: A) Western blot showing knockdown of TET at the protein level. B) Effect of knockdown on FoxP3 depicted by a bar graph showing percentage of FoxP3 (left) and an overlay histogram (right). TET KD – TET knockdown; NT – Non-targeting

Based on these results, we concluded that TET knockdown using siRNA was not strong enough. To get a more stable and stronger TET knockdown, we decided to use a CRISPR/Cas9 based gene edition approach to efficiently knockdown the TET genes. In this CRISPR approach, we are not selecting for a cell clone with a certain gene edition but rather we are analysing the whole cell population containing all different types of gene editions and cells with no gene edition. We nucleofected CD4+CD25- cells with a reaction mixture of TET1, TET2 or TET3- specific gRNA and Cas9 and rest the cells for 24 hours at 37 0C after which the cells were activated and grown in iTreg culturing condition.

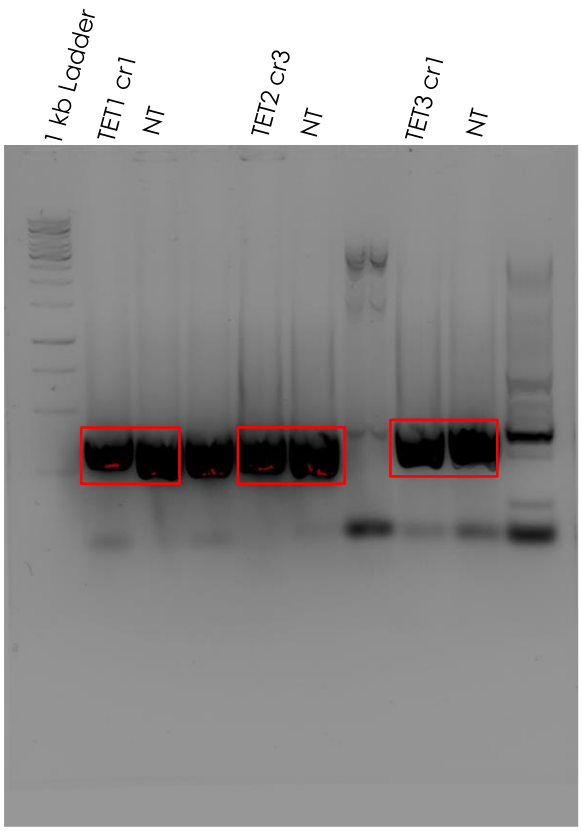
Since the CRISPR/Cas9 approach had not been used for TET proteins previously in the lab, it was first necessary to standardise the procedure and find a suitable crRNA against each TET. For each TET gene, three different crRNAs were validated. Initial experiments (3 replicates) with TET1 crRNAs (TET1-crRNA1, crRNA2 and crRNA3) showed promising results. Specifically, TET1 crRNA1 was successful in knocking the gene out (viewed as a knockdown in western) (Fig. 4 A). The TET1 knockdown persisted for up to two weeks post nucleofection (data not shown), after which the cells began to die. However, since the non-targeting (NT) crRNA also reduced the TET1 expression, we were not sure if the observed TET1 reduction by TET1-crRNA1 was specific. To confirm the specificity of the TET1-crRNA1 mediated TET1 silencing, we decided to sequence the genomic region of TET1-crRNA1 target sequence in the TET1 gene. For this, primers were designed on both sides of the TET1-crRNA1 target sequence and PCR amplification was carried out to amplify the

region. PCR products were analysed on agarose gel (Fig 4. B) and bands of correct size were cut out for DNA extraction and sequencing. Results from DNA sequencing of the NT-crRNA sample showed the targeted TET1 genomic region having clean chromatogram peak sequence free from baseline noise peaks 5´of the Protospacer Adjacent Motif (PAM) sequence (Fig. 4C, upper panel), confirming that the NT-crRNA has at least not influenced the TET1-crRNA1 target sequence. However, in the TET1-crRNA1 sample, the sequence of the same region 5´of PAM contained high baseline noise peaks confirming accumulation of several types of edits (in addition to non-edited cells). This resulted from double stranded DNA break, generated by TET1-crRNA1 some three nucleotides 5´upstream of the PAM sequence, followed by Error-prone Non-Homologous End Joining (NHEJ) (Fig. 4C, lower panel). This experiment was repeated twice and the results were the same. Thus, at least in these experiments, the NT-crRNA seems to have an off-target effect reducing the expression of TET1 and FOXP3. The FOXP3 expression was slightly less in the TET1-crRNA1 targeted sample than the other TET1-crRNA treated samples. However, these experiments have to be repeated using a NT-crRNA showing no off-target effects.

A



B



C

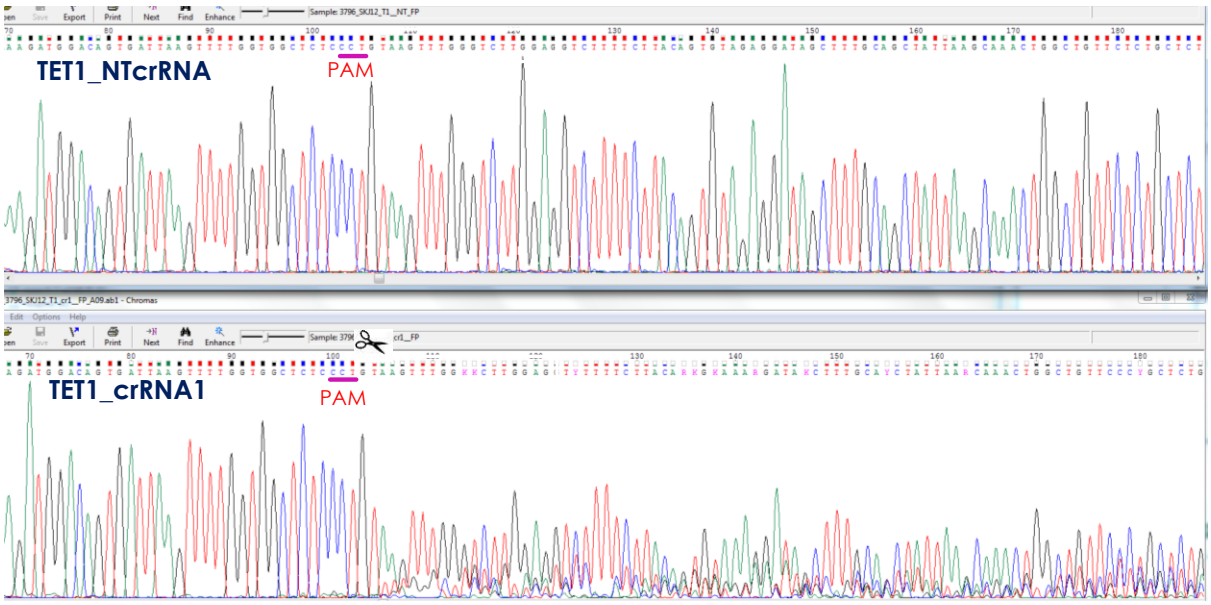
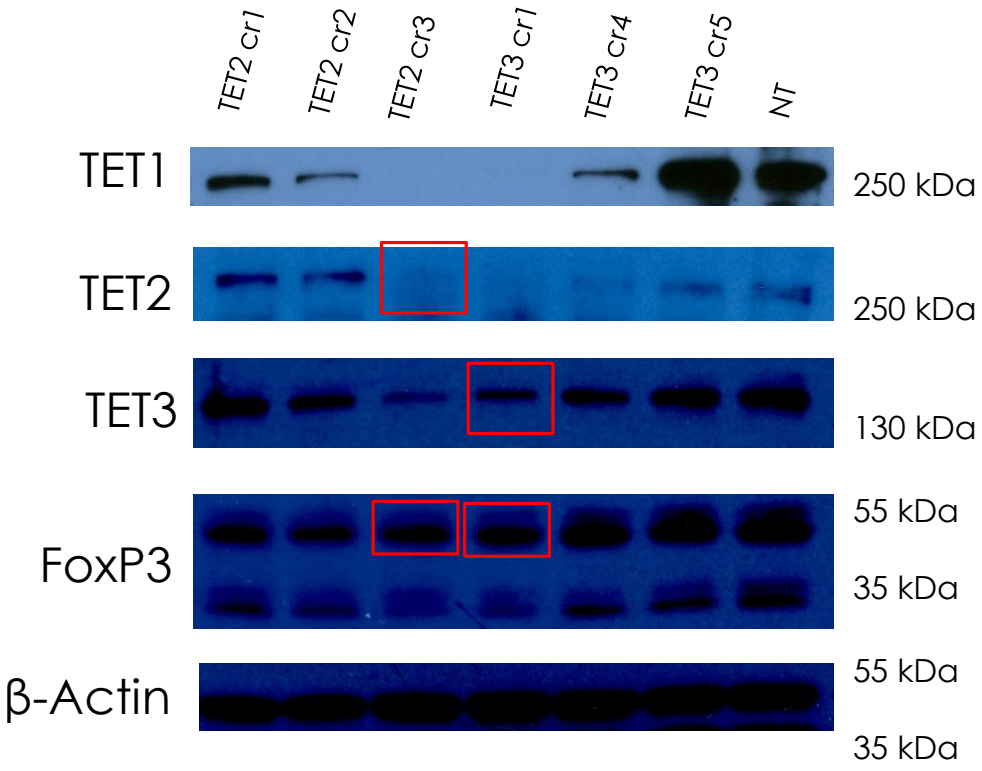


Figure 4: A) Western blot depicting CRISPR/Cas knock out using three different crRNA against TET1. HIC-1 crRNA4 is used as positive control. B) Agarose gel showing the PCR products obtained by amplifying the region around the target of each of the TET crRNAs using the specified primers. Red boxes depict bands which were extracted from the gel to process for sequencing. C) Sequencing result of the target region of the crRNA. NT shows a single sequence towards the right of the PAM. TET1 crRNA1 shows a variety of sequences due to deletions caused by a nick (picture courtesy: Dr. Omid Rasool). NT - Non-targeting; PAM – Protospacer adjacent motif

Following this, we used the same CRISPR-Cas9 approach to target TET2 (using TET2-crRNA1, crRNA2 and crRNA3) and TET3 (using TET3-crRNA1, crRNA4 and crRNA5). Figure 5. A shows a western blot of the TET2 and TET3 CRISPR KO experiment. TET2 crRNA3 and TET3 crRNA1 were found to be successful in knocking out their respective target gene. Moreover, all the three TET2 crRNAs and TET3 crRNA1 seem to slightly reduce the expression of FOXP3 as well. As shown in figure 5A, the NT-crRNA showed less off-target effect compared to TET1 crRNA results of figure 4A. However, in a second replicate of TET2 and TET3 crRNA experiment, the NT-crRNA again showed strong off-target effect similar to the one seen in figure 4A. Thus, these experiments have to be repeated with new NT-crRNAs.

Subsequent PCR amplifications and sequencing confirmed that successful gene edition had taken place in both TET2-crRNA3- and TET3-crRNA1-treated samples (Fig. 5 B).

A



B

Figure 5: A) Western blot depicting CRISPR/Cas knock out using three different crRNA each against TET2 and TET3. HIC-1 crRNA4 is used as positive control. B) Sequencing result of the target region of the crRNA. NT shows a single sequence towards the right of the PAM. TET2 crRNA3 and TET3 crRNA1 show a variety of sequences due to deletions caused by a nick (picture courtesy: Dr. Omid Rasool). NT - Non-targeting; PAM – Protospacer adjacent motif

An interesting observation was that knockout of one of the TETs had an influence on the other two TETs (Fig. 5 A and data not shown). Knockout of one TET brings down the level of the other two. This was quite striking as we expected the contrary to happen. It has been shown by many groups previously that the TETs are

redundant in their functions and the loss of one TET is compensated by the other(s). Further work is needed to study this further.

Another thing to note is the problem we faced with the non-targeting crRNA. It seems to have an effect on all three TETs; TET1 (Fig. 4 A), TET2 and TET3 (data not shown). These NT-crRNA related findings were unexpected since it is commercially provided by Dharmacon as a non-targeting crRNA. One can speculate that the NT-crRNA may indirectly affect TET expression by influencing an upstream protein/s required for TET expression. Thus these experiments have to be repeated with better NT-crRNA which shows no off-target effects.

Now that we have one successful crRNA for each of the TETs, triple knockout of TET1/TET2/TET3 will need to be standardised after which the study can be taken further.

How do CD4+CD25- cells isolated from buffy coat compare to those isolated from cord blood in TET expression?

Cord blood is acquired from the Turku University Hospital. The amount of blood received per day is highly variable due to which sometimes, the yield of cells after CD4+ isolation and CD25- depletion is not enough to set up an experiment. To try to find a way out of this problem we wanted to see if the TETs had the same kinetics in cells isolated from buffy coat (which was easily available and yielded good amount of cells) as those isolated from cord blood.

Figure 6 shows western blots of TET kinetics in iTreg cultures of cells isolated from cord blood (A) and buffy coat (B). As can be seen from the figure, the kinetics of TETs are very different between the two cultures at each of the time-points. Therefore, we concluded that it was necessary to work with cord blood for our experiments.

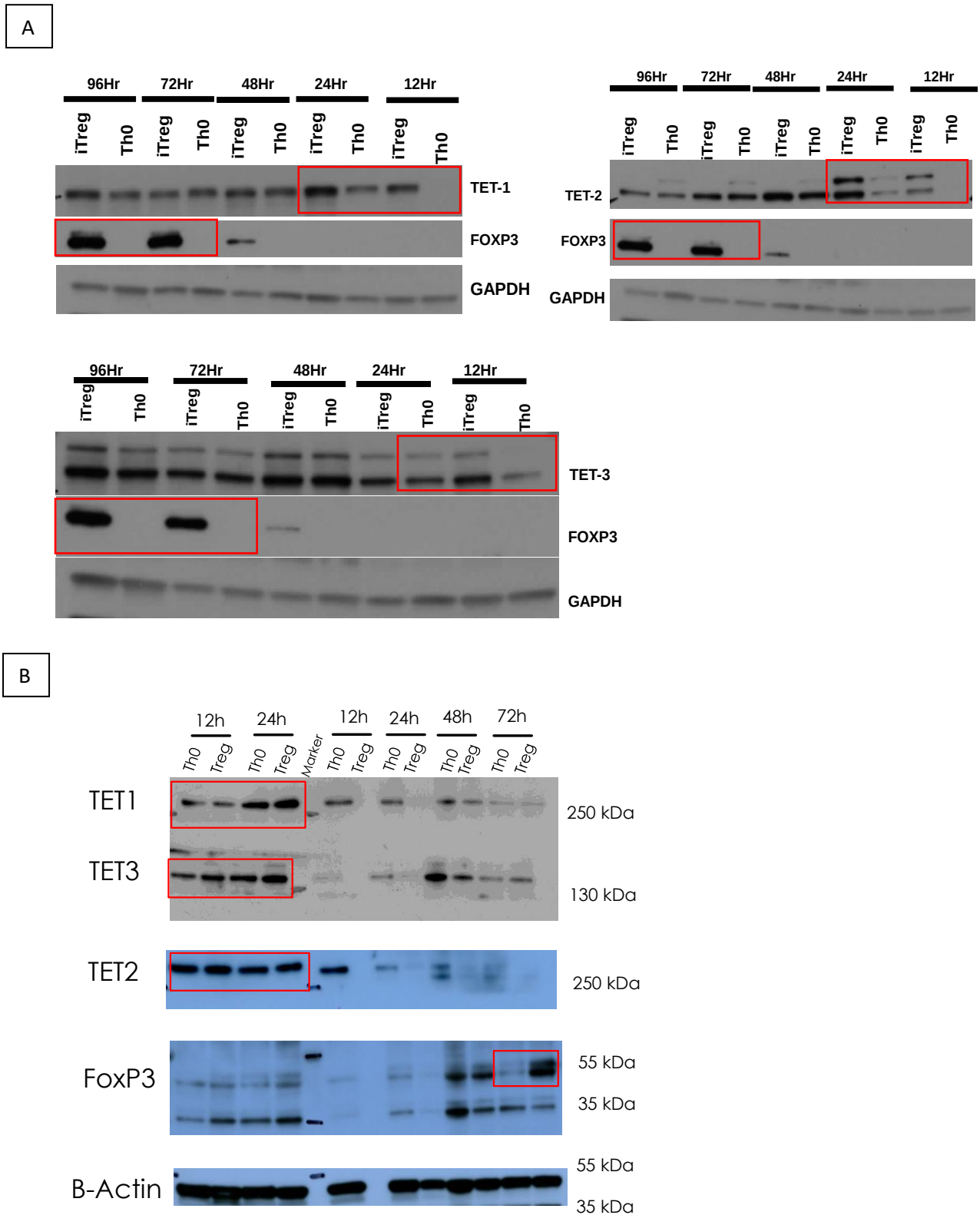


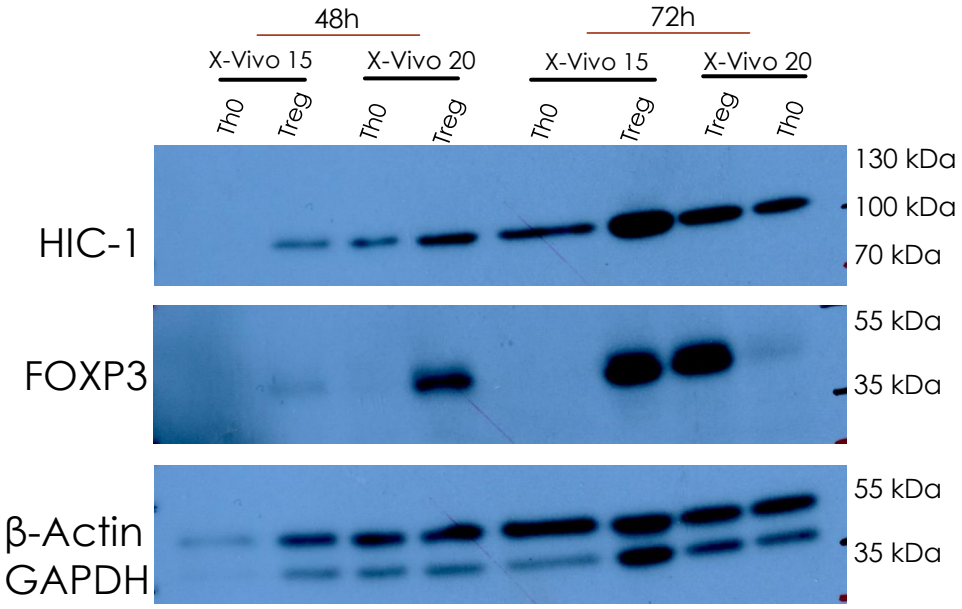
Figure 6: Western blots of TET expression levels in CD4+CD25- cells isolated from A) cord blood (courtesy of Andrabi Syed Bilal Ahmad) and B) Buffy coat at different time-points (12h, 24h, 48h and 72h)

Effect of media on iTreg polarisation (X-Vivo 15 vs X-Vivo 20)

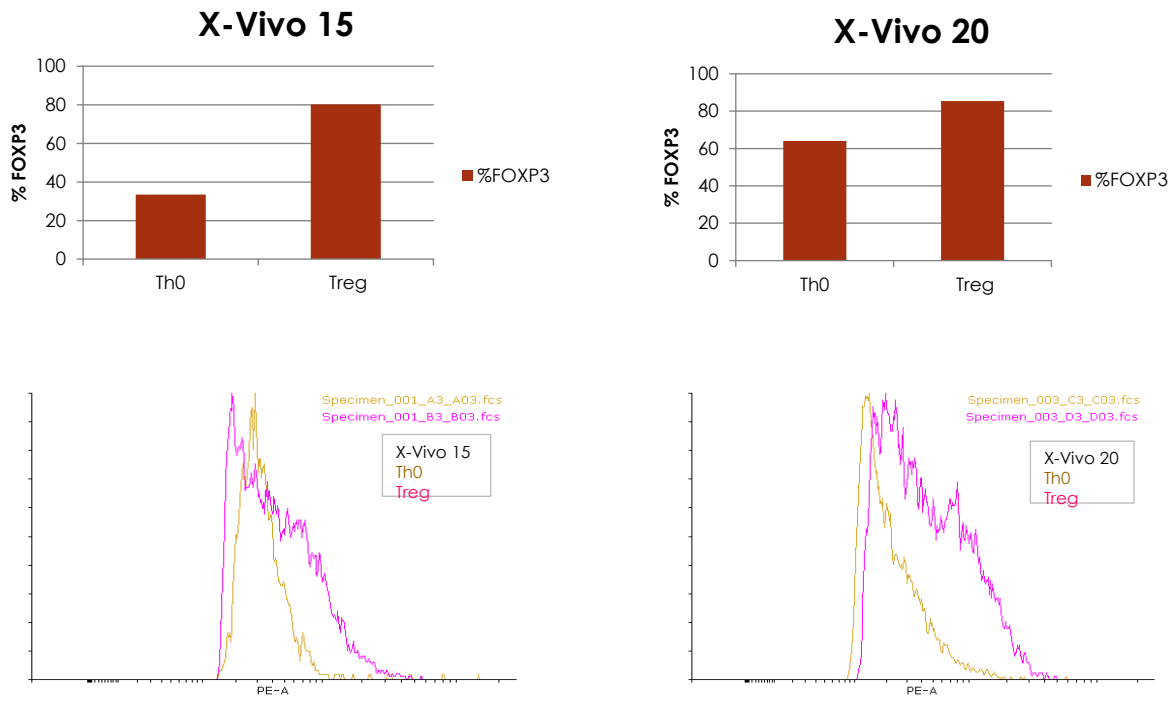
At a point during the course of the project, Lonza (provider of X-Vivo 15) had contamination issues at the X-Vivo manufacturing premises due to which production had come to a standstill and we ran out of X-Vivo 15, the culturing media we used for iTregs. Thus, we decided to compare culturing of iTregs in X-Vivo 15 with culturing in X-Vivo 20 (media used to culture Th17 cells; more widely available).

Figure 7 shows expression of iTreg specific markers (HIC-1 and FOXP3) in cells cultured in X-Vivo 15 and X-Vivo 20 as viewed by western blotting (Fig. 7 A) and FACS (Fig. 7 B and C) (after intracellular staining). Cells grown in both media show similar trends of HIC-1 and FOXP3 expression. Therefore, these results suggest that X-Vivo 20 can be used as a substitute for iTreg culturing when X-Vivo 15 is not available.

A



B



C

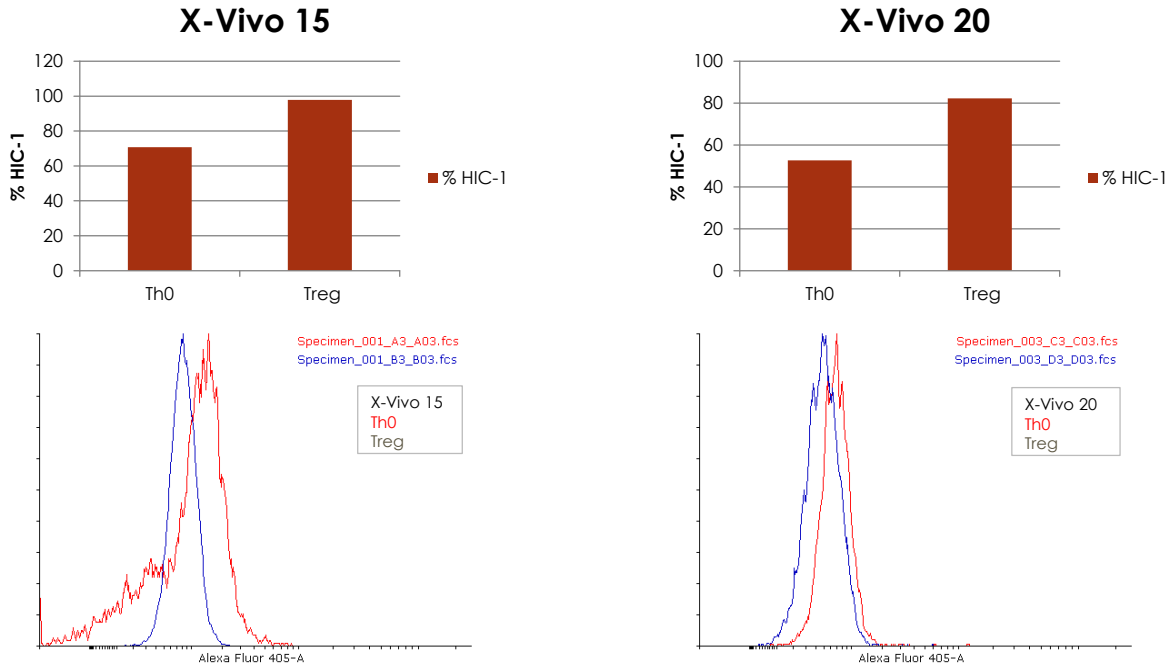


Figure 7: A) Western blot showing expression of iTreg markers (HIC-1 and FOXP3) in cells grown in X-Vivo 15 and X-Vivo 20 at the 48h and 72h time-points. B) Percentage expression of FOXP3 depicted in a bar graph and overlay histogram as viewed by FACS after IC staining. C) Percentage expression of HIC-1 depicted in a bar graph and overlay histogram as viewed by FACS after IC staining.

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