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Characterising interleukin-2-inducible kinase (ITK) inhibitors and their potential for moulding CD4 T-cell plasticity

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Characterising interleukin-2-inducible kinase (ITK) inhibitors and their potential for moulding CD4 T-cell plasticity

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Abstract

Peripheral T-cell lymphomas (PTCLs) are rare diseases with a high mortality that respond poorly to conventional chemotherapy. They represent, therefore, a relatively large unmet medical need. Gene expression profiling has shown that a large subgroup of PTCL are derived from normal follicular helper (Tfh) T-cells. Interleukin-2 inducible T-cell kinase (ITK) is a tyrosine kinase and member of the TEC family of kinases, of which Bruton's tyrosine kinase (BTK), a B-cell signalling protein is also a member, and is expressed in Tfh cells and is important for T-cell receptor (TCR) and chemokine signalling. ITK is highly expressed in Tfh derived PTCL and is, therefore a potential target for treatment. It is inhibited by the clinically active BTK inhibitor, ibrutinib. We have compared siRNA knockdown of ITK and small molecule ITK inhibitors (ITKi) for their effects on proliferation and signalling in human T-cell lines and normal human primary T-cells. There was variation in some effects but all inhibitors repressed proliferation, calcium signalling and cytokine production and caused modest increases in apoptosis. Studies of mice with homozygous disruption of the *Itk* locus, by other researchers, showed that ITK deficiency skewed CD4 T-cell subset differentiation from Th17 to Treg but Tfh differentiation was not investigated. We were able to show that the effects previously reported *in vivo* were reproduced on tonsillar CD4 T-cells cultured under polarising conditions *in vitro* by ITKi. Unexpectedly, we showed that ITK inhibitors prevented Tfh differentiation whilst promoting that of suppressive Treg cells. Blocking IL-21, an important Tfh cytokine regulated by ITK, with IL21R-Fc partially prevented ITKi effects on Tfh and Treg differentiation suggesting a mechanism of action. We also had the opportunity to investigate two cases of primary human T-cell lymphoma and showed that the lymphoma cells had the capacity to differentiate to Tfh cells *in vitro*, which was prevented by ITKi, accompanied by an increase in Tregs. This suggested the exciting hypothesis that ITK inhibitors might alter the T-cell lymphoma environment from activating Tfh to inhibitory Treg. We speculate that in addition to preventing proliferation and enhancing apoptosis, a third potential therapeutic mechanism for ITK inhibitors in PTCL is skewing the microenvironment towards repression. Finally, in an effort to show that ITK inhibitors can be a component of combination treatments we demonstrated synergy with chemotherapy and PI3K inhibitors. The data presented in this thesis supports ITK as a target for therapy of Tfh-derived T-cell lymphomas.

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List of Abbreviations

AITL	Angioimmunoblastic T-cell lymphoma
AKT	Protein kinase B
BCL6	B cell lymphoma 6
BCR	B cell receptor
BH	Btk homology motif
Bmx	Bone marrow expressed kinase
BTK	Bruton's tyrosine kinase
CI	Combination index
CR	Complete remission
CTCL	Cutaneous T cell lymphoma
CTG	CellTiter-Glo
CXCR-4	C-X-C chemokine receptor type 4
CXCR5	C-X-C Motif Chemokine Receptor 5
DAG	Diacylglycerol
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
ERK	Extracellular-signal-regulated kinase
Fa	Fractional affected
FCS	Fetal calf serum
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCs	Germinal centres
G-CSF	Granulocyte-colony stimulating factor
GPCR	G protein-coupled receptor
GVHD	Graft-versus-host-disease
GVL	Graft-versus-leukaemia
HDACi	Histone deacetylase inhibitors
HPRT	Hypoxanthine phosphoribosyl transferase
IC_{50}	The half maximal inhibitory concentration
ICOS	Inducible costimulatory molecule
IFN-γ	Interferon gamma

IL	Interleukin
IP3	Inositol 1,4,5-triphosphate
ITAM	Immune receptor tyrosine-based activation motif
ITK	Interleukin-2 inducible tyrosine kinase
ITKi	ITK inhibitor
LAT	linker for activation of T cells
Lck	Lymphocyte-specific protein tyrosine kinase
MAbs	Monoclonal antibodies
MAPK	mitogen-activated protein kinase
MHC	Major histocompatibility complex
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor κB
ORR	Overall response rate
OS	Overall survival
PCR	Polymerase chain reaction
PD-1	Programmed death 1 protein
PH	Pleckstrin homology
PHA	Phytohaemagglutinin
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5) trisphosphate
PLCγ	phospholipase C-γ
PMA	Phorbol myristate (PHA/PMA
PRR	Proline-Rich Regions
PTCL	Peripheral T-cell lymphomas
PTCL-NOS	Peripheral T cell lymphoma-not otherwise specified
PTKs	Non-receptor protein tyrosine kinases
Regulatory T cells	Tregs
Rlk	Resting lymphocyte kinase
RT-PCR	Real-time PCR
SCT	Stem cell transplantation
SH2	SRC homology 2
SH3	SRC homology 3

SLP-76	Src homology 2 domain-containing leukocyte protein of 76 kDa
SS/L-CTC	Sezary syndrome/leukemic-cutaneous T cell lymphomas
SYK	Spleen tyrosine kinase
TCL	T cell lymphoma
TCR	T cell receptor
Tfh	follicular helper T cells
TGF-β	Transforming growth factor beta
TH	TEC homology
Th cells	T helper cells
TM	Tissue microenvironment
TNF	Tumour necrosis factor
T-PLL	T prolymphocytic leukaemia
Xid	X-linked immunodeficiency
XLA	X-linked agamma-globulinemia
ZAP-70	Zeta-chain-associated protein kinase 70

Chapter I. Introduction

1.1 Peripheral T-cell lymphomas

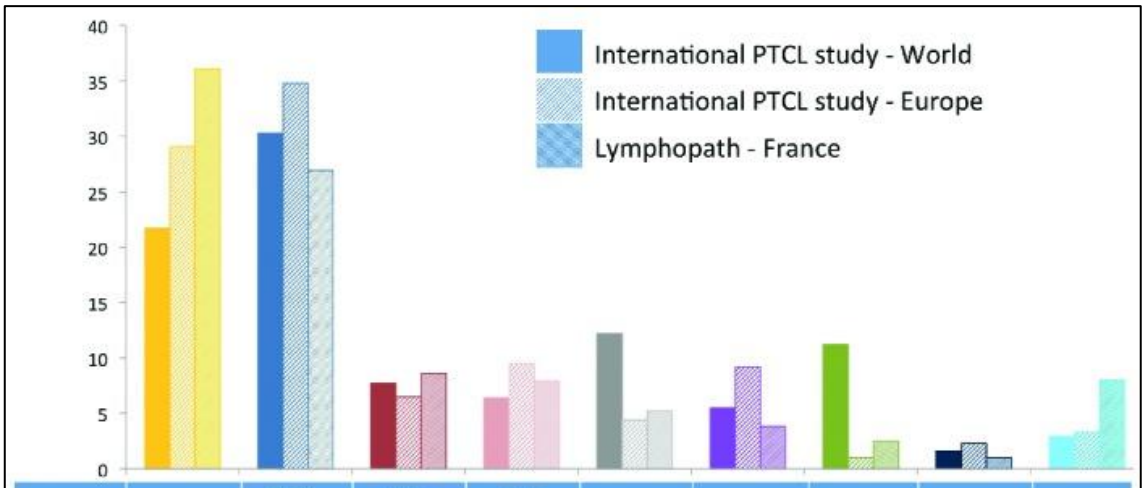
Peripheral T-cell lymphomas-PTCLs are a heterogeneous group of hematologic malignancies originating from mature T lymphocytes, and are generally associated with a poor prognosis (Vose *et al*., 2008). PTCLs remain a challenging entity to create and standardise treatment strategies for. Despite the fact that PTCLs have poor outcome after treatment with conventional chemotherapy, the optimal therapy for these diseases remains to be determined (Corradini *et al*., 2014; Ellin *et al*., 2014). Defining a treatment standard for PTCLs is difficult due to the rarity of these type of lymphomas and heterogeneity of subtypes (Gooptu *et al*., 2015). Thus, it is difficult to perform large prospective clinical trials.

In this chapter, the classification and pathobiology of the most common subtypes of PTCL including angioimmunoblastic T-cell lymphoma (AITL) and peripheral T-cell lymphoma-not otherwise specified (PTCL-NOS) will be explored. Secondly, the role of ITK in signalling downstream of T cell receptor (TCR) and its role in regulation of T-cell function and differentiation will be discussed. This chapter will also describe the phenotypic and functional plasticity of CD4+ T-cells subsets and their plasticity to treat immune-mediated disease.

1.2 The classification of peripheral T-cell non-Hodgkin lymphoma

The World Health Organization (WHO) and European Organization for Research and Treatment of Cancer (EORTC) identify several subtypes of clinicopathologic peripheral T-cell NHLs (Swerdlow *et al*., 2016). Overall these are rare diseases with about 600 new cases of all types in the UK each year (hmrn.org). Broadly T-cell lymphomas can be cutaneous, nodal or extranodal. Peripheral T-cell NHLs include peripheral T-cell lymphoma-not otherwise specified (PTCL-NOS), angioimmunoblastic T-cell lymphoma (AITL), anaplastic large cell lymphoma anaplastic lymphoma kinase-positive (ALCL-ALK+), and ALCL ALK-negative (ALCL-ALK-), which are the commonest subtypes. Rarer subtypes are NK-/T-cell lymphoma (NK/TCL)-nasal type, adult T-cell leukaemia/lymphoma (ATLL), hepatosplenic T-cell lymphoma, enteropathy-associated T-cell lymphoma.

AITL is recognised as one of the most frequent subtypes of peripheral T-cell lymphoma (PTCL) in World Health Organization classifications of haematological malignancies and the REAL (revised European-American classification of lymphoid neoplasms) (Jaffe *et al*., 2001; Swerdlow *et al*., 2008). According to de Leval *et al*. (2015), AITL is the most common PTCL subtypes in France, see figure 1.1. These geographical variations in the distribution of PTCL subtypes was confirmed by WHO and REAL. Another common subtype of peripheral T-cell lymphoma, peripheral T-cell lymphoma not otherwise specified (PTCL-NOS), is involved nodal sites in most patients but may often present in specific extranodal sites such as the spleen, bone marrow, liver and gastrointestinal system (Vose *et al*., 2008).



%	AITL	PTCL-NOS	ALK+ ALCL	ALK- ALCL	NKTCL	EATL	ATLL	HSTL	Others
World	21.7	30.4	7.7	6.5	12.2	5.5	11.3	1.6	2.9
Europe	29.1	34.8	6.5	9.5	4.4	9.2	1	2.3	3.3
France	36.1	26.9	8.6	7.9	5.2	3.8	2.5	1	8

Figure 1.1. Relative frequencies of peripheral T-cell lymphoma (PTCL) subtypes according the international PTCL project. In the Lymphopath registry (France) (n=2046) and (worldwide (n=1314) and European (n=450) statistics). AITL: angioimmunoblastic T-cell lymphomaPTCL-NOS: peripheral T-cell lymphoma, not otherwise specified; ALCL: anaplastic large cell lymphoma; ALK: anaplastic lymphoma kinase; NK/T: extranodal NK/T-cell lymphoma; EATL: enteropathy-associated T-cell lymphoma; ATLL: adult T-cell leukemia/lymphoma; HSTL: hepatosplenic T-cell lymphoma (de Leval et al., 2015).

1.3 Normal T-cell development

In order to understand the most recent ideas on the origins of T-cell lymphomas it is necessary to describe the development of normal T-cells concentrating on those subsets that express the surface marker CD4.

1.3.1 CD4 T-cell development and function in normal immunity

The human immune system is subdivided into innate (natural) and adaptive (acquired) immune systems. The primary functions of immunity are the recognition and elimination of foreign pathogens, establishment of immunologic memory, and maintenance of tolerance to self-antigens. The lymphocyte family is made up of bone-marrow-derived (B cells), thymus-derived lymphocytes (T cells) and the natural-killer cells (NK cells). T-cells and B-cells constitute the adaptive immune system, which functionally collaborates with the innate immunity.

T cells develop in the thymus before being deployed into the secondary/peripheral lymphoid organs for further antigen-mediated differentiation. During thymic development expression of the surface markers CD4 and CD8 alters such that cells move through double positive and double negative stages before expressing either CD4 or CD8. CD4 T-cells, the subject of this thesis, recognise antigen in the context of MHC class II and provide help to B-cells in order to promote the production of high affinity antibodies whereas CD8 T-cells recognise antigen in association with MHC class I and have predominantly cytotoxic effects.

T cell receptor (TCR) is the primary mediator of T-cell activation and is generated by recombination of genomic DNA sequences in the thymus during T-cell development. Each TCR is essential and responsible for each specific T-cells (Germain., 2002, Spits., 2002). Successful rearranged of functional TCR and emergence from thymus enables naïve T-cells to migrate through the secondary lymphoid tissues such as spleen and lymph nodes, and peripheral circulation but they are still not able to produce any kind of response to protect against pathologies. Naïve T-cells require activation in order to produce mediating immune protection. This involves the interaction between T-cell and antigenic peptide on antigen-presenting cell (APC) which bears major histocompatibility complex (MHC) class I or class II molecule (Pennock et el., 2013). The TCR consists of two α and β chains which recognise the peptide antigen in the context of class I or class II MHC. On T-cells, the TCR associates with a complex CD3 for propagating an intracellular

signalling pathways to the TCR ligation. Each TCR, depending on the type of T cell, also associates with either CD8 or CD4 co-receptor and both molecules bind to MHC to further stabilise the interaction between APC and T-cells (Gao et al., 2002). Naïve CD4 T-cells initiate an activation program once they recognise a peptide/MHC combination on the surface of activated dendritic cells.

Naïve CD4+ T cells are activated by interacting with antigen-major histocompatibility complex class (MHC) and differentiate into various subpopulations dependent generally on the cytokine milieu of the microenvironment (Ashkar *et al*., 2000; Tao *et al*., 1997; Bluestone *et al*., 2009). In addition to the classical T helper-1 (Th1) and T helper-2 (Th2) cells (Mosmann and Coffman, 1989), additional subsets have been recognised including regulatory T cells (Tregs), T helper-17 (Th17), follicular helper T cells (Tfh), T helper-9 (Th9) and T helper-22 (Th22), with each entity having a characteristic cytokine pattern (Figure 1.2). For the differentiation of particular phenotypes, a set of cytokine signalling machinery correlated with lineage transcription factor activation and specific epigenetic changes/modifications at proper genes are needed (Luckheeram *et al*., 2012). The effector function of these CD4+ T cells is mediated via the cytokine released by the differentiated cells.

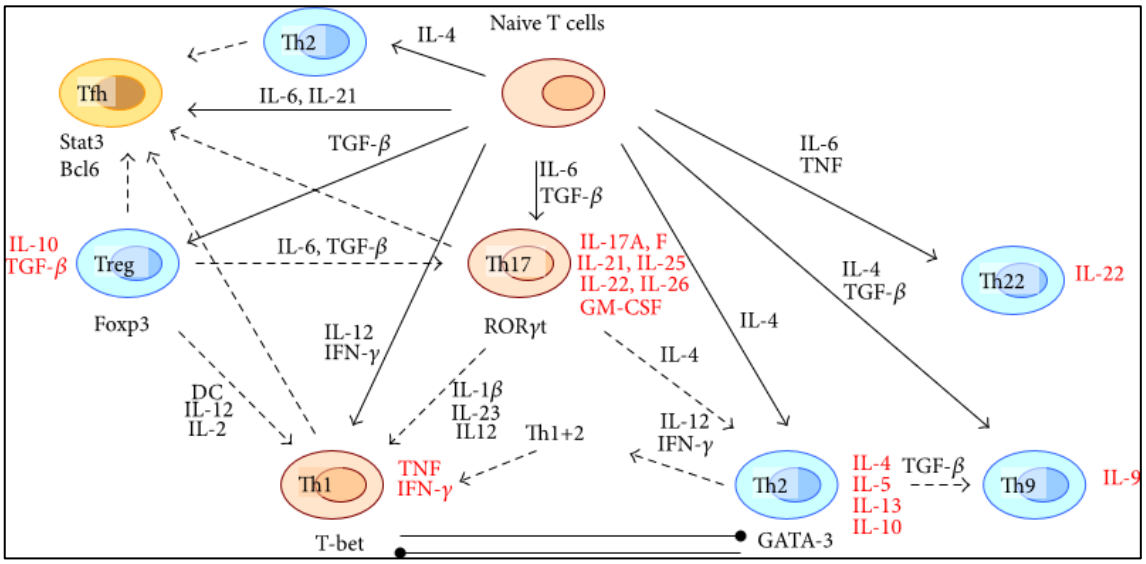


Figure 1.2. Scheme depicting the T cell differentiation pathways and plasticity. All secreted cytokines are represented in red. GM-CSF: granulocyte macrophage colony-stimulating factor; IL: interleukin; TGF-β: transforming growth factor beta; DC: dendritic cells; IFN-γ: interferon gamma; TNF: tumour necrosis factor. (Ivanova and Orekhov, 2015).

1.3.3 T helper lymphocyte subsets and plasticity in normal immunity, autoimmunity and cancer.

1.3.3.1 Tfh cells

T follicular helper (Tfh) cells are the prototypical CD4+ T cell subsets which are specialized in providing help to B cells and supporting the formation of germinal centres (GCs) in secondary lymphoid tissues where a selection of high-affinity B cell clones occurs (Vinuesa *et al*., 2010). In GCs, high affinity maturation of B cells leads to the generation of high-affinity antibodies, long-lived memory B cells, and plasma cells. Tfh cells are phenotypically characterized by the high expression of chemokine receptor (C-X-C motif) CXCR5 that guides their migration to B cell follicles, in addition to being essential for development and differentiation of Tfh (Hardtke *et al*., 2005; Haynes *et al*., 2007). They have high expression of programmed death 1 protein (PD-1) (Haynes, *et al*., 2007), which has been shown to be upregulated by the Tfh population and is also required for their function.

Furthermore, Tfh cells express a variety of receptors, such as, B and T lymphocyte attenuator (BTLA), inducible costimulatory molecule ICOS and CD40L, that are all critical for their development and migration into follicles/or function (Crotty, 2011). Like Th17, Tfh cells also serve a major role in interleukin 21 (IL-21) production (Ma *et al*., 2012; Crotty, 2011) which is essential to promote B cell maturation, survival, affinity maturation and isotype switching (Linterman *et al*., 2010). Tfh cells are important in the production of IFN-γ or IL-4 that can impose (control) isotype class switching to the appropriate immunoglobulin isotype tailored for protection of immunity (Reinhardt *et al*., 2009). It can be noted that the transcription factor B cell lymphoma 6 (BCL6) and STAT3 play a major role in programming Tfh cell differentiation (Nurieva *et al*., 2009; Ma *et al*., 2012; Crotty, 2010).

1.3.3.2 Th1 cells

Th1 cells are induced in response to IL-12 and IFN-γ which has a central role in linking the innate and acquired immunity and is released primarily via dendritic cells (DCs) (Ivanova and Orekhov, 2015). Additionally, IL-12 and IFN-γ are mediated via signal transducer and activator of transcription 4 (STAT4) and STAT1 (Ivanova and Orekhov, 2015). Th1 subsets are identified by expression of the transcription factor, T-bet, which

is encoded by the TBX21 gene and the expression of IFN-γ, which blocks the alternative differentiation programs (Abbas *et al*., 1996; Espinosa and Rivera, 2012). The Th1 phenotype is relatively stable due to the transcriptional circuitry of T-bet, which has the capability to induce its own expression and repress the alternative Th2 differentiation-transcription factor GATA-3 (Mullen *et al*., 2001; Afkarian *et al*., 2002; Oestreich and Weinmann, 2012).

1.3.3.3 Th2 cells

The presence of interleukin-4 (IL-4), can induce Th2 cells through STAT6 signalling. Th2 subsets are characterised by the expression of a master regulator transcription factor GATA-3 which can provide self-reinforcing positive feedback and self-activation (Ouyang *et al*., 2000). Interestingly, GATA-3 and T-bet are mutually antagonistic and favour T-cell polarization towards either the Th2 and Th1 subsets respectively, dependent on the surrounding environment/cytokine profile (Murphy and Stockinger, 2010). Furthermore, Th2 cells release other cytokines such as IL-13, IL-5 and IL-4, and are implicated in humoral immune responses against parasites and extracellular infectious agents (Pearce *et al*., 1991), and involved in the development of allergic reactions and atopy (Robinson *et al*., 1992).

1.3.3.4 Th17 cells

In addition to Th1 and Th2 cells, Th17 are recognised as a separate T-cell lineage (Kleinewietfeld and Hafler, 2013; Park *et al*., 2005; Harrington *et al*., 2005). It has been reported that Th17 polarization results from the presence of IL-21 and TGF- or IL-6, as shown in figure 1.1 (Bettelli *et al*., 2008; Yang *et al*., 2008a). They are not dependent on T-bet and GATA-3 transcription factors and the related signalling for their differentiation, and these cells are regulated by the retinoic acid receptor-related orphan receptors RORt (RORc in humans), ROR, Stat3 and Smad pathways (Hirahara *et al*., 2010; Ivanov *et al*., 2006; Yang *et al*., 2008b). Besides expression of IL-17 and the related IL-17F, Th17 cells also express additional cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF), IL-21 and IL-22 (Korn *et al*., 2009). Apart from its importance in human autoimmune pathologies, IL-23 is also crucial for the survival of Th17 cells and inflammatory potential (Langrish *et al*., 2005). Additionally, it has been revealed that IL-21 and TGF-β drive Th17 differentiation and IL-1β and IL-23 induce their differentiation

from naïve CD4+ T cells and memory T cells, respectively (Yang *et al*., 2008a; Acosta-Rodriguez *et al*., 2007). Th17 are found under normal circumstances in the gut where they can protect against fungal and bacterial infections, albeit these cells are likely to be upregulated in the tissues during inflammation (Ivanov *et al*., 2009; Ouyang *et al*., 2008; Iwakura *et al*., 2011).

1.3.3.5 Tregs cells

According to Josefowicz and Rudensky (2009), regulatory T cells (Tregs), are a subset of T cells that are regulated by the master transcription factor forkhead boxP3 (FOXP3) and are differentiated in response to TGF-beta (Josefowicz and Rudensky, 2009). Although the expression of FOXP3 is also observed in human activated non-regulatory CD4 + T cells (Wang *et al*., 2007), and additional markers are required for Treg identification. It has been shown that CD127 or CD25 could be used as markers for this subset, which allows them to be distinguished from other activated effector T cells. Regulatory T cells are classified into natural and induced (adaptive) Tregs.

Many natural Tregs (nTregs) are constitutively express CD25 which are produced by the normal thymus as a mature and distinct population (Miyara and Sakaguchi, 2007). Development and function of these cells depend on the expression of FOXP3. Roncarolo *et al*. (2006) indicate that adaptive (induced) Tregs are induced from naïve T lymphocytes via special modes of antigen stimulation, particularly in a specific cytokine milieu. Generally, Tregs functional stability is dependent on its origin. For instance, Tregs are considered to be a stable subpopulation derived from the thymus and are less stable in the periphery (Banham *et al*., 2006; Bluestone and Abbas, 2003). However, Tregs can be induced in the periphery by TGF-β and antigen presence, leading to the formation of induced Tregs (Abbas *et al*., 2013). Horwitz *et al*., (2008) indicate that natural and inducible Tregs are similar in functions, but have distinct epigenetic status.

These cells are important to maintain the peripheral tolerance. The function of additional effector T cells and antigen-presenting cells (APCs) can be suppressed by Tregs through cell-cell interactions and secretion of suppressive cytokines such as IL-10 and TGF-β (Sakaguchi *et al*., 2008; Sakaguchi *et al*., 2010; Roncarolo *et al*., 2006).

1.3.3.6 Th9 cells

IL-9 producing helper T-cells (Th9) cells have been identified in the late of 1980s as another distinct subset of T helper cells (Uyttenhove *et al*., 2010). Previous studies show that stimulation of naïve T cells with TGF-β and IL-4 or Th2 with TGF-β can result in the generation of Th9 which is positive for IL-9 (Veldhoen *et al*., 2008; Dardalhon *et al*., 2008). Recently, Kaplan *et al*., (2015) describe the differentiation and *in vivo* function of Th9 in autoimmune diseases and allergy in an excellent review. Th9 cells are generally generated from TGF-β, and IL-4 stimulated naïve CD4+ T cells, albeit IL-4-independent production of IL-9 is likely to be possible in the existence of IL-2, an additional cytokine critical for differentiation of Th9. According to Jia and Wu (2014a), other cytokines promotes IL-9 production such as IL-1, IL-21, IL-25 and IL-33, while IL-27 has the opposite function.

Additionally, Th9 differentiation and elevated IL-9 production have been observed in *in vivo* models of melanoma and allergy (Jones *et al*., 2012; Purwar *et al*., 2012). Wilhelm *et al*., (2011) indicate that IL-9 is mainly produced in innate lymphoid cells (ILCs) in study models, and can also be formed in many cells *in vivo*. This cytokine plays a major role in promoting the proliferation and survival of T-cells and mast cells, stimulating the production certain cytokines and modulating B-cell responses. It is worth mentioning that its effects can be observed in certain non-haematopoietic cell types, and Th9 can produce IL-12 and IL-10, albeit their functions are elucidated. A recent paper shows that increase of IL-9 production has a central role in allergy, antitumor immunity and autoimmune processes (Jia and Wu, 2014).

1.4 Pathobiology of the important subtypes of PTCL: PTCL-NOS and AITL

This section will focus on pathobiology of the most common subtypes of T cell lymphomas. The focus of this thesis is on PTCL-NOS and AITL and, therefore, these diseases will be described in detail.

Recently, numerous studies have focused on a link between multiple cancers and Tfh. Tfh associated cancer generally can be classified into two categories (Ma and Deenick, 2014): (1) those in which the tumour cells have the features of a Tfh-like phenotype, and (2) those such as many types of B-cell lymphoma in which Tfh cells support the malignant B-cells (Figure 1.3). The best described of the former is subtypes of peripheral T-cell

lymphoma (PTCLs) including angioimmunoblastic T-cell lymphoma (AITL) and PTCLs not otherwise specified (PCTL-NOS) (Ma and Deenick, 2014).

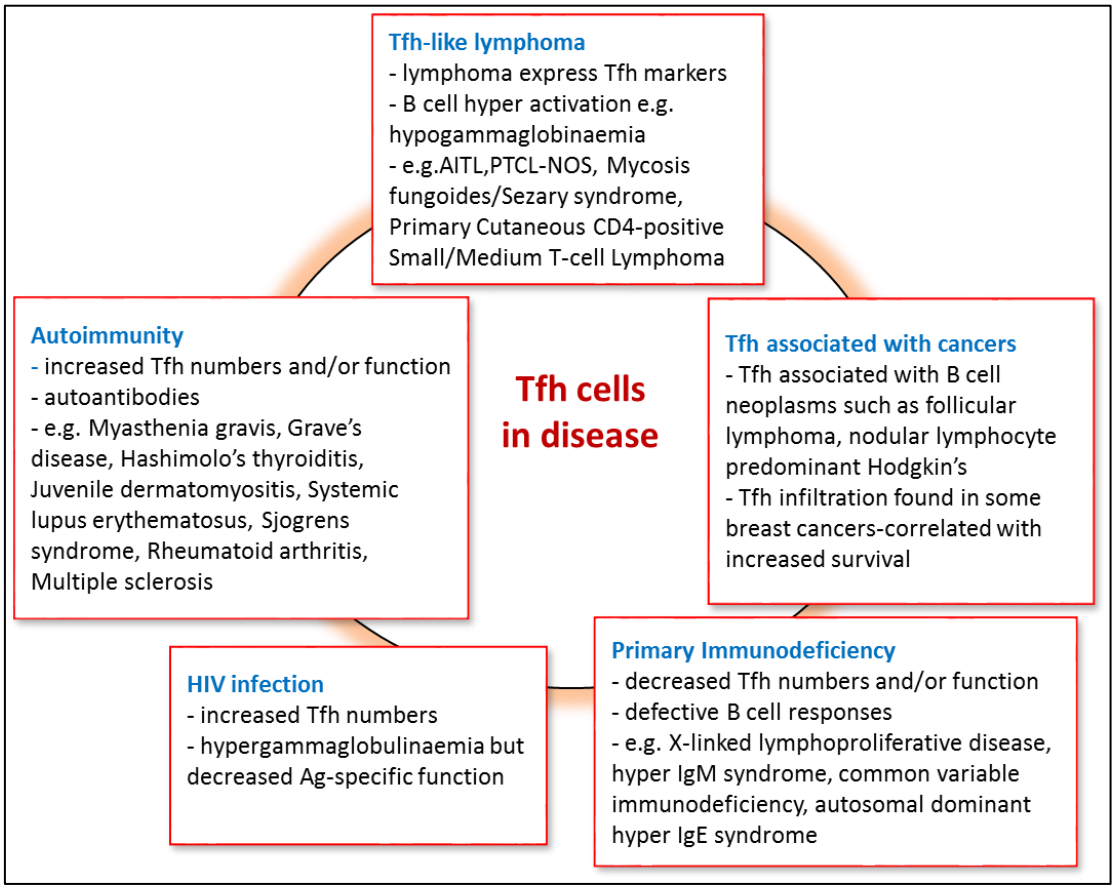


Figure 1.3. Human follicular helper T cells in disease. Tfh cells are implicated in a range of diseases, particularly a decrease of Tfh cells has been associated with various immunodeficiencies, while an increase in this subtype has been observed in acquired immunodeficiency (such as, HIV), autoimmunity and numerous types of cancers such as, AITL and PTCL-NOS. (Ma and Deenick, 2014)

PTCL-NOS constitutes over 25% of cases (Vose, et L., 2008; Bajor-Dattilo, *et al*., 2013). It presents predominantly as nodal, but can affect extranodal, skin and the gastrointestinal tract (Alan, *et al*., 2013). PTCL-NOS show a poorer overall survival rate compared to B-cell NHLs (Vose *et al*., 2008; Mark *et al*., 2013). Figure 1.4 demonstrates overall survival (OS) of patients with the common subtypes of peripheral T-cell lymphoma. The majority of PTCL-NOS patients are adults with median age of 55-60 years (Abouyabis *et al*., 2008) and have an overall poor outcome ranging from 20% to 30% 5-year survival (Gaulard and de Leval, 2014). PTCL-NOS typically displays a highly variable morphology, containing a variety of small and large atypical pleomorphic lymphoid cells expressing

CD3 with common CD7 antigen loss, less frequently CD2 and CD5 (Gaulard and de Leval, 2014).

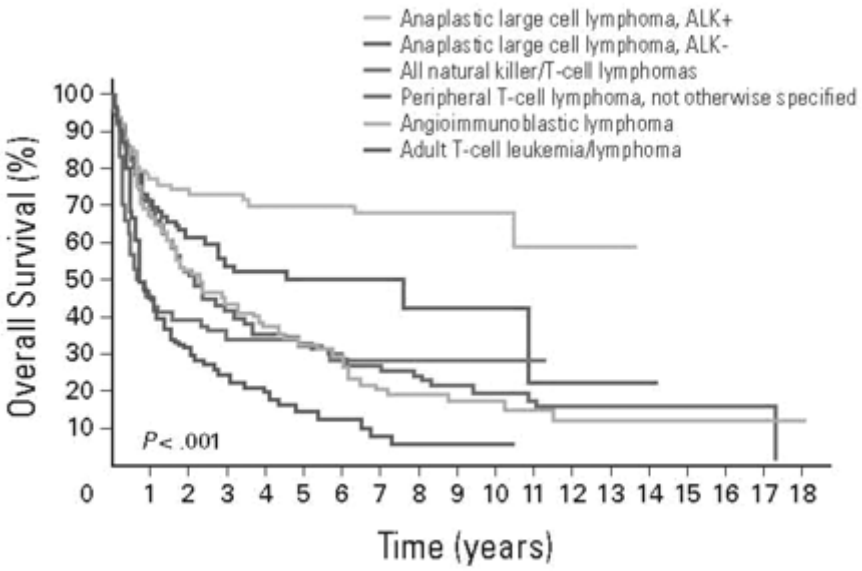


Figure 1.4. Overall survival (OS) of patients with peripheral T-cell lymphoma (PTCL)-common subtypes (Vose et al., 2008).

The cytological spectrum of this pathologic subtype is very broad ranging from monomorphous to polymorphous. Three morphological variants of PTCL-NOS have been identified in this disease, including lymphoepithelioid (Lennert), follicular and T-zone variants. Firstly, the lymphoepithelioid variant has background epithelioid histiocytes and is frequently CD8 positive, and is associated with a better prognosis (Weisenburger et l., 2011). PTCL-NOS with follicular (F-PTCL) or perifollicular growth pattern is rare disease, mimicking follicular lymphoma and marginal zone lymphoma respectively (de Leval *et al*., 2001). Huang*et al*., (2009) show that the neoplastic T-cells are CD3+ CD4+ αβ T cells which actively express Tfh markers (ICOS+ PD1+ CXCL13+ BCL6+ CD10 +/- CD57-/+).

Angioimmunoblastic T-cell lymphoma (AITL) is the most common subtype of PTCL (de Leval *et al*., 2015), and is defined by polymorphous infiltrate including lymph nodes with prominent high endothelial venules (HEV) and meshwork of follicular dendritic cells (FDCs). The disease is derived from the prototypic Tfh neoplasm (Iqbal *et al*., 2009), and affects middle-aged and elderly adults and frequently manifests by peripheral lymphadenopathy and other systemic symptoms such as, autoimmune manifestations, rash and hypergammaglobulinemia (de Leval *et al*., 2010). The neoplastic αβ CD4+ T cells of AITL express numerous Tfh markers such as, ICOS, PD1, CD200, BCL6, SAP

and cMAF, and frequently CD10 (Bisig *et al*., 2012). Generally, ICOS and PD1 are shown to be more sensitive in identifying the neoplastic Tfh cells.

AITL also expresses the chemokine, CXCL13 (Grogg *et al*., 2006, Attygalle *et al*., 2004, Yu *et al*., 2009), leading to expanded B cell recruitment to lymph nodes through adherence to the HEV, plasmacytic differentiation, B-cell activation and expanded FDC meshworks as well as all contributing to the clinical and morphologic features of AITL (de Leval *et al*., 2010). Three morphological patterns (I, II, III) are identified in AITL (Attygalle *et al*., 2002, Attygalle *et al*., 2007), with intense expression of PD1 in the perifollicular tumour cells specifically useful in distinguishing AITL pattern I from paracortical hyperplasia and reactive follicular. Epstein–Barr virus (EBV) is found in up to 97% of patients within the B-cell component in AITL. It has been shown that the most of patients with AITL have clonal rearrangements of TCR genes (Attygalle *et al*., 2007, Tan *et al*., 2006).

Additionally, studies demonstrate that immunoglobulin gene rearrangements could be seen in approximately 25-30 % of patients with AITL (Attygalle *et al*., 2007, Tan *et al*., 2006; Federico *et al*., 2012) presumably due to B-cell expansion in AITL associated with Tfh function of the tumour cells (Dunleavy *et al*., 2007). de Leval *et al*., (2007) show using gene expression profiling studies that AITL is likely to be derived from Tfh cells. PTCL with follicular variant (fPTCL) is another subtype with a Tfh phenotype (Agostinelli *et al*., 2011; Huang *et al*., 2009).

According to Lemonnier *et al*., (2012), *TET2* mutations occur in 58% of PTCLs and 47% of AITL expressing Tfh markers, suggesting that there is overlap between these subtypes and the connection between these neoplasms has subsequently been validated by gene expression analysis (de Leval *et al*., 2007).

1.5 Characterization of ITK-SYK translocation in peripheral T cell lymphoma

The chromosomal translocation t(5;9) (q33;q22) was first reported in 2006 as a recurrent and specific genomic alteration in a small subset of Peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS) (Streubel *et al*., 2006). This translocation fuses the IL-2 inducible T-cell kinase (ITK) and the spleen tyrosine kinase (SYK) genes on chromosome 5 and 9 respectively (Streubel *et al*., 2006). Both ITK and SYK are non-receptor tyrosine kinases required for lymphocyte development, normal antigen-induced lymphocyte activation, and signal transduction. ITK is the dominant Tec kinase in T cells (Berg *et al*., 2005) and possesses a pleckstrin homology (PH) domain at the amino-terminal region which is necessary for plasma membrane recruitment.

Additionally, ITK contains a TEC homology (TH) domain, Src homology SH3-SH2 domains for interaction with T-cell adapter proteins (such as, SLP-76 and LAT) and a kinase domain for T cell receptor downstream signalling (Sahu and August, 2009; Andreotti *et al*., 2010). Sada *et al*., (2001) indicate that high expression of SYK was observed in B lymphocytes and myeloid cells, where it has a crucial role for activation of immune cells. Its expression has also been found in developing thymocytes (Palacios and Weiss, 2007).

In addition, SYK contains the tandem SH2 domain required for ligand-induced connection with B cell receptor (BCR) or other immune receptor tyrosine-based activation motif (ITAM) containing immune-receptors, and a kinase domain which is essential for receptor-proximal molecule phosphorylation as well as subsequent signal transduction. Streubel *et al*., (2006) demonstrate that the chromosomal translocation t(5;9)(q33;q22) results in the fusion of the PH-TH domain of ITK and the kinase domain of SYK. Although the cellular and molecular consequences of ITK-SYK expression in lymphocytes are not cleared. It has been exhibited that this fusion results in a catalytically active tyrosine kinase that is primarily involved in the antigen receptor signalling pathway in T lymphocytes (Pechloff *et al*., 2010). They indicate that in a conditional *in vivo* model, the ITK-SYK fusion kinase induces a highly aggressive T-cell lymphoma with 100% penetrance. Interestingly, the ITK-SYK translocation was found in peripheral T-cell lymphoma, NOS, Follicular Variant (PTCL-F or PTCLTFH) (Streubel *et al*., 2006; Huang *et al*., 2009).

1.6 Current treatments and clinical outcomes of peripheral T-cell lymphoma

Traditionally, the current frontline treatment regimen for most subtypes of PTCL is a combination of chemotherapy agents, such as cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), CHOP with the addition of etoposide (CHOEP), or etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin (EPOCH) (Schmitz and de Leval, 2017). They also indicated that these combination therapies are still the front line therapy of choice for patients with PTCL.

It has been reported that the overall response rates in most of PTCL patients for these regimens are about 75% to 80% (Savage *et al*., 2004; Vose *et al*., 2008). However, the complete response rate is about 40% to 50%. Additionally, retrospective data analyses suggest that achieving durable complete remission with chemotherapy alone occurs in less than 30% of patients, and the majority of patients with PTCL do not achieve a complete response or relapse after their response. Abouyabis *et al*., (2011) demonstrate that 5-year overall survival for patients treated with CHOP or CHOP-like regimens is 38.5%. Recently, several agents have been developed to improve the outcome of PTCL patients, including histone deacetylase inhibitors (HDACi), antifolates, immunoconjugates, immunomodulatory agents, monoclonal antibodies, nucleoside analogues, kinase inhibitors and proteasome inhibitors.

According to Marquard *et al*., (2009), there is high expression of HDAC1 in PTCL, suggesting a mechanism of tumour suppression and sensitivity of T cell lymphoma to HDACi. The US Food and Drug Administration (FDA) have approved two drugs for PTCL, including romidepsin and belinostat (Coiffier *et al*., 2012; O'Connor *et al*., 2015). Romidepsin has a good efficacy and safety profile in relapsed or refractory peripheral T-cell lymphoma (R/R PTCL). In 2011, the FDA approved Romidepsin for the treatment of patients with R/R PTCL who had received ≥ 1 prior therapies. In a phase II study of Romidepsin, the overall response rate (ORR) in 45 patients with relapsed or refractory PTCL was 38%, with 18% showing complete remission (CR). Current clinical trials are combining Romidepsin with chemotherapies, including CHOP in order to improve response rate and overall survival of patients with T-cell lymphoma.

Folates are crucial for synthesis of DNA in cancer cells. Anti-folates were discovered in the 1940s, and have shown to be effective in treatment of cancer. Methotrexate is one of the earliest anticancer drugs, which plays an essential role in the treatment of numerous

types of cancer (Gonen and Assaraf, 2012). Pralatrexate is a derivative of methotrexate and is approved by the FDA for use in R/R PTCL. This novel anti-folate selectively accumulates in cancer cells and blocks the synthesis of purines and pyrimidines. O'Connor *et al*., (2009) demonstrate that among 29 phase I/II T-cell lymphoma patients, the CR was 31%, with 54% ORR and 23% partial responses (PR).

Nucleoside analogues block DNA synthesis and repair, and induce apoptosis (Robak *et al*., 2006). Gemcitabine, fludarabine, cladribine, pentostatin, clofarabine, nelarabine and forodesine are the main agents of the nucleoside analogues. Among them, Gemcitabine, fludarabine and cladribine have exhibited efficacy in PTCL. Gemcitabine is the most effective nucleoside analogue as second-line treatment for patients with relapsed T cell lymphoma (NCCN Clinical Practice Guidelines in Oncology, 2015). Three phase II studies evaluated gemcitabine as single agent in pretreated patients with T cell lymphomas, mainly with PTCL-NOS and the cutaneous disease, mycosis fungoides (MF) (Zinzani *et al*., 1998; Sallah *et al*., 2001; Zinzani *et al*., 2010). In patients with PTCL-NOS overall response rates (ORR) ranged from of 51-69%, with CR rates of 13-30% (Zinzani *et al*., 2010).

Relapse in patients with T cell lymphoma is common and in patients whose performance scores make them eligible, stem cell transplantation (SCT) is used as a consolidation strategy in order to enhance therapeutic results. SCT is within the treatment guideline for PTCL patients in first remission in the US and the UK (Dearden *et al*., 2011). Current patient outcomes suggest the novel therapy regimens and alternative strategies are required to improve disease management and the adverse prognosis, as well as extend the duration of response. New drugs with novel modes of action can help to improve patient outcomes.

1.7 Potential future treatments

1.7.1 SYK Inhibition

The inhibition of the SYK kinase is being investigated in PTCLs as a potential new drug therapy (Wilcox *et al*., 2010), where it is expected that tumours expressing the ITK-SYK fusion transcript will demonstrate high sensitivity to SYK chemical inhibition. Rigby *et al*., (2009) show that the pharmaceutical inhibition of PI3K reduced the activity of ITK-SYK, and ITK-SYK activity was enhanced when co-expressed with constitutively active PI3K. They also reported that selective inhibition of the catalytically active tyrosine ITK-SYK by a SYK inhibitor (Piceatannol) might be beneficial against ITK-SYK-positive PTCL. Notably, the transforming activity of the fusion ITK-SYK was blocked by a functionally inactivated PH domain and was recovered via constitute membrane retargeting in a similar fashion to ITK-SYK signalling. This data suggests that the transforming activity of the fusion t(5;9)(q33;q22) is dependent upon its activation at the plasma membrane. Furthermore, it suggests that the development of inhibitors designed to block the PH domain-mediated membrane association of this fusion shows an alternative therapeutic approach for the treatment of patients with these lymphomas.

1.7.2 ITK inhibition

As mentioned before, ITK plays a central role in the regulation and transduction of signals from surface receptors, and is recognised to be necessary for T cell function and the physiopathology of autoimmune diseases and T-cell malignancies. It is also known to mediate T cell receptor and chemokine signalling, and binds to receptor-proximal signals including Lck, PI3K, ZAP70, SLP-70 and LAT to PLCγ (Grasis *et al*., 2010). Additionally, Itk mediates G protein-coupled receptor (GPCR) signalling through CXCR4 and induces cellular migration and actin polymerisation (GPCRs) signalling through CXCR4 and induced cellular migration and actin polymerisation (Berge *et al*., 2010). With respect to CD4+ T cells, T cells from Itk-deficient mice have defects in T cell development and differentiation (Schaeffer *et al*., 2001), as well as demonstrating profoundly impaired T-cell proliferation, migration, adhesion and F-actin reorganization (Dombroski *et al*., 2005). It can be noted that a few Itk inhibitors have been investigated in certain animal models with promising results such as, asthma, lung inflammation, skin dermatitis (Lin *et al*., 2004), and models for HIV infection (Readinger *et al*., 2008b), albeit they have not entered clinical trials.

Shin *et al*., (2007) demonstrated that Itk is upregulated in the skin lesions of cutaneous T-cell lymphoma (CTCL) patients, and aberrantly activated in T-cell lymphoma (Kaukonen *et al*., 1999). Another study shows that it is engaged in the pathogenesis of T cell lymphoma by regulating its downstream molecules such as, NFAT, MAPK and NFκB (Zhao *et al*., 2010). It is also known that Itk-dependent IL-2 receptor (CD25) signalling machinery has exhibited promising efficiency in T cell lymphoma with anti-CD25 monoclonal antibodies (Dancey *et al*., 2009). Moreover, they further show that targeting CD25 with anti-CD25 antibodies has a therapeutic efficacy in T cell lymphomas.

The identification of the chromosomal translocation t(5;9)(q33; q22) in the PTCL-NOS (see section 1.1.4 for more detailed information) and its important role in the pathogenesis of T cell lymphoma support ITK as a potential therapeutic target of anti-lymphoma drugs. It is worth mentioning that the activation of this fusion is dependent on the PI3K signalling pathway (Hussain *et al*., 2009), suggesting that inhibitors of PI3K inhibitor may be efficacious for treatment of PTCL (Herman *et al*., 2012; Zhong *et al*., 2014). Intriguingly, our data indicate that ITK inhibitors can synergise with PI3K inhibitor in T cell lymphoma cell lines. Guo *et al*., (2012) indicate that the ITK inhibitor, CTA056, has a strong cytotoxic effect on T-cell acute lymphoblastic leukemia (T-ALL) and CTCL cell lines. Furthermore, Ibrutinib is a covalent inhibitor of BTK, which has been approved in clinic for B-cell malignancies, which is also established as a potent ITK inhibitors in T cells (Dubovsky *et al*., 2013).

Interestingly, Carson *et al*., (2015) demonstrate that ITK is aberrantly expressed in melanoma-bearing mice, and induces tumour formation and progression. They also reported that therapeutic targeting of ITK in melanoma with BI 10N (ITK inhibitor) decreased growth of ITK-expressing human melanoma xenografts, suggesting its efficacy in melanoma treatment. More interestingly, Ryan *et al*., (2016) show that Ibrutinib improved the graft-versus-leukaemia (GVL) effect in patients with relapsed chronic lymphocytic leukaemia following allogeneic haematopoietic cell transplant (HCT) treatment without causing graft-versus-host-disease (GVHD) development. This is most likely due to Ibrutinib selectively targeted pre–germinal B cells, depleted Th2 cells and enhanced Th1 cells-mediated GVL effects (Ryan *et al*., 2016). Therefore, it is interesting to study ITK in T-cell lymphoma due to its aberrant activation and heightened expression in these cells.

1.7.3 Therapeutic monoclonal antibodies

A number of new therapeutic agents for the treatment of peripheral T cell lymphomas (PTCLs) have become available to improve the outcome of patients with PTCL. Among these agents, monoclonal antibodies (MAbs) have been shown to be a powerful tool for targeted treatment of PTCLs. The most common class of antibodies (Igs) used in cancer therapy is IgG which is composed of antigen binding fragments (Fab) and Fc domain (Hebb and Kohrt, 2015). MAbs directed at targets on tumour T cells have been reported to have anti-tumour effects through antibody-dependent cellular toxicity, intrinsic pro-apoptotic actions and complement-dependent cytotoxicity (Howman and Prince, 2011). Therapeutic MAbs differ in their targets, structure and mechanism of action. Common mechanisms of action of MAbs include complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity, direct apoptosis, blocking of receptors/signalling pathways, binding to and blocking biologically active molecules and delivery of cytotoxic agents to tumour cells (Hebb and Kohrt, 2015).

Various monoclonal antibodies have emerged as the therapeutic options for PTCLs. Alemtuzumab is a humanised anti-CD52 monoclonal antibody which is active in different haematological malignancies. Studies show that Alemtuzumab has clinical efficacy in PTCL but at the expense of infectious complications and significant immunosuppression (Broccoli *et al*., 2013). Other studies suggest that Alemtuzumab is effective and has clinical activity in T prolymphocytic leukaemia (T-PLL) (Dearden *et al*., 2001; Dearden, 2012) and Sezary syndrome/leukaemic-cutaneous T cell lymphomas (SS/L-CTC), but with strong severe infections (Clark *et al*., 2012, de Masson *et al*., 2014). The combination of Alemtuzumab with other therapies has been investigated (Binder *et al*., 2013, Weidmann *et al*., 2010), and their combinations were active in PTCL, but they were associated with significant toxicity. Brentuximab vedotin is a chimeric immunoconjugate or antibody-drug conjugate directed to CD30 which is typically expressed in Hodgkin's lymphomas and anaplastic large cell (ALCL) (Ansell, 2014). According to Fanale *et al*. (2014), brentuximab vedotin either with replacing vincristine (CHP) or sequentially with CHOP is being moved into front-line therapy in untreated high risk ALCL.

Mogamulizumab is a monoclonal antibody which targets CC chemokine receptor 4 (CCR4) approved for use in cancer therapy. It shows potent anti-adult T-cell leukaemia/lymphoma (ATLL) activity *in vitro* and *in vivo* (Ishii *et al*., 2010). Mogamulizumab was examined as part of initial therapy in Japan for relapsed ATLL,

relapsed CCR4+ PTCL and CTCL in phase I and II study (Ishida *et al*., 2012; Ogura *et al*., 2014). Intensive chemotherapy was given with additional Mogamulizumab, and the response rate was better in the Mogamulizumab arm than chemotherapy alone but at the cost of higher toxicity (Ishida *et al*., 2015). Daclizumab is another therapeutic humanised monoclonal antibody that has shown it safety and efficacy in patients with varying subtypes of ATL (Berkowitz *et al*., 2014). It is a humanized anti-CD25/IgG1 monoclonal antibody that inhibits binding of interleukin-2 (IL-2) to the IL-2 receptor. On phase II study, daclizumab demonstrates activity in chronic ATL but has no efficacy in acute/ lymphomatous ATLL (Berkowitz *et al*., 2010).

1.8 Overview of T-cell receptor signalling and Tec family of protein-tyrosine kinases

1.8.1 Structure and function of the normal T-cell receptor signalling complex

The Tec family is a subfamily of non-receptor tyrosine kinases (PTKs) which is represented by its first member Tec. The Tec kinase family members constitute the second largest family of protein tyrosine kinases after the Src kinase family that are activated in response to lymphocyte development and activation (Schmidt *et al*., 2004). This family has emerged as essential mediators of a signalling pathway in T lymphocytes. The importance of this family was first found in 1993 when mutations in the Bruton's tyrosine kinase (BTK) gene were found to cause the genetic disorder X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (xid) in mice, which are primary immunodeficiencies associated with deficiency in serum immunoglobulins and impaired B cell function and development (Tsukada *et al*., 1993; Vetrie *et al*., 1993; Thomas *et al*., 1993; Rawlings *et al*., 1993). XLA is recognised as the first example of mutations in tyrosine kinases related to a human immunodeficiency, highlighting their important role in antigen receptor signalling pathways and lymphocyte function and development.

The Tec-family protein tyrosine kinases comprise of five family members including Tec, Bruton’s tyrosine kinase (Btk), interleukin-2 inducible tyrosine kinase (Itk, also known as Tsk and Emt), resting lymphocyte kinase (Rlk, or Txk) and bone marrow expressed kinase (Bmx, or Etk), which are expressed principally in immune cells (Readinger *et al*., 2009; Vargas *et al*., 2013; Andreotti *et al*., 2010; Prince *et al*, 2009; Smith *et al*, 2001).

In spite of that fact that both the Tec kinases and Src family kinases have tyrosine-protein kinase catalytic and Src homology domain, the Tec family kinases contain a pleckstrin homology (PH) domain that connects to phosphoinositide 3-kinase (PI3K) products. Thus, this family of kinases are the only kinase proteins which have PH domain and can be organised via PI3K (Berg *et al*., 2005). Three of these kinases, Tec, Itk, and Rlk are expressed in T-lymphocytes and are engaged in transduction of signals from surface receptors to downstream pathways (Debnath *et al*., 1999; Gibson *et al*., 1996; Yang *et al*., 1999; Heyeck *et al*., 1997). ITK is the most predominant among the other kinases expressed in naïve T lymphocytes. mRNA analysis shows that *ITK* is expressed at 3-10 fold higher levels than RLK, and 30-100 fold more than Tec in naïve T lymphocytes (Miller, *et al*., 2004; Colgan, *et al*., 2004).

Analysis of Tec family kinase knockouts in mice suggests that Itk has a dominant role in T cell receptor (TCR) mediated signalling pathways to stimulate IL-2 production and Th2 cytokines such as IL-13, IL-5, and IL-4 (Liao and Littman, 1995; Schaeffer *et al*., 1999; Fowell *et al*., 1999; Schaeffer *et al*., 2001; Gomez-Rodriguez *et al*., 2011). By contrast, RLK deficient mice appear to have a milder phenotype, with loss/lack of this kinase chiefly affecting Th1 cells, probably due to redundancy among Tec family kinase members (Schaeffer *et al*., 1999; Schaeffer *et al*., 2001; Gomez-Rodriguez *et al*., 2011). It is noticeable that Itk/Rlk double-deficient mice have a more prominent phenotype with blunted IL-2 cytokine production, albeit the production of Th2 cytokine is paradoxically not affected (Schaeffer *et al*., 1999; Schaeffer *et al*., 2001; Gomez-Rodriguez *et al*., 2011). Sahu *et al*. (2008) indicate that the loss of Itk function can be compensated by Rlk. Thus, the compensatory mechanisms are likely to contribute to the complexity of phenotypes in Tec family member-deficient mice.

1.8.2 Expression of the Tec kinases in lymphocytes

In addition to the three Tec kinases discussed previously, Woods *et al*. (2001) show that Bmx can be identified in the Jurkat T cell line. These major Tec kinases display specific patterns and expression levels that could reflect their functional significance in distinct subsets of T cells and stages of expansion. ITK, as a predominant T-cell kinase was initially cloned via a degenerate polymerase chain reaction (PCR) screen of specific protein tyrosine kinase (Siliciano *et al*., 1992; Gibson *et al*., 1993; Tanaka *et al*., 1993; Heyeck and Berg 1993; 1993; Yamada *et al*., 1993). It has been observed that at least two forms of Itk have been cloned from mice, which differ via deletion/inclusion of an

encoding six amino acid sequence (Heyeck and Berg 1993; Siliciano *et al*., 1992; Yamada *et al*., 1993). The longer form, which seems to be a splice variant version, is not identified in human cells.

In addition to its presence in T cells, Itk expression is also observed in mast cells and natural killer cells, with the highest-level of expression being found in the mature adult thymus (Heyeck and Berg 1993; Siliciano *et al*., 1992; Tanaka *et al*., 1993; Yamada *et al*., 1993). It is clear that Itk expression is increased in T-cells up on activation and treatment with IL-2 (Siliciano *et al*., 1992; Gibson *et al*., 1993; Tanaka *et al*., 1993). Reports show higher *Itk* mRNA and protein levels in Th2 cells relative to Th1 cells; this may reflect the importance of Itk in the expansion of the Th2 response (Colgan *et al*., 2004; Miller *et al*., 2004). Interestingly, thymocytes and mature T cells from Itk-deficient mice demonstrate defects in phospholipase C-γ (PLC-γ) activation, impaired actin reorganisation and also defects in numerous functions in response to TCR stimulation (Schaeffer *et al*., 2001; Grasis *et al*., 2003; Labno *et al*., 2003).

Resting lymphocyte kinase (RLK) is also expressed in mature T cells and mast cells (Hu *et al*., 1995; Sommers *et al*., 1995). Real-time PCR (RT-PCR) analysis shows 3-10 fold lower levels of Rlk mRNA than Itk in resting mature T cells (Colgan *et al*., 2004; Miller *et al*., 2004). Surprisingly, Rlk expression is dramatically decreased upon TCR stimulation (Hu *et al*., 1995; Sommers *et al*., 1995). The expression of Rlk is increased in primary Th1 differentiation and Th1 cell clones, whereas it remains at a low level in Th2 cell differentiation and cell clones (Kashiwakura *et al*., 1999), indicating that Rlk plays a more substantial role in Th1 cell function. They observed that IL-2 increased the expression of Rlk, while IL-4 decreased its expression in peripheral blood CD4+ T cells. It is worth mentioning that Rlk mutation results in minimal T cell defects, but the exacerbated defects were seen in Itk-deficient cells (Schaeffer *et al*., 2000; Schaeffer *et al*., 1999).

Tec tyrosine kinase was initially cloned from a cDNA library of mouse liver, and has more a broad pattern of expression in immune cells (Mano *et al*., 1990). Relatively, Tec is expressed in very low levels in resting T cells, and its mRNA is approximately 100 fold less than that of Itk. Similar to Itk, Tec is slightly less expressed in Th1 than in Th2 cells (Tomlinson *et al*., 2004). Regarding Tec, no defects have been noted in Tec-deficient T cells. However, mutation of Tec exacerbates defects correlated with Btk deficiency in mouse B cells (Ellmeier *et al*., 2000), indicating its functional redundancy with other members of Tec family kinases. Finally, numerous splice variants of Tec kinase have

been described (Merkel *et al*., 1999), albeit their functional distinctions remain unknown in T cells.

1.8.3 Structure and regulation of Tec kinase

1.8.3.1 Domain structure of Tec kinase

Interleukin-2 inducible tyrosine kinase (ITK), also known as T-cell-specific kinase (Tsk) or expressed in mast cells and T lymphocytes (Emt) was found in the early 1990s (Gibson *et al*. 1993; Heyeck and Berg 1993; Siliciano *et al*. 1992; Tanaka *et al*. 1993; Yamada *et al*. 1993). The domain structure of ITK and Tec kinases family members is similar to other tyrosine kinase families but also displays unique features (Figure 1.5). Shared features include the common Src homology 3 and 2 kinases (SH3-SH2) cassette which is also seen in the Src, Abl and CsK kinases (Williams *et al*. 1998; Tsygankov 2003). Four of the five Tec family tyrosines, BTK, ITK, Tec and Bmx kinases consist of an amino-terminus that contain a Pleckstrin-Homology (PH) domain, followed by a TEC-homology (TH) domain that is composed of a Btk homology motif (BH), and Proline-Rich Regions (PRR) (Andreotti *et al*., 1997; Bunnell *et al*., 2000; Chamorro *et al*., 2001). The exception to this is Rlk /Txk, which contains a cysteine string motif/cysteine-rich region instead of a PH-BH region at its amino terminal region (Schmidt *et al*., 2004).

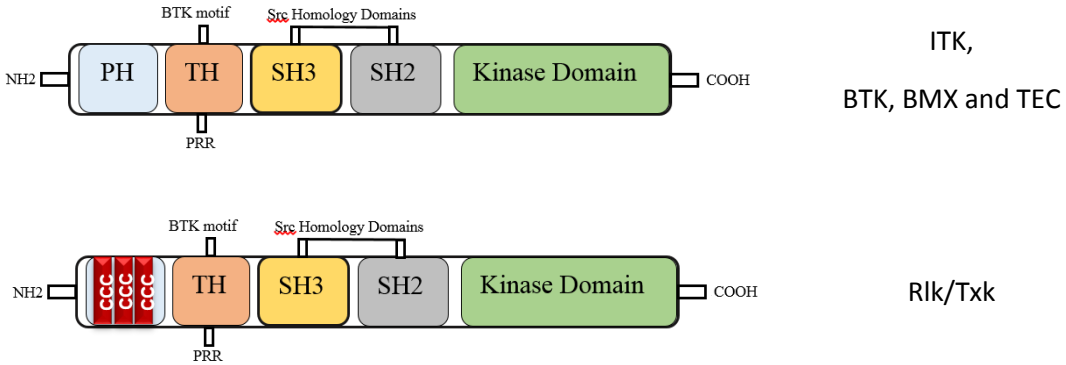


Figure 1.5. Domain structure of Tec Kinases. Tec kinases ITK, BTK, BMX and TEC have five domains: an amino-terminated region that includes a pleckstrin homology (PH) domain, followed by a Tec-homology (TH) domain which composed of BTK motif and Proline Rich Region (PRR), Src homology SH3 and SH2 domain and a kinase domain for TCR downstream signalling. Instead of the PH region, RLK is the only exception in having a cysteine-string motif at its amino terminus. (Sahu and August, 2009).

1.8.3.2 Role of ITK in signalling downstream of T cell receptor (TCR)

Tec kinases are generally engaged in receptor-mediated signalling machinery in multiple cell types (Berg *et al*., 2005, Gomez-Rodriguez *et al*, 2011; Mihara and Suzuki, 2007; Koprulu and Ellmeier, 2009; Readenger *et al*, 2009). It can be noted that significant attention is given to Itk activation via T cell receptor (TCR) stimulation, as seen in figure 1.6. TCR interaction with the peptide-MHC/antigen on antigen presenting cells activates the Src family kinase Lck (Lymphocyte-specific protein tyrosine kinase), resulting in phosphorylation of the immune-receptor tyrosine activation motifs (ITAMs) of the CD3 complex chain (Vargas *et al*., 2013; Andreotti *et al*., 2010). Once zeta-chain-associated protein kinase 70 (Zap-70) binds to the phosphorylated ITAM, it is phosphorylated by Lck, leading to activation of ZAP-70 and subsequent phosphorylation of adaptor proteins linker for activation of T cells (LAT) and Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76) (Au-Yeung *et al*. 2009; Houtman *et al*. 2005; Pitcher and van Oers 2003; Smith-Garvin *et al*. 2009).

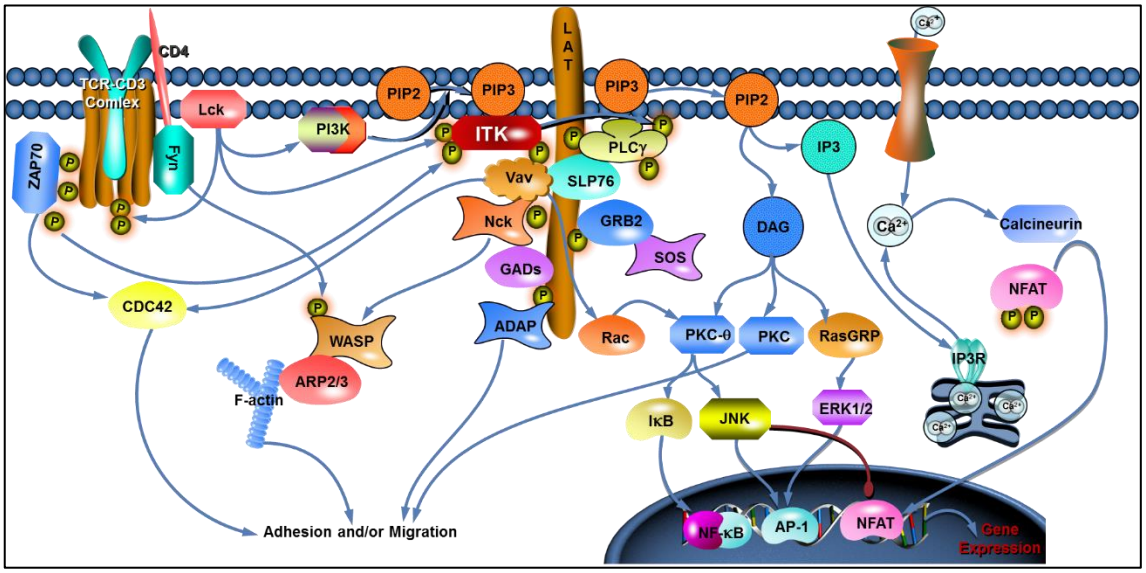


Figure 1.6. T cell signalling regulated by ITK. T cell signalling regulated by ITK. ITK acts downstream of signalling complex downstream of the TCR**.** Activated ITK interacts with the phosphorylated adaptor proteins SLP-76 and LAT via Src homology domain of ITK, and involves in phosphorylation and activation of PLCγ1. PLCγ1 activation then hydrolyse PIP2 to generate second messengers including IP3 and DAG, and regulate Calcium levels, regulate ERK/ MAPK pathways, transcription factor of activated T-cells (NFAT), cytokine release and actin polymerization. The figure was taken from Qiagen Technologies.

Following phosphatidylinositol 3-kinase (PI3k) activation and phosphatidylinositol (3,4,5) trisphosphate (PIP3) accumulation in the plasma membrane, Itk is, via its PH

domain, recruited to the membrane (Ching *et al*. 1999; August *et al*. 1997; Yang *et al*. 2001). When activated, PI3K catalyses Phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5) trisphosphate (PIP3) (Sahu and August 2009). There, the interaction of Itk and phosphorylated adaptor proteins LAT/SLP-76 forma a complex via ITK's SH3-SH2 domains (Wardenburg *et al*. 1996; Shan and Wange 1999; Su *et al*. 1999; Bunnell *et al*. 2000; Sommers *et al*. 2004; Koretzky *et al*. 2006; Zhang *et al*. 1998), which permits Itk phosphorylation at its activation loop, Y511 by Lck (Heyeck *et al*. 1997). Once activated, ITK within its SH3 domain undergoes cis auto-phosphorylation on the tyrosine residue Y180 domain (Joseph *et al*. 2007a; Wilcox and Berg 2003). ITK interacts with and directly involves in phosphorylation of its downstream target, phospholipase-Cγ 1 (PLCγ1) (Perez-Villar and Kanner 1999; Joseph *et al*., 2007c), leading to activation of phospholipase (Liu *et al*. 1998; Schaeffer *et al*. 1999; Wilcox and Berg 2003; Bogin *et al*. 2007).

Houtman *et al*. (2005) indicate the characterisation of many of these early phosphorylation events provides a better understanding of the sequential signalling pathways following TCR engagement. It has been reported that ITK auto-phosphorylation is likely to serve as a trigger for the altering of protein interaction partners and localization occurs either before or following activation of PLCγ1 (Andreotti, *et al*., 2010). This is due to auto-phosphorylation on tyrosine residue Y180 and does not influence the catalytic activity of ITK, but alternatively modulates binding of the SH3 domain within ITK to various targets (Wilcox and Berg 2003; Joseph *et al*. 2007a).

Once activated, PLCγ1 then hydrolyses PIP2 to generate second messengers, including inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Rhee, 2001; Lindvall *et al*., 2005). Downstream consequences of ITK activation include the regulation of protein kinase (PKC) activation and regulation of Ca^{2+} levels (Liu *et al*., 1998; Lewis and Cahalan, 1995; Schaeffer *et al*, 2001; Perez-Viller and Kanner, 1999), activation of ERK/MAPK pathways, Nuclear factor of activated T cells-NFAT activation (Schaeffer *et al*., 2001), cytokine release (Liu *et al*., 1998; Liao and Littman, 1995; Wange *et al*, 1996; Houtman *et al*, 2005; Raab *et al*, 1997) and actin polymerization and reorganization (Labno *et al*., 2003; Sahu and August, 2009). The consequences of this downstream signalling by TCR activation are not ablated by the lack of ITK in T cells, but they are substantially decreased (Andreotti *et al*., 2010; Schaeffer *et al*., 2001; Liu *et al*., 1998), resulting in altered development and differentiation of T-cell lineages.

1.8.3.3 Engagement of ITK in CD28 and CD2 costimulatory signalling pathways and chemokine receptors

Aside from the role of Itk in TCR signalling, several reports indicate that Itk could be essential in the CD28 costimulatory signalling pathway (August, *et al*., 1994; Raab *et al*., 1995; Gibson *et al*., 1996; Gibson *et al*., 1998; Marengere *et al*., 1997; King *et al*., 1997). These biochemical studies generally exhibit an interaction between ITK and CD28. The primary study demonstrates that following binding to CD28, Itk becomes tyrosine phosphorylated on a tyrosine residue upon CD28 stimulation in Jurkat cell lines (August, *et al*., 1994). Moreover, *in vivo* studies using recombinant proteins indicated that binding of Itk to CD28 requires the presence of the Src family kinase, Lck (Raab *et al*., 1995). This finding was then confirmed by using Lck-deficient Jurkat cells (Gibson *et al*., 1996).

Furthermore, structure and function analysis of the tyrosine residues in the cytoplasmic tail of CD28 supports the close relationship between Itk and Lck activities after CD28 stimulation (Gibson *et al*., 1998). Regarding the interaction between ITK and CD28, Marengere *et al*., (1997) show that the Itk-SH3 domain binds to proline-rich sequences in the cytoplasmic tail of CD28. Intriguingly, they also showed that *in vitro* peptides corresponding to the proline-rich region in CD28 may activate Itk activity and support a strong mechanism whereby stimulation of CD28 would activate downstream signalling tyrosine kinases. The data further show that Itk phosphorylated all four CD28 cytoplasmic tail tyrosine residues in *in vitro* kinase assays (King *et al*., 1997), supporting evidence for another Itk's essential role in CD28 signalling. In spite of this biochemical data, it is required to fully understand a substantial role of Itk in CD28 signalling.

Itk also plays a major role in the downstream signalling of the T-cell costimulatory receptor CD2. Similar to the CD28 signalling receptor, it was shown that Itk is phosphorylated following CD2 stimulation on peripheral blood T cells or Jurkat cells (King *et al*., 1996). Moreover, Itk activation requires the activity of Lck following CD2 stimulation and is dependent on the PRR of the cytoplasmic tail of CD2 (King *et al*., 1998). Studies indicate that a kinase-inactive mutant of Itk was able to repress CD2 mediated activation of transcription factor, NFAT in transfected Jurkat cells (Tanaka *et al*., 1997).

It has been reported that the role of Itk in the downstream signalling of the chemokine receptors CXCR4 (Fischer *et al*., 2004; Takesono *et al*., 2004). Their result conclude that

chemokine stimulation of T cells induced Itk and Rlk phosphorylation and translocation of Itk to the plasma membrane (figure 1.7). Similar to Itk activation downstream of the TCR, chemokine-induced Itk localization and phosphorylation are blocked via the Src family kinase (Fischer *et al*., 2004) and by PI3K inhibitors (Takesono *et al*., 2004). Interestingly, these studies show cell migration and actin polymerization were defective in Itk deficient T cells in response to SDF-1α (CXCL12). Furthermore, the impairments are further lessened in the lack of Itk and Rlk (Takesono *et al*., 2004). This result suggests that the Tec kinase members Itk and Rlk contribute to signalling downstream of chemokine receptors.

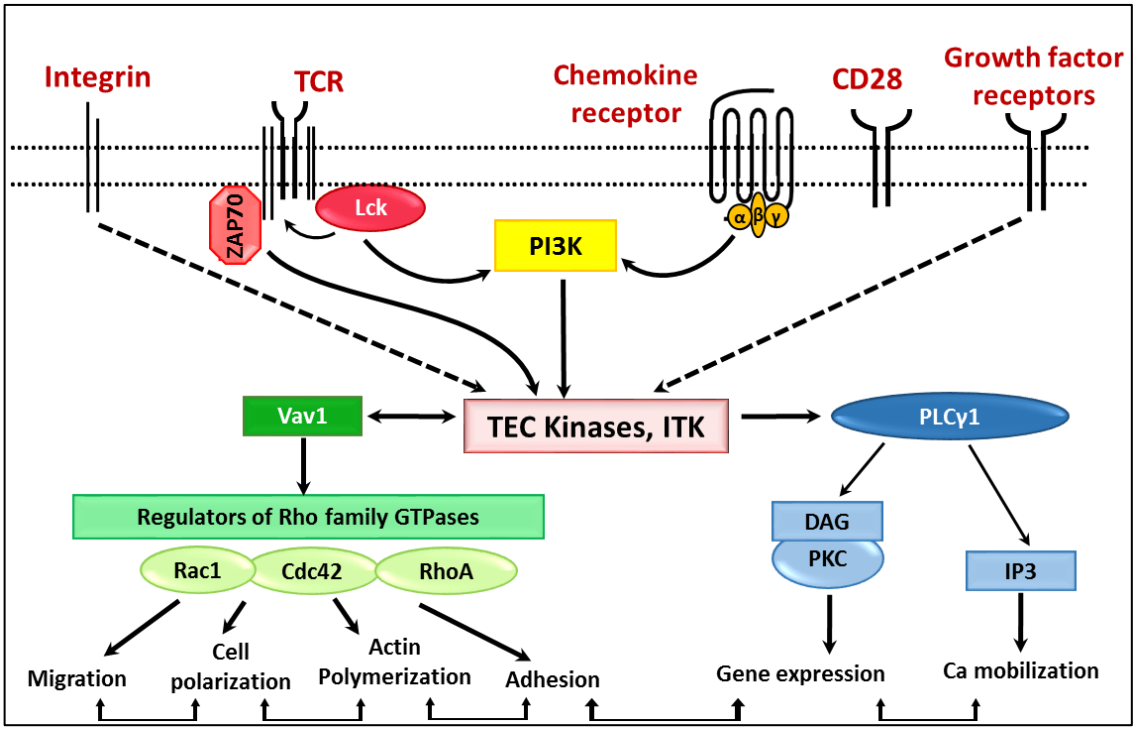


Figure 1.7. The Tec family kinases are involved in chemokine receptors and T-cell receptors signal. TEC family kinases are recruited to the plasma membrane after engagement of the chemokine and TCR receptors. Once activated, TEC family kinases take part in downstream pathways, including $Ca2^{+}$ mobilization, gene expression, migration, actin reorganization and adhesion. The downstream effects are related in the gene expression, adhesion and migration rely on guanine nucleotide exchange factor (Vav1) and proper actin reorganisation in the cell. In addition, $Ca2^{+}$ flux regulates gene expression and adhesion. (Part of this diagram was taken from Schwartzberg *et al*., 2005).

1.8.3.4 The ITK signalling complex

The interaction of Itk and SLP-76 may involve the Itk SH3-SH2 domains targeting a proline-rich stretch at residues 184-195 within SPL-76 and an adjacent SPL-76 phosphorylated tyrosine residue (Y145) respectively (Su *et al*. 1999; Bunnell *et al*. 2000).

In an excellent knock-in mouse study, the tyrosine residue (Y145) within SLP-76 was replaced by a non-phosphorylatable amino acid, phenylalanine to probe the contribution of Itk-SH2 or pY145 binding to signalling (Jordan *et al*. 2008). The Y145 mutation resulted in remarkably reduced phosphorylation of PLCγ1, diminished calcium flux, decreased p-ERK levels and led to T cells that are similar to those lacking Itk (Jordan *et al*. 2008). This is consistent with the observation of Bogin *et al*. (2007), who found that the interaction between Itk and SLP-76 is needed to activate Itk activity in Jurkat cells. This study observes that most of the catalytically active Itk in cells is comprised of only the small fraction of Itk bound to SLP-76 (Bogin *et al*. 2007).

1.8.4 ITK regulation of T cell function and its role in T cell differentiation

The fundamental role of ITK in the LAT/SPL-76 complex suggests that it is an essential element of TCR signalling. Previous studies using Itk deficient mouse models revealed that Itk is needed for robust T-cell activation in response to signals from TCR and costimulatory receptor signalling. However, these investigations showed that Itk is not entirely required for all responses to TCR downstream signalling (Liao and Littman 1995; Liu *et al*. 1998; Schaeffer *et al*. 1999). One of the potential modulatory roles for Itk is partially redundancy between Itk and Rlk, a second member of Tec family kinases. Accordingly, a combination of both Itk and Rlk deficiency results in TCR signalling defects that are substantially more profound than those observed in Itk-deficient T cells alone (Schaeffer *et al*. 1999). In general, the message from these investigations was that Itk is a substantial component of the TCR signalling pathway and functions to amplify TCR signalling.

Despite the modest effect of ITK deficiency on activation of naïve T-cell, many studies have revealed that Itk signalling has a central role in the regulation of T cell effector functions and T cell differentiation, as seen in figure 1.8. In an early study, Fowell *et al*. (1999) demonstrate that Itk deficiency in Balb/c mice were not capable of generating Th2 cells in response to infection with Leishmania major. However, in wild-type Balb/c, the infection was cleared by amounted protective Th1 response. These functions have been studied in detail in both *in vitro* and *in vivo* models, indicating the importance of Itk in generating Th2 responses.

The primary findings that Itk-/- Balb/c mice were not able to mount a Th2 response to infection in some of infectious disease systems such as *Leishmania major* and were not protected from infection (Fowell *et al*. 1999; Schaeffer *et al*. 2001). They further showed

that two parasites *Nippostrongylus brasiliensis* and *Schistosoma mansoni* were eliminated by Th2 dependent granulocyte recruitment and activation. From these infected mice, investigations of cytokine responses for T-cell specific parasites demonstrated that T cells lacking Itk produced reduced IL-10, IL-5, and IL-4 (Schaeffer *et al*. 2001). In a like manner, Itk-/- Balb/c *in vitro* experiments indicated the CD4+ T cells stimulated under Th2 conditions generated less IL-4 than their wild type counterparts (Fowell *et al*. 1999). Moreover, these cytokine responses were associated with defectiveness of a transcription factor, nuclear factor of activated T-cells (NFAT) by T cells lacking Itk, showing a potential reason for the reduction of IL-4 transcription. It can be noted that a series of *in vitro* experiments have been performed with naïve Itk-/- CD4+ T cells as complementary experiments for *in vivo* models. All these data concurred that wild-type T-cells produced substantially more IL-4, IL-5, and IL-14 than CD4+ T cell lacking mice, even after differentiation in a potent Th2 polarizing environment (Au-Yeung *et al*. 2006; Fowell *et al*. 1999; Miller *et al*. 2004; Schaeffer *et al*. 2001). Molecular analyses revealed that impaired effector cytokine production was not due to defects in Th2 differentiation, and indicated that Itk-deficient cells polarized in the Th2 lineage presented all the hallmarks of bona fide effector Th2 cells (Schaeffer *et al*. 2001; Miller *et al*. 2004; Au-Yeung et al. 2006).

In contrast to the indispensable role of Itk signalling in T cells to protect immunity against helminthic parasites, its signalling in protective Th1 responses to intracellular pathogens is dispensable. Therefore, mice that lack Itk clear infections with Leishmania major have a modest susceptibility to a sublethal dose of *Toxoplasma gondii* compared with wild-type mice (Fowell *et al*. 1999; Schaeffer *et al*. 1999). These findings may be relevant to the fact that production of IFNγ is not decreased by Itk-/- T cells to the same extent as cytokine production in Th2 (Au-Yeung *et al*. 2006; Fowell *et al*. 1999; Schaeffer *et al*. 2001; Miller *et al*. 2004).

As described above, the defects of Th2 cells, and less so on Th1 cells, have been observed in T cells lacking Itk. In addition, Itk-deficient cells demonstrate additional alterations in producing cytokines that highlight the complex nature of signalling machinery regulating the effector T cell responses. One of the best examples of this is the regulation of cytokine production via Th17 cells. Korn *et al*. (2009) for first time recognized that CD4 effector T cell lineage differentiated in response to transforming growth factor beta (TGF-β) and Interleukin 6 (IL-6) and express the pro-inflammatory cytokines including IL-17A, IL-21, IL-22, and IL-17F. Th17 cells play a crucial role in antimicrobial activity,

inflammatory response against fungi and bacteria (Gomez-Rodriguez *et al*., 2014) especially in the gastrointestinal system, and hallmarks of inflammation involved in many autoimmune disorders. According to Gomez-Rodriguez *et al*., (2009), Itk is a positive mediator of IL-17A production and importantly the reduction of IL-17A expression was found in Itk-deficient $CD4^+$ T cells differentiated under Th17 conditions. Additionally, they observed that the role of Itk in IL-17A production 17A required robust calcium signalling resulting in activation of NFAT, in the transcription of the IL-17 A gene.

Another study indicated that Itk-deficient CD4+ T cells generated under Th9 condition in culture was failed to express of cytokine IL-9 (Gomez-Rodriguez *et al*., 2016). The same group demonstrated that this defects is linked with reduced levels of pS6, IRF4 and pSTAT5. Finally, decreased numbers of IL-9 producing T cells was detected in Itk-deficient mice (papain hypersensitization model) as well as in human CD4+ T cells treated with Itk inhibitor (BMS-509744), suggesting another way to block Th9-mediated disease (Gomez-Rodriguez *et al*., 2016).

T cell helper development

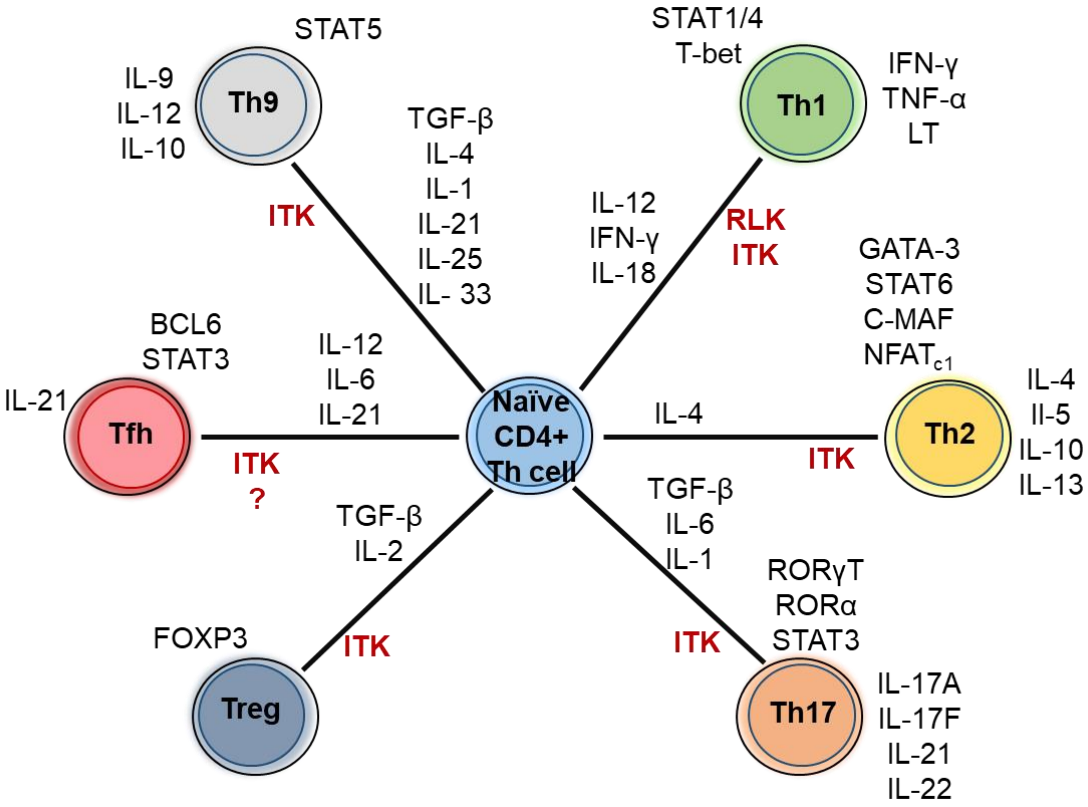


Figure 1.8. The function of ITK in T helper cell differentiation. The six major lineages of effector T cells are illustrated, along with transcription factors and cytokine signals that are essential for regulating their differentiation from naïve CD4+ T-cells. Among the five major helper T cells, ITK is essential for regulation and differentiation of Th2 (Fowell *et al*., 1999), Th17 (Gomez-Rodriguez *et al*., 2009), Treg (Huang *et al*., 2014; Gomez Rodriguez *et al*., 2014) and Th9 (Gomez Rodriguez *et al*., 2016) cells. The mechanism by which ITK regulated Treg cells is not well identified, and the role of ITK in Tfh cells is currently unknown.

1.9 Mechanisms of T-cell Plasticity and potential implications for disease

Multiple researches have observed the presence of certain functional flexibility in T cell lineage commitment. Traditionally, T lymphocyte subsets are determined by the production of cytokine profiles, regulatory transcription factors and the expression of chemokine receptors in certain cases. It has been reported that tissue microenvironment (TM) is required for T-helper-cell lineages function and differentiation (Eyerich and Zielinski, 2014). Bluestone *et al*., (2009) indicate that the functional plasticity of T cell lineages can be described by the activation of regulatory transcription factors. Nevertheless, certain regulations control the transition among CD4+ effector T cells. For instance, CD4+ Th1 and Th2 cells are defined by being the most stable subsets which are regulated by self-reinforcing but mutually repressing transcription factors and cytokine signalling such as, Th1 regulation by T-bet and IFN-γ, and Th2 by GATA-3 and IL-4 (Murphy and Stockinger, 2010).

Additionally, T cells expressing IL-12 receptor from mice and humans are responsive to the IL-12 signalling pathway. IL12-dependent Th17 cells are able to undergo a rapid transition to Th1 state (Martin-Orozco *et al*., 2009; Annunziato *et al*., 2007; Bending *et al*., 2009). Therefore, IL-12 receptor, which comprises IL-12Rβ1 and IL-12Rβ2 chains (Presky *et al*., 1996), is an essential element in the description of the transitional process and pro-inflammatory response. Jones and Vignali, (2011) report that the signal intensity of IL-12 is limited by the presence of the IL-12Rβ2 subunit which is extremely under expressed compared to the IL-12Rβ1 subunit. Interestingly, it has been found that even a stable regulatory T-cell effector lineage, like Th2 cells, are able to re-express IL-12Rβ2 *in vivo* as well as generate IL-4 and IFN-γ in response to infections with virus (Hegazy *et al*., 2010). The induction of a hybrid transition state between Th1 and Th2 has been noticed via type I interferons (IFNs), a subgroup of interferon proteins, in combination with cytokines IL-12 and IFN-γ (Murphy and Stockinger, 2010; Tokoyoda *et al*., 2009).

According to Veldhoen *et al*., (2008), the conversion of Th2 cells into Th9 cells was observed in response to TGF-β. It can be noted that a significant proportion of IL-9 producing cells can acquire the phenotype of Th1 and acquisition of IFNγ production *in vivo* (Tan *et al*., 2010; Umezu-Goto *et al*., 2007). Moreover, a helminthic infection model shows that the Th17 subset has the capability of conversion into Th1 phenotype and the acquisition of IL-4 expressing Th2 phenotype (Annunziato *et al*., 2012). Furthermore,

induced regulatory T cells (iTreg) cells have a functional transition towards Th17 cells in the presence of IL-6 under inflammatory status, indicating iTreg cells are less stable compared with thymus-derived Treg (Murphy and Stockinger, 2010). Stock *et al*., (2004) noticed that there is a possibility of transition from Treg cells to T-bet to Th1 cell-like phenotype co-expressing Foxp3 in mice.

The capability of natural Treg (nTreg) cells to induce the expression of T-bet and IFN-γ resulting in Th1 cell-like phenotype (Dominguez-Villar *et al*., 2011). The existence of such plasticity between Tregs and Th1 cells is relient on IL-2 and IL-12 *in vitro*, and this may have a critical role in autoimmune diseases. Research in humans and mice demonstrates that in the presence of IL-6 and TGF-β Tregs can convert to Th17 cells (Xu *et al*., 2007; Koenen *et al*., 2008). It seems that Tregs and Th17 cells share certain properties with each other and differentiation programmes. Interestingly, TGF-β is responsible for driving Treg differentiation, whereas together with IL-6 or IL-21 cytokines promote differentiation of Th17 and block Treg differentiation (Korn *et al*., 2009). Thus, the flexibility of these cells in differentiation processes and their plasticity allow them to adapt to various environments. Apparently, some transitions do not occur between certain CD4+ effector T cells such as, Th1 to Treg or naïve T cells and Th2 to Th17 or Treg transition (Murphy and Stockinger, 2010). The plasticity in CD4+ effector T cell fates is observed to be advantageous for host immunity against pathogens, and has potential in the study of autoimmune diseases and cancer.

1.10 Harnessing the plasticity of CD4+ T cells for immunotherapy

The hypothesis of Th1 and Th2 subsets of CD4 T cells was developed in 1980 by Mosmann and Coffman, and other studies have provided a framework for understanding the mechanism of how CD4+ T cells direct various immune responses (Mosmann, *et al*., 1986; Del Prete *et al*., 1991). Analysis of clonal populations of CD4+ T-cells reveals that selected patterns of cytokine production were identified by different clones such as interferon γ (IFNγ) being mainly expressed in Th1 cells and interleukin 4 (IL 4) in Th2 cells (Mosmann, *et al*., 1986; Del Prete *et al*., 1991). Importantly, it has been shown that delineating CD4+ T cells into specific subpopulations on the basis of cytokines shows that CD4+ T cells may link to various pathologies and correlated with the control of distinct types of infections (Abbas *et al*., 1996).

Today, the spectrum of CD4+ T cells subsets are broadened into a number of subsets including Th17, Tfh, thymically-derived and peripherally-induced T regulatory cells (tTreg and pTreg cells), Th9 and Th22 (Burkett, *et al*., 2015; Schmitt *et al*., 2014; Crotty, S. T., 2014; Wing and Sakaguchi, 2012). These subpopulations are characterized by their ability to produce certain cytokines and programme the expression of different master transcription factors and function through the production of select cytokines and chemokine receptors to best prevent immune pathology and control specific pathogens. Although modern tools and techniques have shown that the capability of polarized T-cells especially the pTreg and Th17 cell subsets to repolarize toward alternative/mixed fates and modify their phenotype, suggesting the hypothesis that CD4 T-cells can adapt and display phenotypic plasticity capability in response to changing contexts. Thus, understanding the characterization of T-cell subsets and the mechanism of their plasticity and control T cell programming may lead to new therapeutic approaches that may ameliorate cancer and autoimmune diseases.

Identification of a potential therapeutic target for diverse diseases that drive CD4+ effector T cells function and polarization is strongly being pursued to modulate the immune response. Recently, it has been reported that the modulation of IL-2 induced receptor signalling in human T-cells with a low dose of this cytokine can alter the balance between Tregs and inflammatory T-cells (Klatzmann and Abbas, 2015). Numerous studies demonstrate that IL-6 blockade with tocilizumab, a humanized anti-IL-6 receptor antibody, can suppress Th17 differentiation and has been shown to be an effective treatment for certain autoimmune diseases. Many immunologists have indicated that IL-6 has a substantial role in regulating the Th17 and Treg balance. Therefore, any dysregulation in IL-6 signalling is likely to imbalance Th17 and Treg polarisation and subsequently lead to breakage of immunological tolerance and the development of autoimmune diseases.

According to Bluestone *et al*., (2015), the introduction of synthetic mutant cytokines or antibody binding to cytokine (IL-2), induces a change in the cytokine conformation and could be resulting in selective cytokine signalling to distinct T-cell subsets. Other researchers have pointed out that TCR signal strength may control the selective capacity of CD3-specific monoclonal antibody therapy to deplete effector T lymphocyte, whereas sparing regulatory T cells, and preventing the progression of type 1 diabetes mellitus (Herold *et al*., 2002; Penaranda *et al*., 2011). A recent paper shows that modulation of

CD28 co-stimulation with antibody blockade of T-cell molecule cytotoxic T-lymphocyte antigen 4 (CTLA4) and CTLA4–Ig can drive immunity and tolerance (Littman, 2015).

The capacity of targeting key signalling pathways that direct plasticity in T lymphocytes is supported by the expansion of various orally accessible kinase inhibitors. For instance, small molecule JAK inhibitors have been approved by the FDA as potent immunosuppressive agents in patients with autoimmune diseases, albeit their capability in targeting specialized T cell subsets activities is limited due to multi-cytokine receptors using each kinase (O'Shea *et al*., 2015). Other studies pointed that rapamycin, an mTOR activity blocking agent, in combination with IL 2 is likely to promote Treg function in type 1 diabetes (Long *et al*., 2012). However, the enhancement of Tregs with Rapamycin/IL 2 combination therapy is transient (Huynh *et al*., 2015; Shrestha *et al*., 2015), and may be due to the further repression in signalling pathways such as AKT which is most essential in the promotion of the stability and function of Tregs.

Interestingly, suppression of PI3K (p110δ subunit) can have differential effects on inflammatory Tregs by disrupting their stability and subsequently promoting an anticancer immune response (Ali *et al*., 2014). Additionally, Putnam *et al*., (2009) indicate that inhibitors can be used to improve the functional stability of T cells following transfer into patients during *in vitro* expansion for adoptive T cell transfer treatments. Furthermore, studies show that inhibition of acetyl-CoA carboxylase 1 (ACC1) mediated *de novo* fatty acid synthesis with soraphen A blocks the glycolytic-lipogenic pathway leading to the promotion of Tregs and restraint of the development of IL-17 producing T cells (Berod *et al*., 2014). Other studies indicated that inhibition of the glycolysis mechanism with the activated protein kinase (AMPK) agonist metformin in lupus models induced follicular regulatory T cells (Tfr) over T follicular helper cells (Tfh) polarisation (Yin *et al*., 2015).

1.11 Aims of project

The aim of the thesis is divided into three main areas including, characterisation of ITK inhibition in signalling pathways in T cell lymphoma cells, characterisation of the effects of ITK inhibition on plasticity of normal and malignant T lymphocytes, and finally study of synergy between ITK inhibitors and chemotherapy agents

I. Characterisation of ITK inhibitors: Firstly, ITK expression was determined in T-cell lymphoma/leukemia cell lines, primary human tonsillar cells, and patient samples. Secondly, investigation of four ITK agents and their specificity was analysed on cell lines and patient samples. Thirdly, ITK functions were investigated including, its effects on downstream signalling molecules, cytokine secretion, migration and Rho-family GTPases. Additionally, investigation of the functional cross-talk between T-cell receptor signalling machinery and chemokine receptor CXCR4 is required for ITK activation and function in T-cell lymphoma cell line.

II. The role of ITK in CD4 T cell plasticity: The effects of ITK in CD4 effector T cell subsets and its potential role in T cell plasticity were determined using human CD4 T-cell lymphoma cells and normal tonsillar CD4 T-cells.

III. Synergy between ITK inhibitors and chemotherapy agents: The effects of ITK inhibitors on viability of T-cell lines and synergy with other inhibitors of T-cell signalling was determined

Chapter II. Methods and Materials

2.1 Cell line and primary cell isolation and culture

2.1.1 Cell lines and cell culture

T-cell lines representing different tumours were cultured. Jurkat, MOLT-4, K299 and Ramos cells (provided by the University of Leicester) were maintained in culture medium RPMI-1640 supplemented with 10% fetal calf serum (FCS) and 1% glutamine at 37˚C. CCRF CEM cells were grown in RPMI-1640 medium containing 20% FCS and 1% glutamine. HEK 293 cells were maintained in DMEM medium supplemented with 10% FCS, high glucose, L-glutamine, and sodium pyruvate. The cell line maintenance and conditions with their passage numbers (passage no.) are illustrated in table 2.1.

2.1.2 Cell viability assay

Viability of cells and growth was measured by using the CellTiter-Glo® (CTG) luminescent cell viability assay (CellTiter Glo, Promega, Madison, WI, USA). It is worth noting that the assay system is designed for use in multi-well plate formats. It relies on the feature of a proprietary thermostable luciferase and generates a stable luminescent signal to the amount of ATP. The CTG is a homogeneous approach to determine the number of viable cells present in culture depending on quantitation of the ATP, which signals the existence of metabolically active cells. Additionally, the amount of ATP present is directly proportional to the number of cells present in culture. Briefly, CTG reagent (100 µl) was added to 100 µl of medium containing cells, usually equal volume of the reagent is added to the volume of cell culture medium for a 96-well plate. After adding the reagent, the contents of each well were mixed on an orbital shaker to induce cell lysis for about 2 minutes. Then the plate was incubated to stabilize the luminescent signal at room temperature for 10 minutes. Inhibitor potency of the drugs in ITK was determined using the Wallac VICTOR^2 multilabel counter (PerkinElmer, Ramsey, United States) for measuring luminescence at room temperature. Percent cell viability was calculated to dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, United States) used as a vehicle control.

Cell line	Cell type	Tissue	Medium	Surface Marker Characteristics
Jurkat (passage no. 11)	T lymphoblast	Lymphoma	RPMI-1640, 10% FBS	IL-2 is produced in Jurkat cells after stimulation with phorbol esters and either lectins or PHA directed against the T cell antigen receptor
K299 (passage no. 18)	T cell lymphoma cell	Human Anaplastic Large Cell Lymphoma	RPMI-1640, 10% FBS	This cell line was established in 1986 from the peripheral blood of a 25-year-old man with T cell non-Hodgkin's lymphoma; now classed as CD30+ anaplastic large cell lymphoma (ALCL).
CCRF-CEM (passage no. 16)	T lymphoblast	Acute lymphoblastic leukemia	RPMI-1640, 20% FBS	CCRF-CEM cells were derived from the peripheral blood of a child (CEM) with T-cell acute lymphoblastic leukemia who had originally presented with lymphosarcoma.
MOLT-4 (passage no. 19)	T lymphoblast	Acute lymphoblastic leukemia	RPMI-1640, 10% FBS	MOLT-4 was derived from CD4 T-cells (ATCC CRL-1552). Antigen expression: CD1 (49%), CD2 (35%), CD3 A (26%) B (33%) C (34%), CD4 (55%), CD5 (72%), CD6 (22%), CD7 (77%)
Ramos (passage no. 16)	B lymphocyte	Human Burkitt's lymphoma	RPMI-1640, 10% FBS	The cells were derived from an EBV negative Burkitt lymphomae,. The cells have surface IgMκ.
SUDHL-6 (passage no. 21)	B lymphocyte	peritoneal effusion; derived from metastatic site: peritoneal cavity	RPMI-1640, 10% FBS	SUDHL-6 were derived from a B-cell non-Hodgkin lymphoma (B-NHL). CD3 -, CD10 +, CD13 -, CD19 +, CD20 +, CD34 -, CD37 +, CD38 +, CD80 +, CD138 -, HLA-DR +, sm/cyIgG -, sm/cyIgM +, sm/cykappa +, sm/cylambda.
HEK 293 (passage no. 19)	Human embryonic kidney	Embryonic kidney	DMEM medium, high glucose, L-glutamine, and sodium pyruvate, 10% FBS	The cells express cell surface receptor for vitronectin which composed of the integrin beta-1 and the vitronectin receptor alpha-v subunits.

Table 2.1. List of cell lines used throughout this project.

2.1.3 Inhibitors

Jurkat cell, K299, MOLT-4 and CCRF CEM lines were seeded in a flat-bottomed 96-well plate (Greiner Labortechnik, Germany) at 5×10^4 cells in 100 µL of medium per well and incubated with various concentrations of ITK inhibitors ONO-7790500 (*ONO Pharmaceutical*) (figure 2.1), BMS-509744 (5009), PF-06465469 (4710) and Ibrutinib (S2680) in different time points 24, 48 and 72 hours as shown in Table 2.1 and 2.3.

Drugs	Company	Reference number	Characteristics
PF-06465469	Tocris	4710	PF-06465469 is potent and covalent inhibitors of Itk with half maximal inhibitory concentration (IC50) values of 2 nM. It shows inhibitory activity against BTK with IC50 of 2 nM.
Ibrutinib (PCI-32765)	Selleckchem	S2680	Ibrutinib is a potent and selective Btk and ITK inhibitors (Pan *et al*., 2007; Dubovsky *et al*., 2013). It binds irreversibly to cysteine 442 residue in Itk and cysteine 481 in Btk and supresses downstream of TCR and BCR activation respectively. This compound inhibits ITK downstream targets, including PLCγ1, NFAT, IKBα and JunB downstream activation (Dubovsky *et al*., 2013). It has significant clinical activity in CLL and mantel cell lymphoma (MCL) treatment (Byrd *et al*., 2013; Wang *et al*., 2013; Woyach *et al*., 2014), and been approved for the treatment of these two malignancies by the U.S. Food and Drug Administration (FDA).
BMS-509744	Tocris	5009	BMS is ATP competitive inhibitor and it binds to the ATP binding pocket of the ITK domain. It is selective and potent ITK inhibitor with IC50 value 19 nM. This compound blocks TCR-induced PLCγ1 phosphorylation, reduces T cell proliferation, calcium mobilization and IL-2 production *in vitro*. BMS efficiently attenuated lung inflammation in a mouse model-allergic asthma and reduced the establishment of HIV infection.
ONO-7790500	ONO -Pharmaceutical	M110824045	ONO-7790500 is potent and selective ITK inhibitor. It inhibits ITK downstream molecules (TCR-induced PLCγ1, calcium flux and cytokine secretion) and reduces T cell proliferation. It is efficiently reduced migration and GTPase family members Cdc42 and Rac1 in primary CD4+ T cells and Jurkat T cell line.

Table 2.2. List of ITK inhibitors used throughout this project**.**

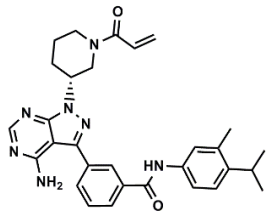
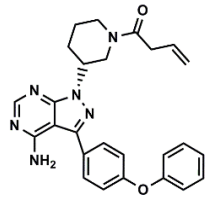
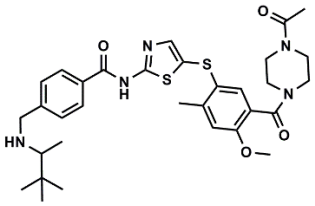
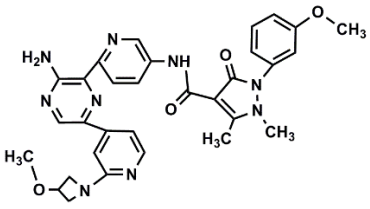
ITK inhibitors	Chemical Structure	Formula
PF-06465469		**$C_{30}H_{33}N_7O_2$**
Ibrutinib		**$C_{25}H_{24}N_6O_2$**
BMS509744		**$C_{32}H_{41}N_5O_4S_2$**
ONO-7790500		**$C_{31}H_{31}N_9O_4$**

Table 2.3. Chemical structure of PF-06465469, Ibrutinib, BMS509744 and ONO-7790500**.** Chemical structures of ITK inhibitors were drawn by ChemDraw Professional 15.1 (provided by the University of Leicester).

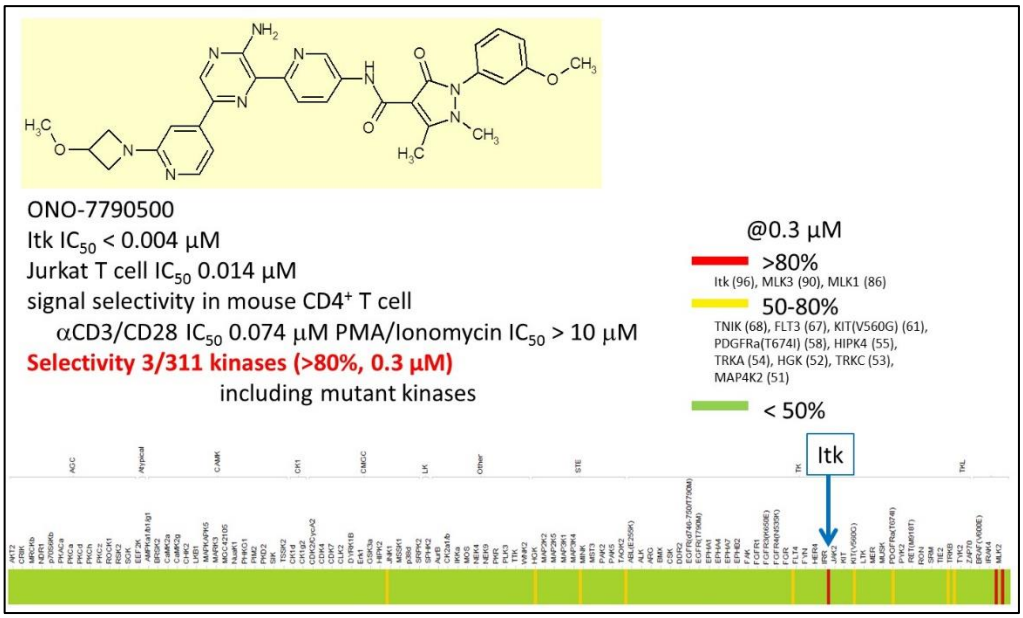


Figure 2.1. N-[6-[3-amino-6-[2-(3-methoxyazetidin-1-yl) pyridin-4-yl] pyrazin-2-yl] pyridin-3-yl]-1-(3-methoxyphenyl)-2,3-dimethyl-5-oxopyrazole-4-carboxamide (ONO-7790500) is a novel ITK inhibitor with extremely high selectivity in a broad kinase panel (Itk IC50< 0.004 μM). In addition, it showed an excellent signal selectivity in mouse CD4+ T cell (αCD3/CD28 IC50 0.074 μM, PMA/Ionomycin IC50 > 10 μM; αCD3/CD28 stimulates upstream of Itk in the TCR cascade while PMA/Ionomycin does downstream) and mouse *in vivo* IL-2 inhibition by oral administration. (ONO Pharmaceutical Company, Osaka, Japan).

2.1.4 Isolation of human peripheral blood mononuclear cells from whole blood

Ficoll histopaque was performed as the standard procedure to separate of white blood cells (WBCs) from fresh whole blood in a suitable cell culture facility. The following steps were undertaken:

1. Remove histopaque solution (17-1440-03, Ficoll-Paque PLUS) and RPMI 1640 (supplemented with 10% FCS, Glutamine and Pen/Strep) to warm at room temperature.
2. After labelling all tubes and lids with sample number, add 15 ml of histopaque into a 50 mL falcon tube (Fisher Scientific, Hampton, New Hampshire, United States).
3. Next, gently layer blood (up to 30 mL) onto histopaque and avoid mixing blood with histopaque layer.
4. The sample was centrifuged at 400 g without acceleration or deceleration at 20 °C.
5. Collect WBCs into a falcon tube (Fisher Scientific) using a proper pipette and add the re-warmed fresh culture media to make up to 30 mL.

6. After spinning down at 200 g for 10 minutes, discard the supernatant and resuspend the cell pellet in about 10-50 ml of media (depending on the pellet size).
7. Count cells using hemocytometer and TC20 automated cell counter-based trypan blue staining (Bio-Rad, Hercules, California, United States).

2.1.5 Preparation of single cell suspension from tonsil

Human tonsils were obtained from Leicester Royal Infirmary - University Hospitals of Leicester. Tonsils are a rich source of B lymphocytes (70%) and T lymphocytes (30%). Briefly, tonsils were cut into small fragments in Petri dishes using a scalpel. The fragments were transferred into a 70 µm cell strainer placed on top of a 50 ml Falcon tube (Fisher Scientific). Tissue was gently pushed through the mesh so as not to break it with the rubber end of a 2 ml syringe, and flushed with 10 ml of cold PBS. After centrifuging the cells at 300 g for 5 mins at 4˚C, the cell pellet was resuspended in 5 ml Red Blood Cell (RBC) lysis buffer (420301, San Diego, United States) and incubated for 15 mins at room temperature. Then, the pellet was resuspended in 5-10 ml PBS or PBS containing 2-3% FCS, and passed through a 40 µm cell strainer into a fresh 50 ml Falcon tube (Fisher Scientific) and count cells were counted using a hemocytometer or a TC20 automated cell counter-based trypan blue staining (Bio-Rad).

2.1.6 Negative isolation of CD4+ T helper cells

CD4+ T helper cells from human tonsils were isolated using negative selection (Human CD4+ T Cell Isolation Kit, 130-096-533, Miltenyi Biotec, Bergisch Gladbach, Germany). The kit contains two main components: $CD4^+$ T Cell Biotin-Antibody Cocktail and $CD4^+$ T Cell MicroBead Cocktail. The $CD4^+$ T Cell Biotin-Antibody Cocktail consists of cocktail of biotin-conjugated monoclonal antibodies against CD19, CD36, CD16, CD56, CD132, CD8a, CD14, CD15, TcRγ/δ, and CD235a (Glycophorin A). The $CD4^+$ T Cell MicroBead Cocktail consists of MicroBeads conjugated to monoclonal anti-CD61 antibody (isotype: mouse IgG1) and monoclonal anti-biotin antibody (isotype: mouse IgG1). Cells were isolated from Single-cell suspensions as follows:

I. Immunomagnetic labelling (Working fast, keep cells cold and use pre-cooled solution):

1. Determination of cell number (for up to 1 x 10^7 total T-cells).

2. Centrifugation of cell suspension at 300 g for 10 min at 4˚C. Aspirate supernatant completely and resuspend cell pellet in 40 µL of Magnetic-activated cell sorting (MACS) buffer (phosphate-buffered saline (PBS), 2 mM EDTA and 0.5% bovine serum albumin, pH 7.2).
3. Add 10 µL of $CD4^+$ Biotin-Antibody cocktail. Mix well and incubate for 5 minutes in the fridge (2-8 ˚C).
4. Then, add 30 µl of MACS buffer and 20 µL of CD4+ T cell microbead cocktail.
5. Mix the mixture well and incubate in the fridge for 10 minutes.
6. Proceed to magnetic cell separation.

II. Magnetic cell separation (with LS column-130-042-401, maximum number of cells per column 10^8 cells):

1. Place column in the magnetic field of a QuadroMACS Separator (130-090-976).
2. Prepare Column by rinsing with 3 mL of PBS buffer.
3. Before applying cell suspension, rinse the column with 3 ml of buffer, and then collect flow-through unlabelled cells which represents enriched CD4+ cell.
4. Wash the column with 3 ml of the buffer and combine unlabelled cells that pass through the column with the effluent from step 3.

III. Evaluation of CD4 T-cells purity:

Purified $CD4^+$ T cells are stained with anti-CD4-FITC antibody (564419) and analysed by using flow cytometry using BD FACSCanto™ II (San Jose, United States. BD Biosciences.

2.1.7 siRNA Transfection analysis

Efficiency of siRNA transfection in Jurkat cells was optimized using Block-i™ Fluorescence Oligo (Life technology) with DharmaFECT 1 transfection reagent (T-2001-01, Dharmacon). The cells at the density 2, 4 and 8 X 10^5 cells/mL were transfected with 100 nM Block-iT using various amount of DharmaFECT reagent (1, 3, 6, 9 12 and 16 µL) overnight. The transfected rate was over 50 % by conducting 3 µL DharmaFECT in 2-4 x 105 cell/mL per well in 24 well plates. For siRNA transfection specific human ITK, Jurkat cells were seeded in 24 well plates at the density of 4 X 10^5 cells per well. On the day of transfection, final concentration of 100 nM siRNA specific ITK s223953 and s223954 (life technology) in Opti-MEM reduced serum Medium (Invitrogen), and in combination were incubated at room temperature for 5 min, and DharmaFECT was then incubated for 5 min. Following the incubation, a complex of siRNAs and the reagent was

further incubated for 20 min at room temperature. The complex was transfected into Jurkat cells overnight. Cell viability, suppression of Ca2+ influx and apoptosis were determined using a CellTiter-Glo® luminescent cell viability assay, FluoForte Calcium Assay and Annexin-V FITC kit respectively. The depletion of ITK analysis was performed using a western blotting. The detail of techniques was described in sections (2.6, 2.7, 2.8, 2.9, 2.10 and 2.14). For knockdown of Vav1, PI3k and ZAP70, 100 nM Silencer Select-siRNA VAV1 (s14752) was transfected by DharmaFECT 1 reagent for 48 h in 96 well-plate, 100 nM ON-TARGETplus PIK3R1 siRNA (L-003020-00-0005) using HiPerFect Transfection Reagent (Qiagen, 301705)/ DharmaFECT 1 and 200 ng ON-TARGETplus ZAP70 siRNA (L-005398-00-0050) using HiPerFect Transfection Reagent. It is worth noting that the cells were activated by plate-bound anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) at 30 °C for 30 minutes or with 100 ng/mL CXCL12 (350-NS-010) for 5 minutes.

2.1.8 Analysis of drug combinations

Synergy between ITK inhibitors and other chemotherapy or novel agents was formally assessed. The anti-tumour activities of PF-06465469, Ibrutinib or ONO-7790500 in combination with Cyclosporine A (S2286), Romidepsin (S3020), Pralatrexate (S1497), Gemcitabine (S1714), Pictilisib (S1065) and Doxorubicin (44583) were assayed in a single 96-well plate, see Table 2.5.

Jurkat cells were treated with five concentrations of both compounds for 48 h at 37 °C with 5 % CO_2. The concentration ranges of drugs were nearby the IC50 of drugs to generate a sigmoid-shaped growth inhibition curve based on a fixed constant ratio and non-fixed ratio. The fractional affected (fa) was calculated from the percentage growth inhibition by the formula Fa= 1- (% growth/100). The Fa values were entered separately into the Calcusyn software program (Biosoft) for single agents and drug combinations. This method provides quantification of synergism (CI < 1) and antagonism (CI > 1) at different dose and effect levels as shown in Table 2.4. The drug interactions were indicated by a variety of graphs including combination index (CI), median-effect plot and isobologram.

Range of combination index	Description	Graded symbols
<0.1	Very strong synergism	+++++
0.1–0.3	Strong synergism	++++
0.3–0.7	Synergism	+++
0.7–0.85	Moderate synergism	++
0.85–0.90	Slight synergism	+
0.90–1.10	Nearly additive	±
1.10–1.20	Slight antagonism	-
1.20–1.45	Moderate antagonism	--
1.45–3.3	Antagonism	---
3.3–10	Strong antagonism	----
>10	Very strong antagonism	-----
Simplified CI values and their indication		
< 0.8	Synergism	
0.8-1.2	Additive	
> 1.2	Antagonism	

Table 2.4. Description and symbols of synergism and antagonism in drug combination studies analysed with the combination index values.

Drugs	Company	Reference number	Characteristics
Cyclosporine A	Selleckchem	S2286	Cyclosporin A (CsA) is an immunosuppressive drug that binds to the cyclophilin. CsA forms a Cyclophilin-Cyclosporin A complex after binding to the cyclophilin in T cells. The agent suppresses calcineurin with an IC50 of 7 nM (Fruman *et al*., 1992), and then inhibits nuclear factor of activated T cells-NFAT (Flanagan *et al*., 1991).
Romidepsin	Selleckchem	S3020	Romidepsin is a potent and selective inhibitor of histone deacetylases 1 and 2 (HDAC1 and HDAC2), but only weakly inhibits HDAC4 and HDAC6.
Pralatrexate	Selleckchem	S1497	Pralatrexate is an antifolate agent and is structurally a folate analog. It shows time and concentration dependent cytotoxicity against a panel of T cell lymphoma cell lines. This drug shows anti-tumour activity which is superior to other antifolates activity (Molina, 2008).
Gemcitabine	Selleckchem	S1714	Gemcitabine is a very specific and potent deoxycytidine analogue which is used as a chemotherapy agent. The drug results in 50% of cell growth inhibition in CCRF-CEM cell line with IC50 of 1 ng/ml.
Idelalisib	Selleckchem	S2226	Idelalisib is a selective PI3K inhibitor with IC50 of 2.5 nM, this inhibitor has selectively against p110δ, and is not sensitive to p110α, p110β, and p110γ (PI3K class I subunits).
Pictilisib	Selleckchem	S1065	Pictilisib is a selective PI3K inhibitor with an IC50 of 3 nM, which has selectivity against p110γ (25 fold) and p110β (11-fold). The compound is potent against PI3Kδ, PI3Kα and PI3Kα mutants H1047R and E545K, showing modest selectivity level against PI3Kγ and PI3Kβ, and better selectivity level against class II, III, and IV PI3K, including mTOR, Vps34 and DNA-PK.
Doxorubicin	Sigma-aldrich	44583	Doxorubicin is an antitumor antibiotic agent that induces apoptosis and DNA damage through inhibition of DNA topoisomerase II and macromolecular biosynthesis in tumour cells. The agent alters DNA and produces free radicals through DNA damage to trigger apoptosis of tumour cells.

phospholipase C (PLC) inhibitor (U73122)	Selleckchem	S8011	73122 is a potent PLC inhibitor, and reduced agonist induced calcium increase. It has been shown to block PLCγ1-dependent processes in cells (Li *et al*., 2005, Thomas *et al*., 2003; Thompson *et al*., 1991; Smith *et al*., 1990)

Table 2.5. List of drugs used throughout this project**.**

2.2 RNA and Protein

2.2.1 Total RNA Extraction

Total RNA was extracted from cells in suspension, including Jurkat, K299, MOLT-4, CCRF CEM, Ramos and HEK293 cell lines, human tonsil, $CD4^+$ T cells from human tonsillar cells and cells from patients with T-cell prolymphocytic leukaemia (T-PLL). The cells (1 x 10^6 cells/mL) were incubated in 1 mL lysis buffer (TRIZOL Reagent-Invitrogen) for 5 min for dissociation of nucleoprotein after harvested by centrifugation at 350 x g for 5min. Total RNA was extracted using PureLink™ RNA Micro kit (12183025) and PureLinkTM DNase/ carrier RNA (12185010, Invitrogen) according to the manufacturer's instructions.

2.2.2 RNA measurement

NANODROP 2000 spectrophotometer (lab tech) was used to analyse 1 µL of extracted RNA to quantify the amount present and dilute it to 50 ng/ mL in PCR mix to 96-well PCR plates for ITK estimations. The concentration of RNA was determined by quantification of the optical density (OD) at 340 nm.

2.2.3 Real-Time PCR

Convential and real-time PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, United States). PCR was carried out with Taqman Universal Master MIX II, with UNG (Catalogue number: 4440038, Applied Biosystems). The PCR mixtures were pre-heated at 50 °C for 2 min and then at 95 °C for 15 min, followed by 40 °C cycles of amplification at 95 °C for 15 sec. A final extension step was performed at 60 °C for 1 min. Real-time PCR results were also

analysed using the ABI 7500 software (Applied Biosystems). Quantitative RT-PCR was performed in 96-well plates using human-specific Taqman primers (*ITK*, Hs00950634_m1 [4331182]; *RLK*, Hs00177433_m1 [4331182]; *HPRT1*, Hs02800695_m1 [4331182]; *IL-21*, Hs00222327_m1 [4331182], *IL-17A*, Hs00174383_m1 [4331182], Applied Biosystems, Foster City, California, United States) and probe with ABI Prism 7500 sequence Detection system. Each sample was assayed in triplicate and it is also essential to include the appropriate minus or no RT control reaction in the experimental design to ensure that there is no cross contamination/contamination in the PCR reagents. *HPRT* expression of target genes was quantified by comparing Ct values for *HPRT* with CT values for the transcript of interest (*ITK*/*RLK*) using relative quantification method (Pfaffi *et al*., 2001).

2.2.4 Reverse transcription PCR (RT PCR)

A variety of mathematical models can be used in the calculation of the expression of a target gene in connection with an adequate reference gene. Calculations are determined by a variety of methods based on the comparison of the distinct cycle, including crossing points (CP) and threshold values (Ct) at a constant level of fluorescence; or crossing points according to an established mathematic algorithm (Tichopad *et al*, 2004; Pfaffi *et al*., 2002). Two categories of relative quantification models among several mathematical models to determine the relative expression are available:

1) Without efficiency correction (Pfaffi, 2001)
    a. $R= 2^{-[\Delta CP\ sample- \Delta CP\ control]}$
    b. $R= 2^{-\Delta\ \Delta CP}$
2) With kinetic PCR efficiency correction. (Pfaffi *et al*., 2002)

$$\text{Ratio} = \frac{(Etarget)^{\Delta}cptarget\ (control-sample)}{(Eref)^{\Delta}cpref\ (control-sample}$$

$$\text{Ratio} = \frac{(Etarget)^{\Delta}cptarget\ (control-sample)}{(Eref)^{\Delta}cpref\ (Mean\ control-MEAN\ sample}$$

Reverse transcription (RT) was performed with SensiFAST^{TM} cDNA synthesis kit using the manufacturer's protocol (Bioline). A 20 µl RT reaction included 50 ng of total RNA, 4 µl of 5x TransAmp buffer, 1 µl of reverse transcriptase (RT) and DNase/RNase-free water up to 20 µL. Semi-quantitative real-time PCR was used to evaluate *ITK* and *RLK*, *IL-17 and IL-21* mRNA expression relative to a reference gene (hypoxanthine

phosphoribosyl transferase-*HPRT*) in T-cell lymphoma cell lines, human tonsillar cells and patient cells.

2.2.5 Western blot analysis

2.2 5.1 Protein Extraction

To evaluate the expression level of ITK in Jurkat, K299, CCRF CEM and MOLT-4 cells, cell pellets were washed twice with PBS and lysed in radioimmunoprecipitation buffer (RIPA) (50 mM Tris-Hcl, pH 8.0, 150 mM Nacl, 1% sodium deoxycholate, and 0.1 % SDS) containing protease (sigma) and phosphatase (sigma) inhibitors. To extract total proteins, cell lysates were centrifuged at 15,000 xg for 10 min at 4 ℃ to remove debris, after incubated on ice for 10-15 min. Bicinchoninic acid (sigma) and copper (II) sulfate solution (sigma) was used to determine protein concentration according to the manufacturer's instructions.

2.2.5.2 Western blotting

Jurkat, K299, CCRF CEM and MOLT-4 cell lines and primary cells were lysed with radioimmunoprecipitation-RIPA buffer (50 mM Tris-Hcl, pH 8.0, 150 mM Nacl, 1% sodium deoxycholate, and 0.1% SDS) supplemented with protease and phosphatase inhibitors (sigma). Cell lysates were separated by gel electrophoresis on 7.5% Mini-protean TGX Precast gels (BIO-RAD), and transferred onto a polyvinyldine difluoride (PVDF) membrane (Mini Format, 0.2 µM PVDF Single Application, BIO-RAD). The blot was incubated in blocking buffer-TBST (20 mM Tris-Hcl, pH 7.6, 136 mM Nacl, and 0.1% Tween-20) supplemented with 5% skim milk (OXOID) for 1 h at room temperature. Then the membrane was incubated with primary antibodies in blocking solution at 4℃ overnight as seen in Tables 2.6 and 2.8. After three to five washed with TBST, the blot was incubated with secondary antibodies IgG horseradish substrate with shaking for 1 h at room temperature, see Table 2.7. Blots were washed five times for 10 min, and signals were detected with chemiluminescent HRP substrate (BIO-RAD) and imaging system (Konica Minolta SRX-101A X-ray) using medical X-ray film (34089, thermo scientific).

For downstream signal analysis detected by western blotting, cells were pre-treated for 30 min in the case of primary and T-PLL cells, and for 24 h for Jurkat cell line with ITK inhibitors. The cells were then stimulated with 10 µg/ml plate-bound anti-CD3 (16-0038-

85, eBioscience, San Diego United States) and 1 µg/ml soluble anti-CD28 (16-0289-85, eBioscience) for 45 minutes. The blot was reprobed with blot stripping buffer (46430, thermo scientific) for additional primary antibodies and anti-GAPDH antibody as a loading control. Densitometry analysis was performed using ImageJ analysis software (National Institutes of Health). Raw density was normalized to the intensity of vehicle-treated and anti-CD3/anti-CD28 stimulated cells.

Target	MW (kDa)	Company	Reference number	Species reactivity	Dilution
ITK	72	abcam	ab32039	Human	1:1000
Phospho-BTK/ITK	75	eBioscience	14-9015-82	Human, Mouse	1:1000
PLCγ1	150	Cell Signaling Technology	5690	Human, Mouse, Rat, Monkey	1:1000
Phospho-PLCγ1	155	Cell Signaling Technology	2821	Human, Mouse, Rat	1:1000
AKT	60	Cell Signaling Technology	4691	Human, Mouse, Rat, Monkey, D. melanogaster	1:1000
Phospho-AKT	60	Cell Signaling Technology	4060	Human, Mouse, Rat, Hamster, Monkey, D. melanogaster, Zebrafish, Bovine	1:1000
MEK	45	Cell Signaling Technology	8727	Human, Mouse, Rat, Monkey, D. melanogaster	1:1000
Phospho-MEK	45	Cell Signaling Technology	9154	Human, Mouse, Rat, Monkey, D. melanogaster	1:1000
PARP	116, 89	Cell Signaling Technology	9532	Human, Mouse, Rat, Monkey	1:1000
Vav1	95	Cell Signaling Technology	D45G3	Human	1:1000
Phospho-Vav1	101	abcam	ab76225	Human	1:1000
PI3K	110	Cell Signaling Technology	5405	Human, Mouse	1:1000

ZAP70	70	Cell signal	3165	Human, Mouse	1:1000
GAPDH	37	Cell signal	14C10	Human, Mouse, Rat, Monkey, Bovine	1:10,000

Table 2.6. List of primary antibodies used for western blot**.**

Target	Company	Reference number	Dilution
Anti-rabbit IgG, HRP-linked antibody	Cell Signaling Technology	70745	1:2000
Anti-mouse IgG, HRP-linked antibody	Cell Signaling Technology	7076	1:2000
Anti-Goat IgG	Sigma-aldrich	A5420-1ML	1:2000

Table 2.7. List of secondary antibodies used for western blot**.**

Range of combination index	Component
PBS	PBS buffer (pH 7.4) was prepared by dissolving one tablet of PBS (Sigma Aldrich) in 100ml deionised water (ddH2O).
10x TBS	40g NaCl, 1g KCl, 15g Tris Base pH 7.4 for 500 mL
1x Wash Buffer	1x TBS, 0.05% Tween 20
1x Blocking Buffer	1x Wash Buffer, 5% Milk (w/v)
3X Loading Buffer	2.4ml 1M Tris, pH6.8, 3ml 20% SDS, 3ml Glycerol, 1.6ml β-mercaptoethanol, 600µl Bromophenol blue (BPB)
RIPA Buffer	150mM NaCl, 1% NP-40, 0.5% Na-Doc (Sodium Deoxycholate), 0.1% SDS, 50mM Tris, pH6.8
Buffers 5x Running Buffer	144g Glycine (1.92 M), 5g SDS (0.5%), 30g Tris Base (250 mM) for 1 Litre

Table 2.8. Buffers prepared in this study for immunoblotting analysis.

2.3 Flow Cytometry

2.3.1 Determination of apoptosis by flow cytometry

Jurkat, K299, CCRF CEM and MOLT-4 cells were treated with ITK inhibitors at the indicated concentrations for 24 h to induce apoptosis. Cell pellets were harvested at 200 xg for 5 minutes at room temperature, then the pellets were washed twice with cold PBS prior to analysis and resuspended with 100 µL annexin binding buffer and 5 µL annexi-V. The cells were incubated in 20 minutes at room temperature, and then 400 µL of 1 X annexin binding buffer was added to the cells and kept on ice. For apoptosis staining in

CD4 T cells, cells were stimulated with plate-bound anti-CD3 (10 µg/ml) and soluble anti-CD28 (1 µg/mL) at 37°C for 48 h in the presence of ITK compounds in RPMI media. Cells were stained with CD4 FITC (564419), CXCR5 PerCP-Cy5.5 (582781) and PD1 PE (557946) prior to analysis to purify Tfh and Non-Tfh subsets. Flow cytometric data were analysed with FlowJo software (vX.0.7). Apoptotic cells were subsequently determined using a FITC Annexin V Apoptosis Detection kit I (Bioscience) and Draq7TM (BioLegend). The acquisition and analysis of laboratory data report was automatically generated using BD FACSCanto^{TM} II. Cell apoptosis was also detected by using anti-PARP antibody as seen in section (2.6.2). PARP, a nuclear poly (ADP-ribose) polymerase, seems to be involved in a number of cellular processes such as, DNA repair in response to environmental stress and programmed cell death. It has been shown that PARP plays an important role in maintaining cell viability and serving as a marker of cells undergoing apoptosis as well as cleavage of PARP also facilitates cellular disassembly (Oliver *et al*., 1998).

2.3.2 Intracytoplasmic staining of cytokine analysis

$CD4^+$ T cells were isolated from human tonsillar cells by negative selection using a CD4+ T Cell Isolation Kit, as indicated in sections 2.1.5 and 2.1.6. Cells (1 x 106 cells/ml) were cultured in complete RPMI 1640 media under different conditions (as illustrated in table 2.9) in a flat-bottomed 96-well plate (Greiner Labortechnik, Germany) at 5×10^4 cells/100 µL. Cells were activated with 1 µg/mL plate-bound anti-CD3 and 3 µg/mL soluble anti-CD28 antibodies (eBioscience) or PMA (50 ng/mL) and ionomycin (1 µg/mL) in the absence and presence of 1 µM Ibrutinib and ONO-7790500 for 2 days.

Cell subsets	Cell different conditions	cytokine and transcription factor staining
Th1	10 ng/ml IL-12 (219-IL-005/CF),10 mg/ml anti–IL-4 (204-IL-010)	$CD4^+$ FITC (564419), IFN-γ APC (17-7319)
Th2	10 ng/ml IL-4 (204-IL-010), 10 mg/ml anti–IFN-γ (285-IF-100)	$CD4^+$ FITC, IL-4 APC (17-7049)
Th17	20 ng/ml IL6 (206-IL-010/CF), and 5 ng TGF-β1 (240-B-002/CF)	$CD4^+$ FITC, IL-17A APC (17-7179)
Treg	TGF-β1 (240-B-002/CF)	$CD4^+$ FITC, FOXP3 PE (12-4777), CD25 APC (17-0259)

Table 2.9. Subsets CD4+ T cell studied throughout this project**.**

Firstly, 50 µL of differentiated $CD4^+$ T cells (containing 1 x 10^6 cells/mL) was aliquoted into 50 µL of flow cytometry staining buffer (00-4222-26) containing antibodies in 1.5 ml eppendorf tube. 5 µL of each antibody (such as CD4 or CD25) was added to the staining buffer and pulse vortex gently to mix. Stained cells were incubated for 30-60 minutes at 2-8 °C or on ice in the dark place. Next, cells were washed 2X with 200 µL of FC staining buffer and centrifuged at 400-600 xg for 5 min at room temperature. After washing, the sample was pulse vortexed to completely dissociate the pellet. 1X Fixation/Permeabilization working solution (00-5223-56) was added to fix cells, pulse vortex and incubated for 30-60 minutes at room temperature in the dark. After washing with 1X Permeabilization buffer (00-833-56) and centrifugation, 2% normal mouse or rat serum was added to the cells and incubated 15 minutes at room temperature. Then, cells were stained with 5 µL fluorochrome-conjugated antibody (FOXP3, IL17A, IFN-γ or IL-4) in 100 µL 1X Permeabilization buffer. Finally, the cells were incubated for 30-60 minutes, then washed 2X with the buffer. The stained cells were resuspend in 500 µL of FC staining buffer and acquire data on a flow cytometry using BD FACSCantoTM II. The data were analysed with FlowJo software (vX.0.7).

2.4 Calcium flux assay

Intracellular calcium mobilisation was detected in Jurkat cells by using FluoForte Calcium Assay Kit (Enzo Life Sciences, Farmingdale, United States). Jurkat cells (1.25 x 10^5 cells/mL) were treated with IC50 of PF- 06465469 and Ibrutinib, BMS-509744 and ONO-7790500 for 4 hours at 37 ˚C in growth medium RPMI 1640 with 10% FCS. After the treatment of cells, the cells were centrifuged at 200 xg for 2 minutes. Then the cell pellets were re-suspended in 80 µl FluoForte dye-loading solution, and plated in 96-well black tissue culture plates (655086, Greiner Bio One, Kremsmünster, Austria). Cell plates were incubated for 45 minutes at 37˚C, then 15 minutes at room temperature before adding activation agents. Cells were activated by adding 5 µL Dynabeads Human T-Activator anti-CD3/CD28 beads (11132D, Gibco by life technology, Waltham, United States) in a final volume of 20 µL PBS and then activating by 1 µg of ionomycin (I3909, St. Louis, United States) in a final volume of 20 µl PBS. Fluorescence of the calcium flux was measured at Excitation = 490 nm/ Emission = 525 nm by using Infinite M200 PRO (Tecan, Männedorf, Switzerland). Before stimulation, Dynabeads were washed in buffer (containing phosphatase buffered saline and 0.1% bovine serum albumin with 2 mM

EDTA pH 7.4). The washed Dynabeads were then resuspended in the same volume of RPMI medium 1640 with 10% FCS and 2 mM L-glutamine.

2.5 Enzyme linked immunosorbent assay (ELISA) for cytokine measurement

Jurkat cells were cultured in RPMI 1640 plus 10% fetal calf serum containing 10 mL penicillin/streptomycin. Cells were incubated at 37°C for 20 hours in the absence or presence of 1 µg/mL phytohemagglutinin (PHA) (11082132001, Sigma-Aldrich) and 50 ng/ml phorbol 12-myristate 13-acetate (P1585, Sigma-Aldrich), and 2 µg/mL PHA and 50 ng/mL PMA for IL-21. For testing IL-21 in human tonsillar $CD4^+$ T cells, cells were stimulated with with 10 µg/mL plate-bound anti-CD3, 2 µg/mL soluble anti-CD28 (eBioscience) and 10 ng IL-12 (219-IL-005/CF). The stimulated cells then treated with IC50 of PF- 06465469, Ibrutinib, BMS-509744 and ONO-7790500 for 4 hours after changing half of the medium to fresh medium. Cell culture media was centrifuged at 2,000 xg for 10 minutes to remove debris. The curve was prepared by loading standard by preparing a fresh set of positive controls according to the manufacturer's instructions. After centrifugation, the clarified broth was tested for IL-2 and IL-21 production using an IL-2 (Interleukin 2) + IL-4 (Interleukin 4) Human SimpleStep (ab176137, abcam) and IL-21 (Interleukin 21) Human SimpleStep ELISA™ Kits (ab119542, abcam) according to the manufacturer's instructions.

2.6 CellTrace Violet Cell Proliferation Assay

Purified $CD4^+$ T cells were stained with CellTrace violet (CTV) at a concentration of 5 µM in pre-warmed PBS at 1 x 10^6 cells/mL, and incubated at 37 °C for 20 min. After incubation, stained cells were washed and cultured in a 96 well round bottom plate for 6 days in the presence of ITK inhibitor (1 µM) and DMSO. The cells were activated with (10 µg/mL) and soluble anti-CD28 (1 µg/mL) at 37°C with or without IL-12 (219-IL-005/CF) or PMA (50 ng/mL) and ionomycin (1 µg/mL). For identification of Tfh ($CD4^+$ $CXCR5^{high}$ $PD1^{high}$) and Non-Tfh ($CD4^+$ $CXCR5^-$ $PD1^-$) subpopulation groups, cells were stained for 10 minutes at 4°C or on ice for 30 minutes for surface markers with the following antibodies: FITC-anti-CD4 (564419), PE-anti-PD-1 (557946) and PerCP-Cy5.5-anti-CXCR5 (582781), all purchased from BD Biosciences. Isotype controls (PE Mouse IgG1 κ Isotype-555749, BB515 Mouse IgG1 κ Isotype-564416 and perCP-Cy5.5

mouse IgG1 κ Isotype- 552834) controls and Fluorescence Minus One (FMO) controls were used to set positive staining gates. Flow cytometric analysis was performed for proliferation using a BD FACSAria II, and analysis of cells was performed using FlowJo software (vX.0.7).

2.7 Chemotaxis Assay

Briefly, Jurkat and CD4 T cells were performed by using 24-well transwell chambers with 5-µm polycarbonate membrane (catalog no. 3421; Costar, Kennebunk, USA). The membrane was pre-coated with PBS containing 10 µg/mL Fibronectin (F1141-1MG, Sigma-Aldrich). Then, Jurkat and CD4 cells were pretreated with ITK inhibitors with 2 µM for 24 h and 1 h respectively in serum-free RPMI 1640 media. A chemotaxis medium with and without 100 ng/ml SDF-1α/CXCL12 (Catalogue number 350-NS-010, R&D Systems) was added in the lower chamber. The chemotaxis medium consists of RPMI 1640 medium containing 25 mM HEPES buffer and 1% BSA. Later, 100 µL cell suspension (1 x 10^6 cells/100 µL) were placed in the upper wells. The cells were allowed to migrate for 3 hours at 37 °C in 5% CO_2 and cells, which migrated cells across the membranes were collected in the lower compartment and counted by using haemocytometer or automated cell counter (TC20) based trypan blue staining (Bio-Rad).

Summary of protocol:

1. Reconstitute fibronectin with 1 ml sterile H_2O/mg of protein.
2. Dilute fibronectin in PBS, and coat 5 µm polycarbonate membrane surface with a minimum volume of RPMI containing fibronectin at 4°C overnight.
3. Wash twice with PBS and allow to air dry for at least 45 mins at room temp.
4. The chemotaxis (migration)/ binding medium (RPMI 1640 medium containing 1% BSA, 10 mM HEPES buffer, pH 6.7) with and without SDF-1α/CXCL12 is placed in the lower chamber of trasnwells. (Usually add 600 µL to the well for 24-well plate).
5. Place transwell insert in 24-well plate with sterile forceps (in Tissue Culture hood).
6. Place 50-100 µl of the cell suspension in chemotaxis medium (serum-free medium) in the upper wells.
7. After incubation at 37°C for 3 h in humidified air with 5% CO_2, migrated cells are collected and counted using a haemocytometer and Trypan Blue Solution.

2.8 Rho GTPase Activity Assays

To determine Rho, Rac, and Cdc42 activities, which are downstream of ITK, Jurkat and CD4 T cell lysates were analysed using a G-LISA activation assay (BK124, BK128, and BK127 G-LISA kits; Cytoskeleton). Briefly, Jurkat cells were serum starved for 24 h for Jurkat cells and 1 h for CD4 cells (so that basal Cdc42, Rac1 and RhoA activity is low prior to stimulation), in the absence and presence of ITK inhibitors at the concentrations of 3 and 6 µM. After incubation for 1 h, the cells were stimulated with 100 ng/mL CXCL12 for 5 min and the cell activation was stopped by washing the cells with cold PBS, and lysed in 200 µL of lysis buffer containing protease inhibitors. Rac1, Cdc42 and RhoA GTPase activities were measured by using Infinite M200 PRO (Tecan).

Summary of protocol:

Prepare lysate

1. Retrieve culture dish from incubator immediately and place on ice (aspirate off media).
2. Wash with a cold PBS (pH 7.2, filtered) to remove serum proteins (Aspirate off all PBS buffer completely.
3. Place lysates on ice 4°C.
4. Resuspend in amount of lysis buffer containing protease inhibitor (100 µL).
5. Immediately harvest cell lysates with a cell scraper.
6. Transfer lysate into pre-labelled and pre-chilled 1.5 mL Eppendorf on ice.
7. Immediately clarify lysates by centrifugation at 10,000 xg at 4˚C for 1 minute.
8. Measure lysate protein concentration and equalize all cell lysate to the optimal concentration [0.25-1.0 mg/mL for Cdc42 and Rac1, 0.4-2.0 mg/mL for RhoA].
9. Snap-freeze 120 µL aliquots of all cell lysate in liquid nitrogen after harvest and clarification. Store at -70˚C for no longer than 30 days.

Measure lysate protein

1. 20 µL lysate + 1 mL Precision Red Advanced Protein Assay Reagent.
2. Incubate 1 min at room temp.
3. Blank spectrophotometer with lysis buffer at 600 nm.
4. Read absorbance of lysate samples.
5. Multiply the absorbance by 5 to obtain protein concentration in mg/mL.

6. Calculate how much ice-cold lysis buffer is needed to equalize the cells conc. (0.4 mg/mL Cdc42, 0.6 mg/mL Rac1 and RhoA).

A-B/B x volume of A= µL

[A is the higher conc. mg/mL; B is the conc. of the dilute sample mg/mL]

Assay procedure

1) 120 µL of Lysis buffer was aliquoted into a labelled 1.5 ml Eppendorf as a blank control.
2) Positive control was prepared (36 µL of Rac1 control protein plus 84 µL lysis buffer in 1.5 ml labelled Eppendorf, 12 µL of Rho control protein plus 48 µL cell lysis buffer with 60 binding buffer, 24 µL of Cdc42 control protein mixed with 96 µL 0f lysis buffer)
3) The plate of assays was washed with 100 µL ice-cold water to dissolve the powder in the wells.
4) After completely removing the water, 50 µL of blank buffer, positive controls and cell lysates were added to wells in replicate, and placed the plate on microplate shaker (200-400 rpm) at 4°C for 30 min.
5) Primary antibodies were prepared in antibody dilution buffer (dilute ant-Rac1 antibody to 1/50, anti-RhoA antibody to 1/250, anti-Cdc42 antibody to 1/20 in the buffer).
6) After washing the plates, 200 µL antigen presenting buffer was placed into each wells and incubated at room temperature for 2 minutes.
7) Wash the wells 3x with 200 µL of wash buffer, removing wash buffer.
8) 50 µl of diluted anti-Rac1 (1/50), anti-RhoA (1/25) and anti-Cdc42 (1/20) primary antibodies were added to each well and the plates left on the microplate shaker (200-400 rpm) at room temperature for 45 minutes.
9) Wash 3x with 200 µL of wash buffer.
10) Add 50 µL of diluted secondary HRP labelled antibody to each well and place the plate on a shaker (200-400 rpm) at room temperature for 45 minutes.
11) After washing the wells 3x with 200 µL of wash buffer, 50 µL of the mixed HRP (secondary antibody) detection reagent was pipetted into each well and incubate at room temp for 20 minutes.
12) Finally, 50 µL of stop buffer was placed in each well (free of bubbles), the signal was read by measuring absorbance at 490 nm using a microplate spectrophotometer (Tecan, Männedorf, Switzerland).

2.9 TCRβ Gene Clonality Assay

DNA extraction:

Genomic DNA were extracted from purified $CD4^+$ T-cells from patients with T cell lymphoma using QIAamp DNA Mini Kit (51304, Qiagen, Hilden, Germany), and resuspended DNA to a final concentration 10 µg in 1X TE buffer (1 mM Tris-HCl, pH 8.0; 0.1 mM EDTA).

Amplification for T cell clonality:

PCR assay was used for identification of clonal T-cell populations in patients. Samples were amplified using the following PCR program by PCR Thermal Cycler (Applied Biosystems) using TCRβ Gene Clonality Assay (9-205-0011, Invivoscribe Technologies, San Diego, USA). PCR conditions for AmpliTaq Gold DNA Polymerase (4398813, thermofisher) are as follows:

Step 1: 95°C for 7 minutes
Step 2: 95°C for 45 seconds
Step 3: 60°C for 45 seconds
Step 4: 72°C for 90 seconds
Step 5: Go to step 2; 34 more times
Step 6: 72°C for 10 minutes
Step 7: 15°C forever

Reagent Preparation

TCRβ clonality kit contains four master mixes including TCRβ Tube A and B target "the framework regions" within variable (V) and joining (J) region of the TCR beta chain locus (Table 2.10). TCRβ Tube C targets the diversity (D) and joining (J) regions (Figure 2.2). Additionally, a master mix, the Specimen Control Size Ladder, targets multiple genes to generate series of amplified products (amplicons) of approximately 100, 200, 300, 400 and 600 basepairs.

1) 45 µL each master was aliquoted to individual microcentrifuge tubes.
   For TCRB Tubes A (Vβ + Jβ1/2) **and B (**Vβ + Jβ2)**:** add 0.45 µL at 5 units/µL of AmpliTaq DNA polymerase per reaction to each master mix.

For TCRB Tube C (Dβ + Jβ1/2) **and Specimen Control Size Ladder:** add 0.25 µl at 5 units/µL of AmpliTaq DNA polymerase per reaction. Then gently vortex to mix.

2) Aliquot 45 µL of the appropriate master mix plus AmpliTaq DNA polymerase solution was added into individual wells in a PCR tube for each reaction.
3) For sample DNA, negative control DNA (IVS-0000 Polyclonal Control DNA), positive control DNA (IVS-0009 Clonal Control DNA), or no template (water), 5 µL of appropriate template was added to each wells which contain the respective master mix solutions. Total reaction volume is 50 µL.

Catalog #	Description
2-205-0011	TCRB Tube A – 6FAM & HEX
2-205-0021	TCRB Tube B – 6FAM
2-205-0031	TCRB Tube C – 6FAM & HEX
2-096-0021	Specimen Control Size Ladder – 6FAM
4-088-0490	IVS-0009 Clonal Control DNA
4-088-0190	IVS-0004 Clonal Control DNA
4-092-0010	IVS-0000 Polyclonal Control DNA

Table 2.10. Materials (primers, positive and negative controls) provided with TCRβ clonality assay.

ABI Fluorescence Detection

0.5 µL of Red DNA size standard-fluorescence-labelled with carboxy-x-rhodamine (Rox dye) (DSMR-100, MCLAB, South San Francisco, USA) was mixed with 15 µL of Hi-Di Formamide (4311320) per well for each PCR reaction. 1 µL of each PCR reaction was transferred to the wells containing Red DNA size standard plus Hi-Di Formamide in 96-well plates (4483485, Applied Biosystems). The PCR plates were covered and the samples processed to heat denature at 95ºC for 2 minutes and chill on ice for 5 minutes before analysis by GeneScan. T-cell clonal expansion was detected by analysis of TCRβ gene rearrangement. TCRβ T-cell clonality was assayed at Vβ + Jβ1/2, Vβ + Jβ2, Dβ + Jβ1/2 and Multiple Genes (Specimen Control Size Ladder). T-cell clonality of fluorochrome-labelled PCR products was analysed by capillary electrophoresis using GeneScan-ABI 3100 (Applied Biosystems).

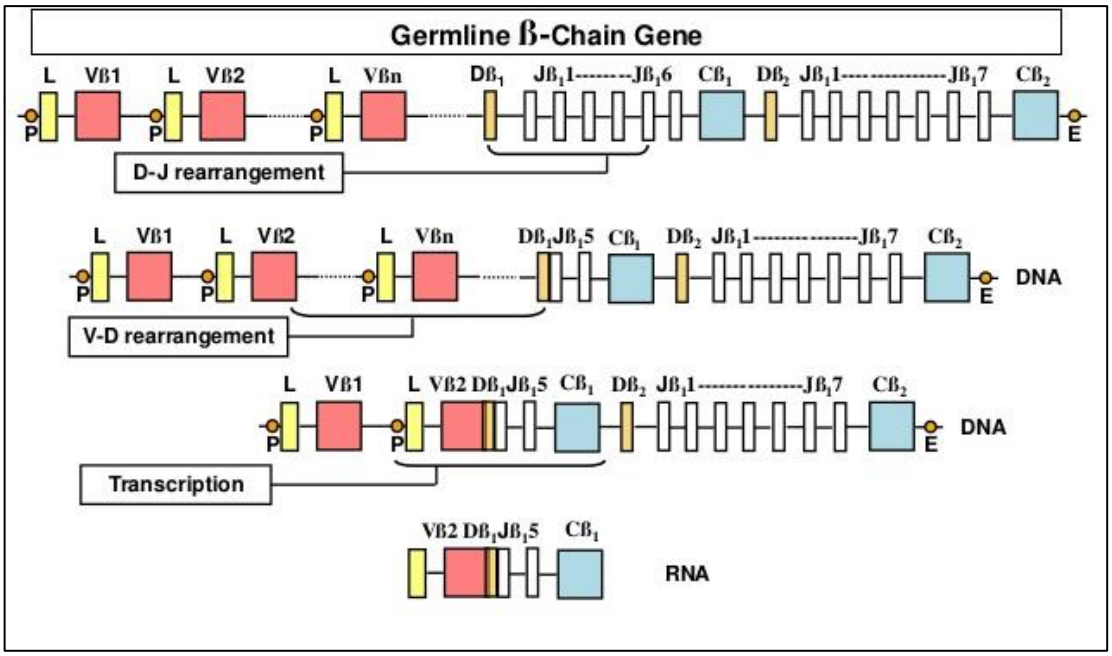


Figure 2.2. Organisation and rearrangement of the T cell receptor β (TCRβ)-chain genes (Gene and Jennifer, 2017). The TCRβ-chain gene consists of discrete segments that are joined via somatic recombination during T-cell development. TCRβ is composed of a V and a C region, and it also contains D gene segments and J gene segments. For the β chain, the variable domain is encoded in three gene segments including V_β, D_β and J_β. A functional VDJ_β V-region exon is generated from rearrangement of these gene segments. The exon is transcribed and spliced to join to C_β, and the resulting mRNA is translated to yield the TCRβ chain. Type of gene segment: V, variable; D, diversity; J, joining; C, constant.

2.10 Agarose gel electrophoresis

Gels were casted with 1% agarose (BP160-500, fisher scientific, Hampton, New Hampshire, USA) in tris-acetate EDTA (TAE: 2 M Tris, 50 mM EDTA, 57.1% (v/v) glacial acetic acid) containing Syber Safe DNA gel stain (S33102-Invitrogen, Carlsbad, California, USA). Agarose gels were run in gel electrophoresis (Bio-Rad) with TAE buffer at 120 V. DNA samples (see section 2.9) were mixed DNA loading dye (R0611, thermofisher scientific) before gel electrophoresis and loaded alongside Hyper ladder (DNA ladder) 1 kb (H1-315106, Bioline, London, UK). Pictures were acquired with Bio-Rad Gel Doc (Bio-Rad).

2.11 Statistics

Statistical analyses were undertaken using the GraphPad Prism version 7.00 (GraphPad Software, San Diego, CA, USA). A two-tailed student's *t*-test was performed to determine statistical differences among sample groups. Due to the sample groups are unpaired, an unpaired *t*-test using non-parametric test was applied for comparison between unpaired samples such as the comparison between unstimulated and stimulated or stimulated samples with DMSO/ drug treatment groups. All experiments were performed in duplicate/ triplicate, and the data were expressed as mean ± SEM. The p-value was considered statistically significant if $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and NS represents statistically non-significant.

Chapter III. Role of ITK and characterisation of ITK inhibitors in T cell lymphoma cell lines

3.1 Introduction

Activation of T cells depends on T cell receptor (TCR) and chemokine receptor-mediated signalling (Berg *et al*., 2005, Schwartzberg *et al*., 2005; Kumar *et al*., 2006; Soldevila and García-Zepeda, 2003; Luther and Cyster, 2001), therefore, agents that can interrupt these pathways may produce significant clinical benefit. Given that ITK is a key component of both TCR and CXCR4 signalling, ITK inhibitors are likely to provide useful small-molecule tools to impact T-cell lymphomas, which have functional TCR and chemokine signalling pathways.

Over the past decade, considerable efforts have been made to develop small molecule inhibitors against ITK to treat autoimmunity or allergy/hypersensitivity (Vargas *et al*., 2013). In this research, four different ITK inhibitors - PF-06465469, Ibrutinib, BMS509744 and ONO-7790500 have been compared side-by-side. The first three inhibitors are commercially available; these compounds were found to be potent, with an IC50 (for the purified enzyme) of 2 nM for PF-06465469 (Zapf *et al*., 2012), 2.2 nM for Ibrutinib (Dubovsky *et al*., 2013) and 19 nM for BMS509744 (Das *et al*., 2006). ONO-7790500 is a new class of ITK inhibitor provided by ONO Pharmaceutical company. It showed a high selectivity and potency against ITK with an IC50 0.4 nM (ONO Pharmaceutical company, Osaka, Japan). The chemical structure and formula of all four ITK inhibitors are demonstrated in the Table (2.3).

In this chapter, following an analysis of ITK knockdown in a T-cell line, the effects of small molecule ITK inhibitors (Table 2.3) will be determined in T cell lymphoma cell lines, normal primary human tonsillar T-cells and primary human T-cell leukaemia. Effects on proliferation and apoptosis, and their effects on T cell receptor (TCR) and chemokine receptor-mediated signalling will be described. Finally, a model of ITK action that integrates some novel findings with respect to TCR and chemokine signalling will be presented.

3.2 Results

3.2.1 Expression of ITK in T cell lymphoma/leukaemia cell lines, primary $CD4^+$ T cells and T-cell Prolymphocytic Leukaemia (T-PLL) cells.

ITK expression in four different T cell lymphoma/leukaemia cell lines - Jurkat, MOLT-4, CCRF CEM and K299 was examined in this study (see M and M, section 2.3.3). Total RNA from cells was interrogated for *ITK* and *HPRT* mRNA by using semi-quantitative RT-PCR. The data showed that *ITK* was expressed at very variable levels in these cell lines, as shown in Figure 3.1A. *ITK* expression was higher in MOLT-4 and Jurkat cells compared to CCRF CEM cells, whereas expression was essentially undetectable in K299 cells, and their Ct values are demonstrated in Supplemental Figure A. Therefore, of the four cell lines chosen for this study two, Jurkat and MOLT-4, have high-levels of *ITK* expression, one has a middling expression (CCRF CEM) and one (K299) has low/undetectable expression. It is worth mentioning that Ramos cells (a Burkitt lymphoma cell line) and HEK293 cells (a human embryonic kidney cell line) that contain no *ITK* were used as a negative control.

In line with the RT-PCR results, Western blots showed high ITK protein expression in Jurkat, MOLT-4 and CCRF CEM cells and undetectable levels in K299 (Figure 3.1B and C). Therefore, there seems to be a reasonable correlation between mRNA and protein levels in the cell lines selected for this study although some differences in post-transcriptional regulation cannot be excluded.

RLK is a TEC kinase related to ITK in structure and the known ITK inhibitors may also inhibit RLK (Zhong *et al*., 2014). Effects on RLK might, therefore, be confounding factors in functional assays. To quantify *RLK* expression levels, quantitative RT-PCR was performed on cDNA from the cell lines. The level of *RLK* mRNA expression is ~100-fold lower than that of *ITK* in Jurkat, MOLT-4 and CCRF CEM cells, but is ~50-fold higher in K299, see Figure 3.1A.

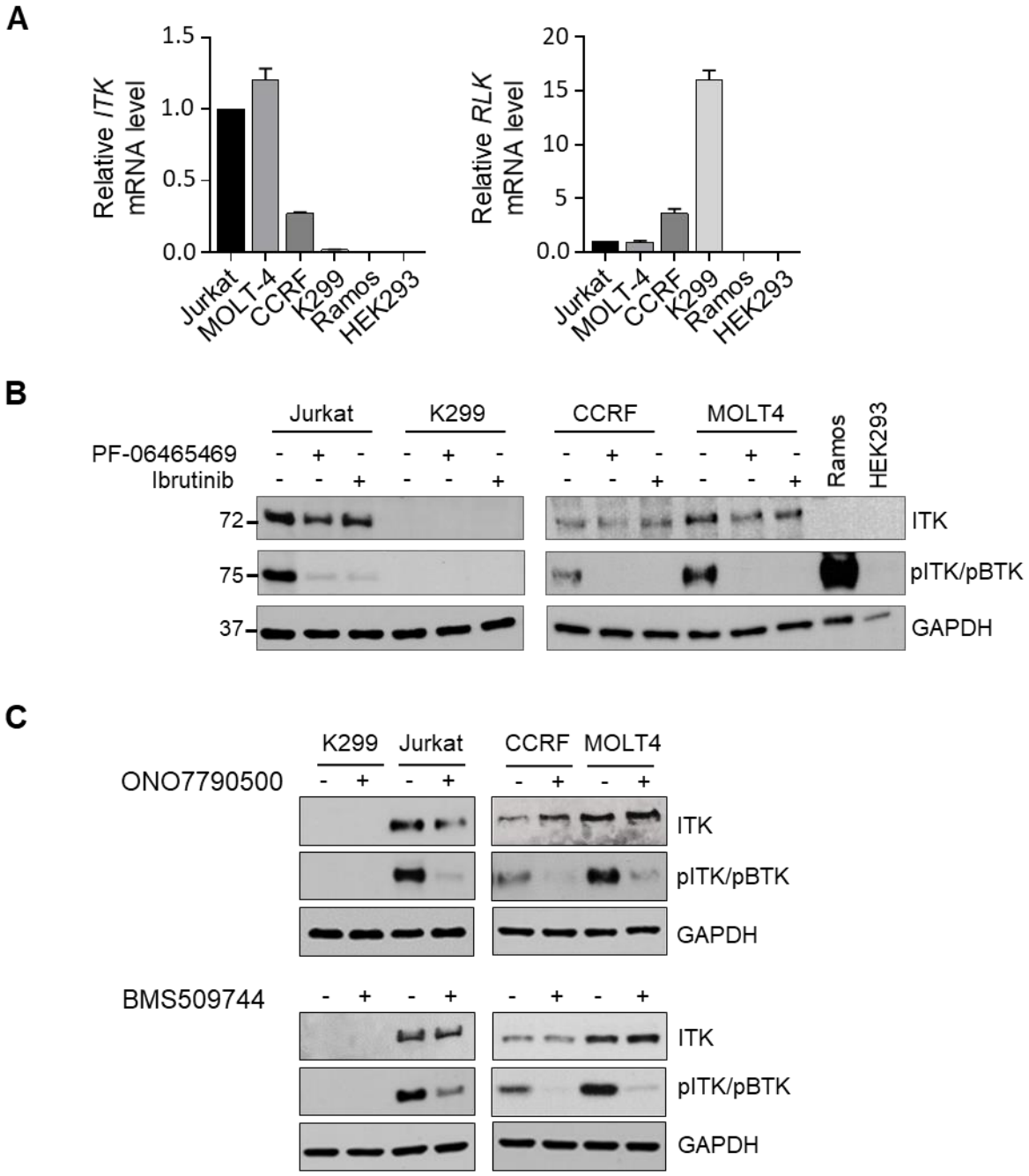


Figure 3.1. Expression profile of ITK in Jurkat, MOLT-4, CCRF CEM and K299 cells, and Inhibition of ITK phosphorylation in Jurkat cells with all four ITK inhibitors. (A), total RNA from cells was interrogated for *ITK*, *RLK* and *HPRT* mRNA by using semi-quantitative RT-PCR. *HPRT* (housekeeping gene) was used as a control. (B and C), Immunoblot analysis of lysates from MOLT-4, Jurkat, CCRF CEM and K299 cells was performed to measure the expression of ITK and phospho-ITK protein. phospho-ITK expression was determined after treatment with a concentration of drug close to the IC50 (see Table 3.1) for 24 hours. Ramos and HEK293 cell lines were used as negative controls. Blots were probed with anti-phospho-ITK/BTK and anti-ITK, and anti-GAPDH as a loading control. The results presented are representative of three experiments.

mRNA expression of *ITK* and *RLK* was examined in leukemic T-cells from T-cell Prolymphocytic Leukaemia (T-PLL) patients and CD4 T cells from human tonsil to explore *ITK* and *RLK* expression in primary cells. T-PLL patient cells (n=11) and CD4 T cells (n=3) had similar (indistinguishable) *ITK* mRNA. Levels of *ITK* mRNA are ~20-fold higher than *RLK* levels in primary CD4+ T cells and T-PLL cells (Figure 3.2A and B), suggesting ITK is an important component of signalling pathways in T lymphocytes and may be a potentially useful target for antitumour therapy. ITK, therefore, appears to be a predominant TEC kinase in primary T-cells (both normal and malignant).

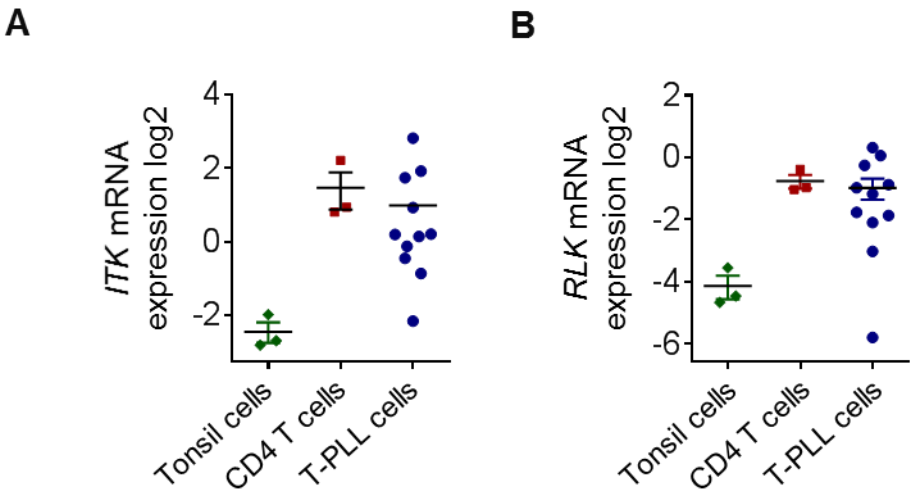


Figure 3.2. Expression profile of ITK in primary T-cell-prolymphocytic leukaemia (T-PLL), human tonsil cells and purified CD4 T cells from human tonsil. (A and B), total RNA from cells was interrogated for *ITK*, *RLK* and *HPRT* mRNA by using semi-quantitative RT-PCR. The figures represent a relative *ITK* or *RLK* mRNA level. *HPRT* expression was performed as control. Log2 expression levels are shown.

3.2.2 Function of ITK in cell lines: Silencing of endogenous ITK expression by short interfering RNA (siRNA)

The efficiency of siRNA transfection in Jurkat cells was optimised using a Block-iT^{TM} Fluorescence Oligo with DharmaFECT 1 transfection reagent. The transfection efficiency was approximately 60% (supplemental Figure B). To investigate whether ITK protein knockdown in malignant T cell lines leads to impaired T cell function and activation, Jurkat cells were transfected with two *ITK* specific siRNA's (s223953 and s223954) either separately or together. Mock and negative siRNA were performed as controls. Immunoblot analysis shows that siRNAs s223953 and s223954 were efficient in mediating ITK protein knockdown (Figure 3.3).

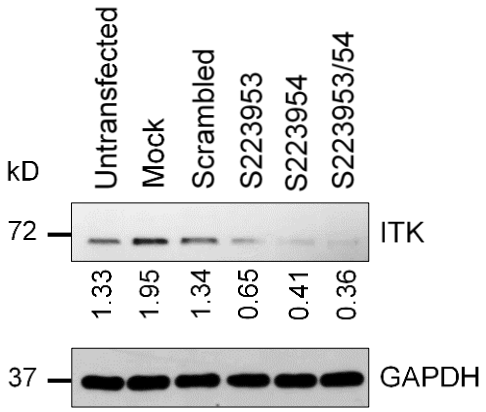


Figure 3.3. Expression of ITK in siRNA-induced silencing of human ITK in Jurkat cells. Jurkat cells were transfected overnight with 100 nM siRNA specific ITK (s223953 and s223954), see section 2.1.7 in mthods and materials. ITK expression was determined using an ITK-specific antibody in western blotting. GAPDH was used as loading control.

3.2.2.1 Cell viability

After ITK expression was examined in a panel of T-cell lymphoma/leukaemia cell lines, the effect of ITK was determined on cell viability. Jurkat cells were transfected with ITK-specific siRNA and the viability was evaluated using the CellTiter-Glo® luminescent cell viability assay. Figure 3.4A shows that Jurkat T cells lacking ITK exhibited a significant reduction in cell viability compared to scramble siRNA control after 24 h. This data suggests that ITK plays an essential role in viability of T cell lymphoma cells and may be a promising target for treatment of T cell lymphomas.

IL-2 plays an important role in maintaining normal proliferation of T-cells and can promote T-cell survival. T-cells require cytokines including IL-2 along with calcium signaling and NFAT activation for their development and differentiation (Schaeffer et al., 1999; Schaeffer et al., 2001; Fowell et al., 1999; Schwartzberg et al., 2005; Berg et al., 2007; Prince et al., 2009; Hu et al., 2008). Additionally, IL-2 family cytokines have been shown to have a critical role in regulating T-cell apoptosis (Kelly et al., 2002). In the absence of Itk, the development of CD4+ and CD8+ are altered and T-cell lacking Itk was linked to reduced IL-2 secretion (Liao and Littman, 1995; Liu et al., 1998). Due to ITK positively regulates IL-2 production and calcium signaling machinery (Figures 3.5 and 3.6), therefore, the reduction in Jurkat cell viability caused by the knockdown of ITK can be linked to cytokine secretion and calcium signaling.

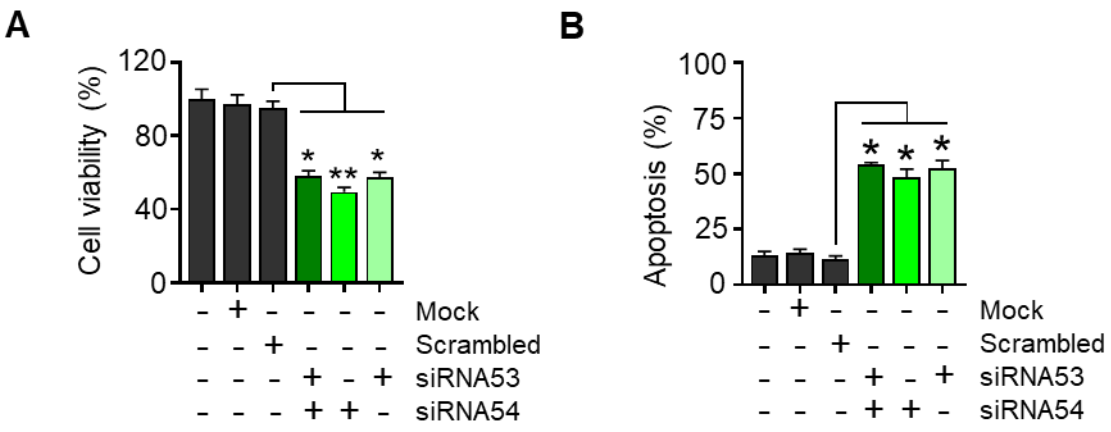


Figure 3.4. Silencing of ITK via siRNA causes reduction of viability and induction of apoptosis in Jurkat T cells. Jurkat cells were transfected using 100 nM siRNA specific ITK (s223953 and s223954). Untransfected cells, Mock and negative siRNA were performed as controls. (A) After transfection, cell viability was determined using CTG luminescent cell viability assay. (B) Cell apoptosis was measured using FITC-annexin V and Draq7. Data shows mean ± SEM and P-values were calculated by using a two-tailed, unpaired t-test, $*P < 0.05$, $**P < 0.01$.

3.2.2.2 Apoptosis

Apoptosis, or programmed cell death, is required for normal development and aberrant apoptosis is one of the mechanisms driving carcinogenesis. Following disruption of the outer mitochondrial membrane and release of cytochrome c into the cytoplasm, the membrane phospholipid phosphatidyl serine (PS) is translocated from the inner leaflet to the outside of the plasma membrane. Annexin V is a calcium-dependent phospholipid-binding agent, which is conjugated to fluorochromes and has a high affinity for PS. Therefore, it is a sensitive probe for detection of apoptosis analysis by flow cytometry. The objective of using Annexin V FITC assay is to detect whether or not ITK inhibitors induced apoptosis in malignant T cell lines.

To confirm that T cells undergo apoptosis in response to ITK knockdown, Jurkat cells were treated with *ITK* specific siRNA. We examined apoptosis in this cell line using Annexin V/Draq7. After transfection, a significantly high percentage of apoptosis (approximately 50%) was observed in *ITK* siRNA treated cells than in mock and scramble treated cells (Figure 3.4B). Together these data show that ITK regulates an anti-apoptotic program in T cell lymphoma/leukaemia cells and block of ITK leads to induction of apoptosis.

3.2.2.3 Calcium Signalling

CD3-TCR complex signalling has been shown to stimulate an increase in calcium (Ca^{2+}) resulting from pre-treatment of T cells with an anti-CD3 antibody (Wacholtz and Lipsky, 1993). According to Feski *et al*. (2001), Ca^{2+} is essential for the regulation of many T cell genes including cytokines. Previous data demonstrated that elevation of intracellular calcium is crucial for NFAT activation (Timmerman *et al*., 1996), and production of IL-2 (Wacholtz and Lipsky, 1993; Wülfing *et al*., 1997). Studies showed that IP3 plays an important role in inducing calcium mobilization from intracellular and extracellular stores (Berridge,1984; Kuno and Gardner, 1987), resulting in increased free calcium. Calcium ionophores have been demonstrated to mimic the effects of IP3. Ionomycin is a selective and potent ionophore agent (Liu and Hermann, 1978; Kauffman, 1980), and acts as a motile calcium carrier and induces calcium influx via stimulating store-regulated cation entry across the plasma membrane (Morgan, 1994). Lobo (1999) indicated that ionomycin at the micromolar level can activate calcium, calmodulin dependent kinase and phosphatase in order to stimulate gene expression. Further studies showed this agent can induce hydrolysis of phosphoinositides in human T cells and activates Protein Kinase C (PKC) to mediate T cell activation (Chatila, 1989).

The effects of ITK knockdown on Ca^{2+} were then determined. The result revealed that both siRNA alone and in combination inhibited Ca^{2+} flux compared with untransfected cells and controls (mock transfected and transfected with scramble siRNA control). However, an increase of calcium was observed in untransfected cells stimulated with anti-CD3/CD28, (Figure 3.5). The result also showed that no reduction of calcium flux was observed in Jurkat cells after applying ionomycin due to ionomycin is bypassing ITK.

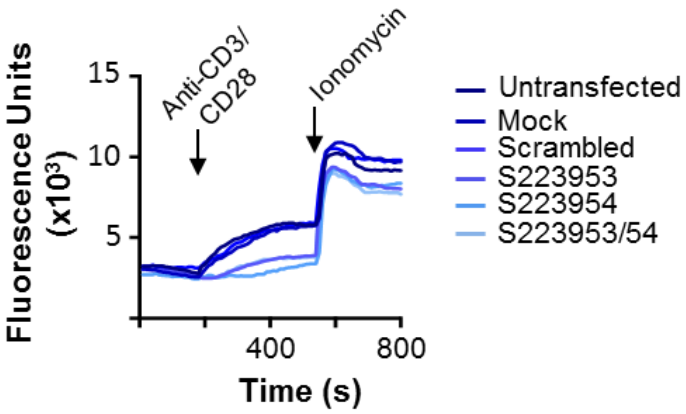


Figure 3.5. Silencing of ITK blocks calcium mobilization in response to TCR signalling in Jurkat cells. Cells were transfected with siRNA specific for ITK. Cells were stimulated with antiCD3/CD28 and 1.5 μM ionomycin. These experiments were repeated twice. Calcium flux was detected using FluoForte Calcium Assay.

3.2.2.4 IL2 Production

To study whether ITK knockdown in Jurkat cells leads to impaired cell activation and function in a manner comparable to cells treated with ITK inhibitors, Jurkat cells were activated with 1 μg/ml PHA and 50 ng/ml PMA for 24 h. The cells then were transfected with s223953 and s223954. As seen in Figure 3.6, ITK knockdown inhibited IL-2 production in response to PHA/PMA. Interestingly, the observed reduction in IL-2 production with siRNA knockdown is less than that seen in cells treated with the ITK inhibitors. This may be either because the knockdown was not complete or because of "off-target" effects by the inhibitors.

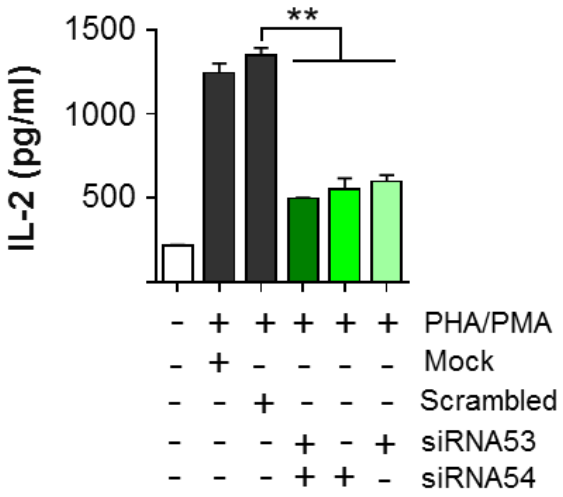


Figure 3.6. Silencing of human ITK by siRNA abrogates IL-2 secretion. (a) Jurkat cells were transfected using siRNA specific ITK (s223953 and s223954) and DharmaFECT 1 transfection reagent. After overnight post-transfection, cells were activated with PMA/PHA for 24 h, and then IL-2 in the supernatants was determined by ELISA. Data was shown as mean ± SEM. Significance was measured by two-tailed P value with unpaired t test $^{**}P < 0.01$.

3.2.2.5 Activation of RHO GTPases

The Rho family of small GTP-binding proteins belongs to the subgroup of the Ras superfamily of small GTP-binding proteins (GTPases) and comprises 23 members. Among the 23 Rho family members in the human genome, the best studied Rho proteins are Rac1, Cdc42 and RhoA (Rathinam *et al*., 2011). Rho family GTPases are essential intracellular signalling proteins that regulate proliferation and motility and diverse cellular functions relevant to cancer, including tissue invasion, actin cytoskeletal organization, transcriptional regulation, vesicle trafficking, cell cycle progression, apoptosis, epithelial cell polarity, cell survival and angiogenesis (Ridley, 2006; Mack *et al*., 2011). Consequently, they have been implicated in tumour progression and progression of other diseases by many studies (Lazer and Katzav, 2011; Parri and Chiarugi, 2010; Mulloy *et al*., 2010; Pai *et al*., 2010; Vega and Ridley, 2008; Tang *et al*., 2008; Karlsson *et al*., 2009).

Previous data showed that ITK is required for activation of Rac and Cdc42 (Takesono *et al*., 2004). To investigate the effects of ITK on the Rho family of GTPases (RhoA, Rac1, and Cdc42) in response to the chemokine CXCL12, Jurkat cells were transfected with siRNA specific ITK (s223953 and s223954). siRNA knockdown of ITK blocked RAC1 and Cdc42 activity and partially inhibited CXCL12 induced activation of Rac and Cdc42. However, RhoA activity was not significantly reduced (Figure 3.7).

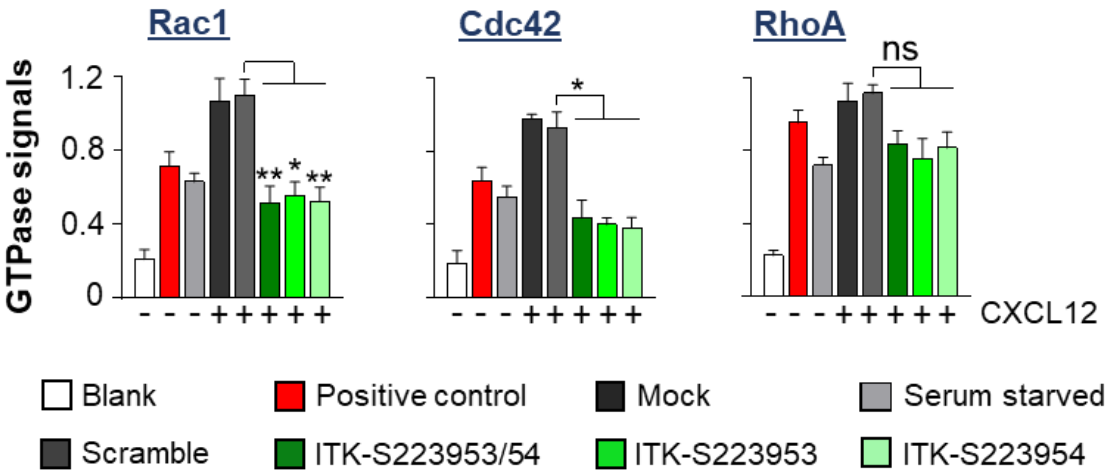


Figure 3.7. siRNAs induced silencing of human specific ITK abolishes the activity of Rho GTPase, Rac1 and Cdc42. siRNAs were transfected into Jurkat cells 100 nM siRNA specific ITK (s223953 and s223954) and DharmaFECT 1 transfection reagent. Following overnight post-transfection in 96-well plate, cells were stimulated with CXCL12 for 5 min at room temperature, then Rho, Rac, and Cdc42 activities were analysed from Jurkat cell lysates by using a G-LISA activation assay. All experiments were repeated in duplicate/triplicate, and the data reported as mean ± SEM. Significance was determined by two-tailed P value with unpaired t test $*P < 0.05$, $**P < 0.01$ and NS represents Not Significant.

3.2.3 Potency and selectivity of ITK inhibitors in malignant T cells

3.3.3.1 ITK inhibitors reduce the proliferation of T cells *in vitro*

Ibrutinib is a small molecule inhibitor of the TEC family kinase, BTK, which has found clinical use in the treatment of B-cell lymphoproliferative disorders. ITK and BTK have structurally similar active sites. Dubovsky *et al*., (2013) demonstrate that Ibrutinib is an irreversible ITK inhibitor, and pre-clinical work demonstrates that it has potential for the treatment of parasitic infections and other infectious diseases such as *Listeria monocytogenes*. Furthermore, the ITK inhibitor CTA056 has been suggested to induce apoptosis in T-cell malignancies (Guo *et al*., 2012).

	ITK inhibitors IC50 (μM)				
Cell lines		PF06465469	Ibrutinib	ONO7790500	BMS509744
	Jurkat	6.2	5.22	2.6	2.2
	MOLT-4	8.8	11.1	4.6	4.6
	CCRF CEM	10.1	15.1	5.1	2.5
	K299	15.27	22	12	9

Table 3.1. This table shows IC50 (EC50) values of all four ITK inhibitors**.** ITK inhibitors were tested in a flat-bottomed 96-well plate using the CellTiter-Glo (CTG) luminescent cell viability assay. All inhibitors were tested against ITK, and IC50 values of the inhibitors (for 48 hours) were calculated from the concentration versus the percentage viability as a function of inhibitor concentration, see Figure 3.8.

Cells were incubated with PF-06465469, Ibrutinib, BMS509744 and ONO-7790500 to determine the effect of the drugs on viability, which was assessed using the CellTiter-Glo® luminescent cell viability assay. The results revealed that the Jurkat cell line was most sensitive to PF-06465469 at 6.2 μM, Ibrutinib at 5.2 μM, BMS509744 at 2.2 μM and ONO-7790500 at 2.6 μM *in vitro* as seen in Figure 3.8. These drugs were effective at inhibiting the growth of MOLT-4 at 8.8 μM PF-06465469, 11.1 μM Ibrutinib, 4.6 μM ONO-7790500 and μM 4.6 BMS509744, and CCRF CEM cells at 10.1 μM, 15.1 μM, 5.1 μM and 2.1 μM for PF-06465469, Ibrutinib, BMS509744 and ONO-7790500 respectively (Table 3.1). By comparison, K299 cells were insensitive to all four drugs *in vitro* in a dose and time-dependent function. This is most likely due to the low ITK expression in K299 cells, see Figure 3.1A, B and C. It is worth noting that there is, to some extent, an effect of ITK inhibitors on the viability of K299 cells. One possibility for this effect is that the inhibitors might be active against RLK in the K299 cell line which expresses a higher level of RLK than other cell lines in this research.

Moreover, the inhibitory effect of the inhibitors on all malignant T cell lines was examined at different time points (24, 48 and 72 h). As expected, all agents showed highest growth inhibition to the cells after 72 h as seen in figure 3.3. This data suggests that there is a strong correlation between malignant T cell viability *in vitro* and ITK inhibition. The data further strongly suggests that the *in vivo* targeting of Itk is needed for the growth inhibition in T-cell malignancies.

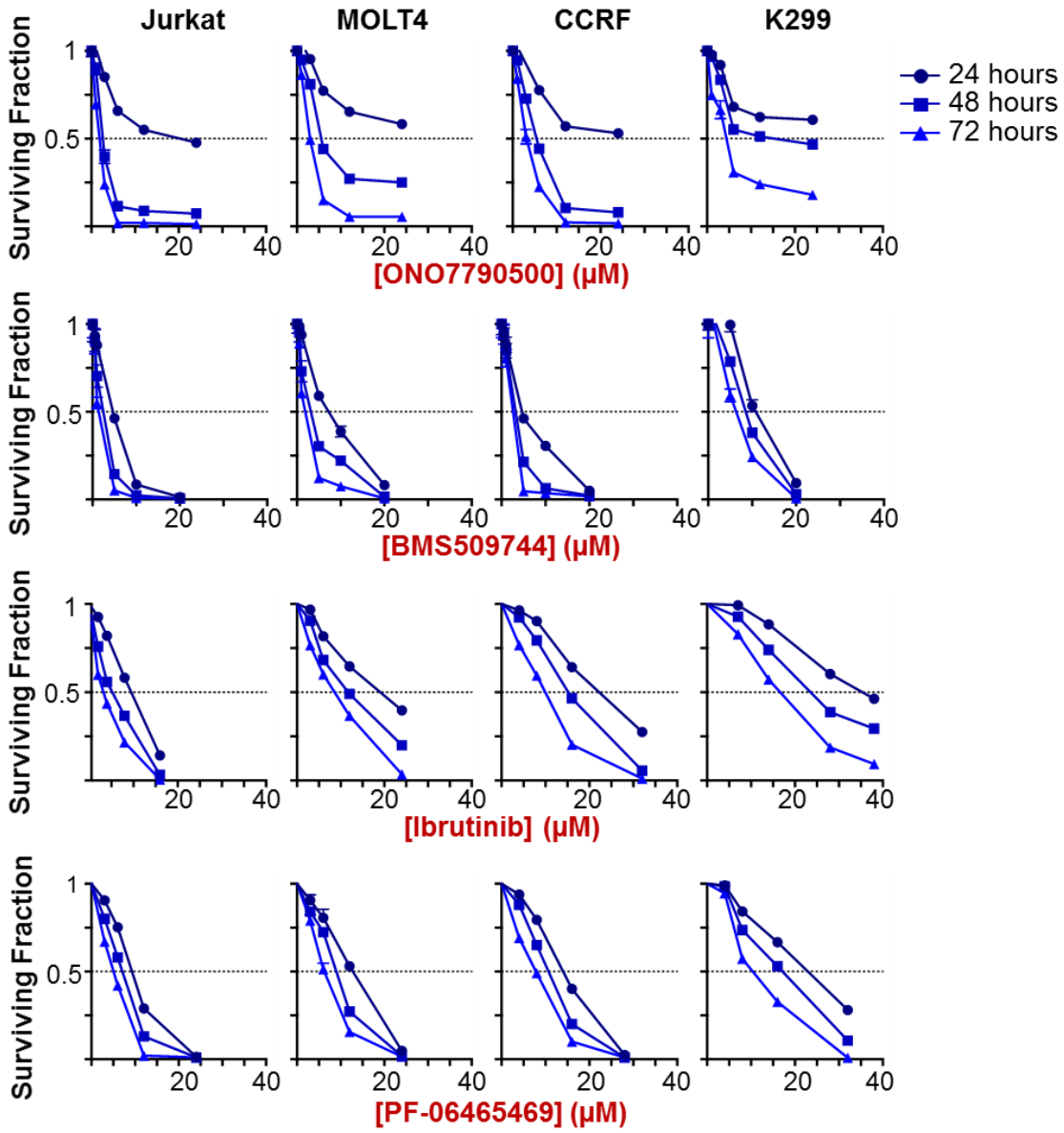


Figure 3.8. Effect of ITK inhibitors on the viability of Jurkat, MOLT-4, CCRF CEM and K299 cell lines. Cell viability was assayed following treatment of cell lines with PF-06465469, Ibrutinib, BMS509744 and ONO-7790500 for different time points, and cell viability was evaluated by using the CellTiter-Glo® luminescent cell viability assay. The graphs present that Jurkat cells were more sensitive to the ITK inhibitors than MOLT-4 and CCRF CEM cells, whereas K299 cells are more resistant to the inhibitors in particular with Ibrutinib and ONO-7790500 compared with other cell lines *in vitro* experiments. Cells were treated for 24, 48 and 72 h with indicated concentrations of the drugs. The experiments were repeated in triplicate.

To confirm the specificity of ITK inhibitors, non-T cell cancer cell lines H460 (a lung cancer cell line), Ramos (a Burkitt lymphoma line) and SUDHL6 (a diffuse large B-cell lymphoma cell line) were treated with all ITK inhibitors for 48 hours as indicated in Supplemental Figure C and cell viability was determined by CTG assay. Interestingly, BMS509744 and ONO-7790500 were less active against B cells, supporting their greater specificity as compared to PF-06465469 and Ibrutinib. H460 cells seem to be more resistant to ONO-7790500 and Ibrutinib than PF-06465469 and BMS509744, suggesting that both PF-06465469 and BMS509744 might target additional kinase in this cell line.

3.3.3.2 ITK inhibitors induces apoptosis in T-cell lymphoma cell lines

In line with the siRNA results, flow cytometry was used to characterise the growth inhibition induced apoptosis by ITK inhibitors (PF-06465469, Ibrutinib, BMS509744 and ONO-7790500) on Jurkat, MOLT-4, CCRF CEM and K299 cells. The cells were stained with Draq7 (BioLegend) after treatment with the inhibitors for 24 hours. The populations of viable cells, early apoptotic, late apoptotic, and necrotic cells were determined by Annexin-V FITC/APC and Draq7, respectively. The flow cytometric analysis revealed that all ITK inhibitors induced apoptosis of the cells in a dose-dependent manner in the ITK expressing lines (Figure 3.9A). The data further demonstrated that these drugs caused lesser degrees of apoptosis in K299 cells compared to other cell lines.

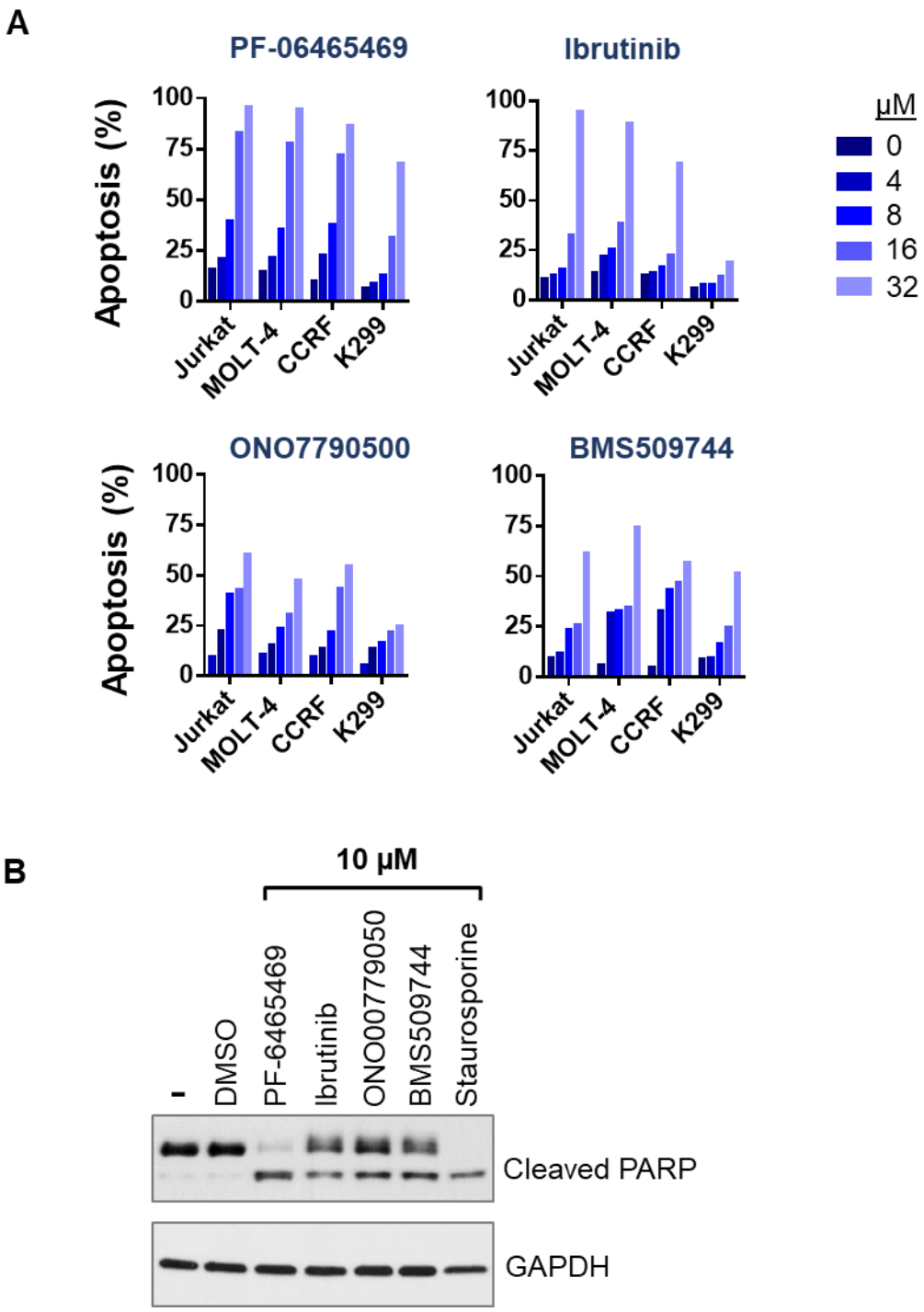


Figure 3.9. Induction of apoptosis of MOLT-4, Jurkat, CCRF CEM and K299 cells after treatment with PF-06465469, Ibrutinib, ONO-7790500 and BMS509744. (A), cells were seeded at $1x10^6$ cell/ml in a 24-well plate and treated with all four ITK inhibitor for 24 hours at the indicated doses. Apoptosis was assayed using Annexin-V kit and Draq7. (B), immunoblot analysis of lysates from Jurkat cells was performed to measure cell apoptosis after treatment with 10 μM of the compounds, and cell apoptosis was detected by using anti-PARP antibody. The experiments were performed in duplicate.

In addition to Annexin-V/Draq7 assay, in this study cell apoptosis was also investigated by using an anti-PARP1 antibody for measurement of apoptotic cells with cleaved PARP1. Measurement of PARP1 cleavage by western blot confirmed that the inhibitors induced apoptosis on the Jurkat cell line but to a lesser extent than the non-specific kinase inhibitor, staurosporine as demonstrated in Figure 3.9B.

3.2.4 ITK inhibitors block downstream signalling by PLCγ1

As mentioned earlier, ITK expression was detected in a panel of T-cell lymphoma/ leukaemia cell lines. To determine the effect of PF-06465469, Ibrutinib, BMS509744 and ONO-7790500 on signalling pathways in Jurkat, MOLT-4, CCRF CEM and K299 these cells were incubated with the ITK inhibitors for 24 hours. The inhibitory activity of ITK inhibitors against auto-phosphorylation of ITK in these cells was determined by western blot. ITK phosphorylation in Jurkat cells was effectively inhibited at a dose equivalent to the IC50 of the compounds although BMS509744 appears slightly less effective. In addition, all inhibitors were very effective at inhibiting ITK phosphorylation in MOLT-4 and CCRF CEM. ITK was not detectable in the K299 cell line, (Figure 3.1A, B and C). Table 3.1 shows the IC50 for the drugs.

To further determine the effects of ITK inhibitors on signalling molecules downstream of the T-cell receptor (TCR), Jurkat cells were treated with inhibitors for 24 hours, before activation by anti-CD3 and anti-CD28 antibodies for 40 minutes. The inhibitors suppressed the phosphorylation of phospholipase C γ1 (PLCγ1) and mitogen-activated protein kinase (MEK1/2), as demonstrated in Figure 3.10A. It is worth noting that BMS509744 and ONO-7790500 only partially blocked MEK1/2. Moreover, PF-6465469 and Ibrutinib reduced levels of phosphorylated protein kinase B (AKT) in response to stimulation but by contrast ONO-7790500 and BMS509744 were not able to inhibit phosphorylation of AKT (Figure 3.10A), suggesting that phosphorylation of AKT may not be entirely dependent on ITK activation.

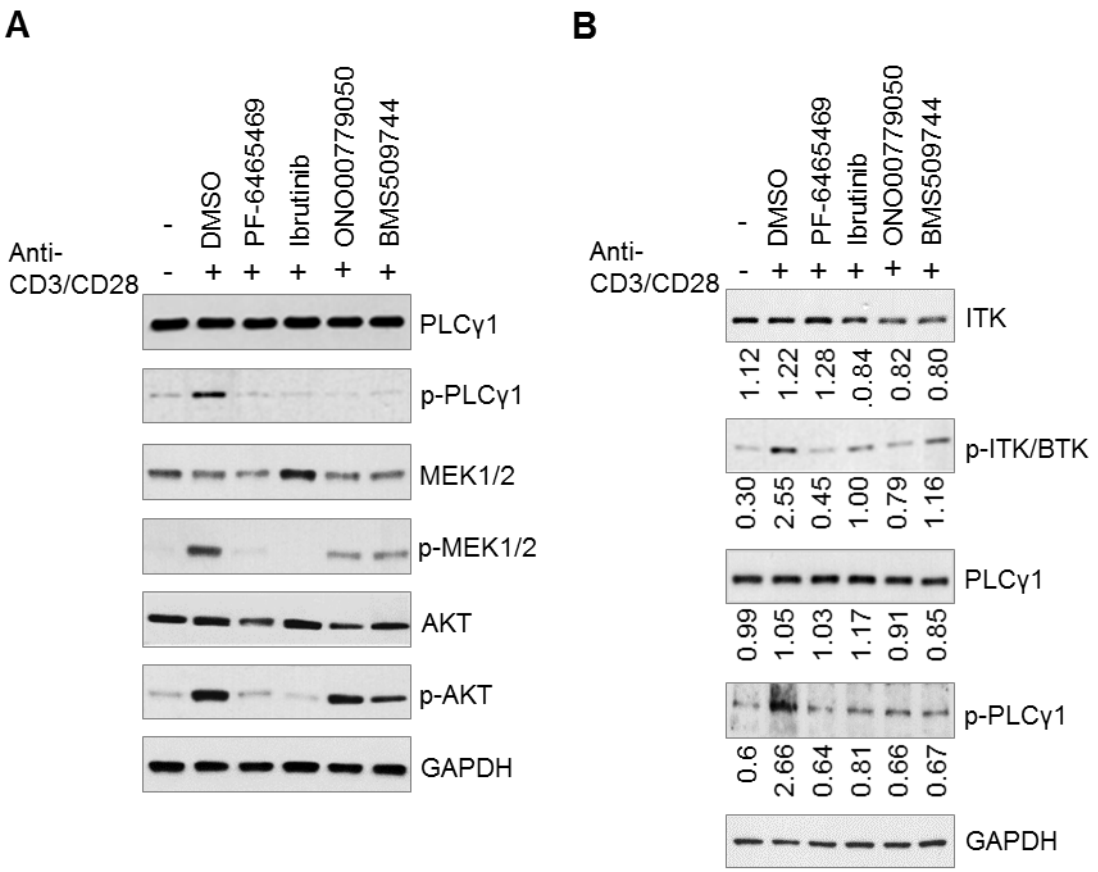


Figure 3.10. PF-06465469, Ibrutinib, ONO-7790500 and BMS509744 block the Formation of Phospho-ITK in Jurkat cells and T-cell prolymphocytic leukaemia (T-PLL). TCR-proximal signalling is inhibited by the inhibitors, abrogating ITK downstream Signals**.** (A), Jurkat cells pretreated with IC50 of the inhibitors for 24 h or vehicle (DMSO) and then the cells stimulated with anti-CD3 and anti-CD28 antibodies for 45 min were measured by western blot. (B), T-PLL cells were pre-incubated with 1 µM of all four inhibitors or vehicle (DMSO) in the present of anti-CD3/CD28 antibodies for 45 min. ITK, p-ITK, PLC-γ1, p-PLC- γ 1, MEK1/2, p-MEK1/2, AKT and p-AKT levels were detected using the corresponding antibodies through immunoblot. For immunoblots B, densitometric ratios are provided between phosphoprotein and total or loading control. Raw density was normalized to the intensity of vehicle-treated stimulated cells using ImageJ analysis software. The experiments were performed in duplicate.

To study the effects of all four ITK inhibitors in primary cells, CD4+ T cells were isolated from T cell lymphoma cells (n=2) and T-cell prolymphocytic leukaemia (T-PLL) (n=2). Purified cells were pretreated with 1 µM of the inhibitors for 30 minutes and then TCR stimulated for 45 minutes with plate-bounded anti-CD3 antibody and soluble anti-CD28 antibody. Treatment of primary cells with ITK compounds resulted in the inhibition of ITK phosphorylation. Immunoblot analysis revealed that all four inhibitors could significantly block downstream TCR signalling of phosphorylation of PLCγ1 as shown in Figure 3.10B and C, supporting the hypothesis that the ITK-inhibiting activity of PF-6465469, Ibrutinib, BMS509744 and ONO-7790500 was driving its effect. These data *in*

vitro suggest a potential clinical application of ITK inhibitors in the treatment of T-cell lymphoma.

3.2.5 ITK inhibitors block calcium signalling in Jurkat cells

In experiments described here T-cells were stimulated with anti-CD3 and anti-CD28 either in the presence or absence of drug and at the end of the experiment, ionomycin was used to control for the continuing responsiveness of cells. In cells stimulated with anti-CD3/CD28, an increase of calcium was observed (Figure 3.11) and this was reduced by all four small molecule inhibitors. Together, these data demonstrated that the inhibition of ITK significantly decreases intracellular calcium flux based on ITK inhibitors responses to TCR activation, whereas ionomycin bypasses the function of ITK.

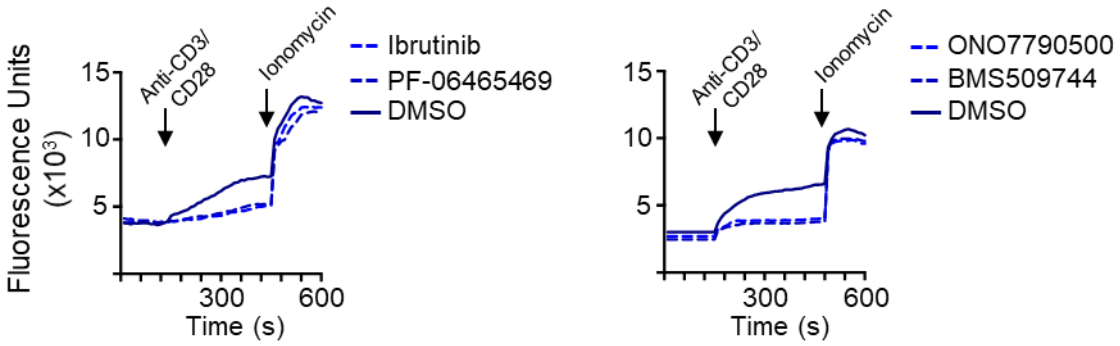


Figure 3.11. PF-06465469, Ibrutinib, ONO-7790500 and BMS509744 inhibit anti-CD3/anti-CD28 induced calcium signalling in Jurkat cells. Intracellular calcium flux was detected in Jurkat cells using FluoForte Calcium Assay. Cells were pretreated with IC50 of the inhibitors for 4 hours before addition of anti-CD3/anti-CD28 (as indicated by the black arrow). Ionomycin was added at the time indicated in order to maximally raise the level of intracellular calcium. The red arrow indicates the reduction in fluorescence between DMSO control (solid line) and ITK inhibitors (dashed line). These experiments were repeated twice.

3.2.6 Inhibition of ITK reduces IL-2 and IL-21 cytokine release in Jurkat cells

PLCγ1 catalyses the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 2,4,5-trisphosphate (IP3) and diacylglycerol (DAG) upon antigen presentation in T cells, which stimulates Ca^{2+} release. The rise in the cytoplasmic concentration of free Ca^{2+} triggers cytokine production, cell proliferation and transcription activation. IL-2 secretion was severely reduced in Itk deficient mice (Schaeffer *et al*., 1999; von Bonin *et al*., 2011) and, therefore, the effects of these inhibitors on the downstream functions of PLCγ1 including IL-2 secretion were investigated.

After incubation with ITK inhibitor, the mRNA levels of *IL-2* and IL-2 protein secretion in the culture supernatant were measured using real-time PCR (RT-PCR) and ELISA assay, respectively. PF-06465469 and Ibrutinib significantly reduced PMA/PHA-induced IL-2 release in Jurkat cells at both mRNA and protein levels (Figure 3.12A). Similar results in a reduction of IL-2 were found in the treatment of Jurkat cells with BMS509744 and ONO-7790500, although with apparently slightly reduced potency.

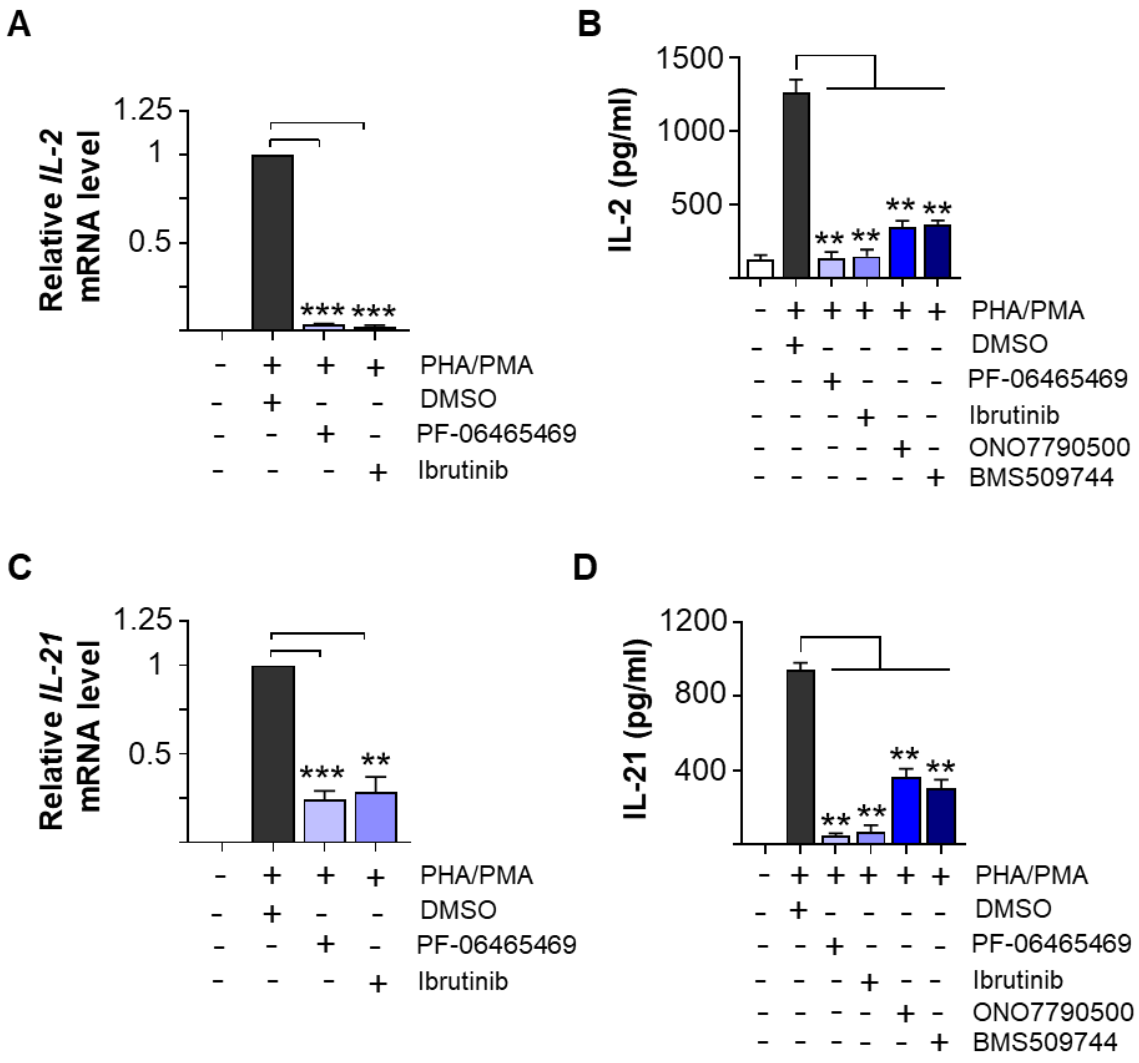


Figure 3.12. Inhibition of Il-2 and IL-21 secretion level in Jurkat cells after treatment with PF-06465469, Ibrutinib, BMS509744 and ONO-7790500. Jurkat cells were cultured in RPMI 1640 supplemented with 10% FCS containing 10 mL/L penicillin. Cells were incubated at 37°C for 24 h in the absence or presence of 1 µg/mL phytohaemagglutinin (PHA) and 50 ng/mL phorbol 12-myristate 13-acetate (PMA). The stimulated cells then treated with a concentration corresponding to the IC50 of the drug for 4 hours after changing half of the medium. (A) IL-2 mRNA levels following phytohaemagglutinin/phorbol myristate (PHA/PMA) stimulation alone and in the presence of ITK inhibitors. (B) IL-2 protein in culture supernatants determined by ELISA after PHA/PMA stimulation alone or in the presence of ITK inhibitors. (C) IL-21 mRNA levels after PHA/PMA stimulation alone or in the presence of ITK inhibitors. (D) IL-21 protein in culture supernatants determined by ELISA after PHA/PMA stimulation alone or in the presence of ITK inhibitors. The experiments were performed in duplicate and the data represented as mean ± SEM. Significance was determined by two-tailed P value with Unpaired t test $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Interleukin-21 (IL-21) is produced by follicular helper T (Tfh), and to a lesser extent by Th9 and Th17 cells (Yi *et al*., 2010; Spolski and Leonard, 2014; Vegran *et al*., 2014). Previous studies show that IL-21 regulates the functions and differentiation of certain subsets of CD4 T cells including Tfh (Vogelzang *et al*., 2008; Nurieva *et al*., 2008), regulatory T cells (Treg) (Attridge *et al*., 2012; Schmitz *et al*., 2013), Th17 cells (Nurieva *et al*., 2007; Korn *et al*., 2007; Zhou *et al*., 2007), Th1 cells (Wurster *et al*., 2002; Suto *et al*., 2006; Kastirr *et al*., 2014) and Th2 cells (Pesce *et al*., 2006; Frohlich *et al*., 2007; Lajoie *et al*., 2014; Coquet *et al*., 2015). One of the most common T-cell lymphomas, AITL, is derived from Tfh cells and therefore, it is valuable to evaluate the effect of ITK inhibition on IL-21 secretion. Both the mRNA levels and secretion of IL-21 were significantly decreased after treatment with the inhibitors (Figure 3.12B), suggesting that ITK, may regulate CD4 effector T cells including Tfh cells by reducing IL-21 levels.

3.2.7 Inhibition of ITK attenuates CXCL12 (SDF-1α) induced cell migration in CD4+ T cells and Jurkat cells

Cell migration has a major role in a variety of physiological and pathological situations, including immune responses, inflammation, angiogenesis, tumour invasion and metastasis. Chemokines are low-molecular-weight proteins that have the potential to mediate directional migration of T cells (Nishita *et al*., 2002). Polarisation and migration of T lymphocytes in response to chemokines are crucial for appropriate activation of immune response and development of the immune system. Previous studies showed an important role of chemokine signalling for phosphoinositide 3-kinase (PI3K) activation, which is essential for polarisation of membrane association of pleckstrin homology containing proteins (Funamoto, *et al*., 2001, Servant, *et al*., 2000) and activation of the small GTPases of the Rho family that are important for actin reorganisation and cell polarisation (Akasaki *et al*., 1999, Benard *et al*., 1999).

ITK plays an essential role in regulating chemokine signalling pathways in T cells. The chemokine CXCL12, also known as SDF-1α, stimulated a transient membrane association of ITK and induced phosphorylation of ITK and RLK (Takesono *et al*., 2004). Purified T cells from Itk deficient mice showed defects of migration to different chemokines and reduced homing to lymph nodes upon transfer to wild-type mice *in vitro* (Takesono *et al*., 2004).

To determine the effects of chemokine stimulation on phosphorylation of ITK, migration assays and western blot were carried out in Jurkat cells treated with CXCL12 at various doses and times. Jurkat cells were stimulated dramatically by increasing CXCL12 concentrations (Figure 3.13A). ITK and VAV phosphorylation were observed at early time points upon CXCL12 stimulation (Figure 3.13B).

Migration was investigated in Jurkat cells and human tonsillar CD4 T-cells. CXCL12 induced migration was impaired by all four small molecule inhibitors (Figure 3.13C and D). These results suggest that ITK is an important component of the signalling machinery required for T-cell migration in response to the chemokine CXCL12.

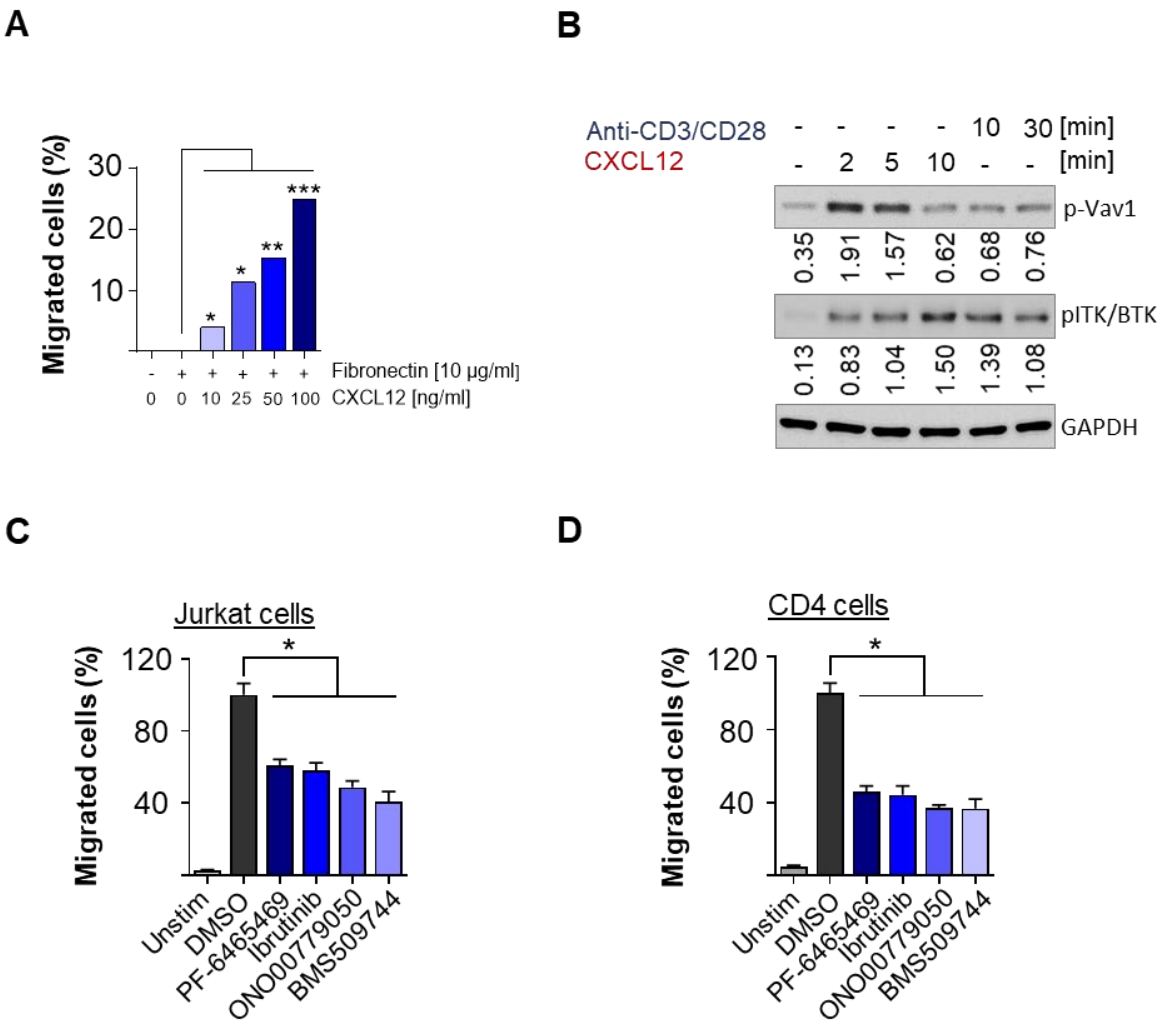


Figure 3.13. CXCL12 activates T lymphocytes, and Inhibition of ITK in Jurkat and CD4 T cells reduced migration in response to CXCL12. (A), Jurkat cells were activated with different doses of CXCL12 for 2 h at 37 °C with CO_2 in transwell plates coated with 10 µM of fibronectin. (B), Jurkat T cells were stimulated with 10 µg/mL anti-CD3 and 2 µg/ml anti-CD28 antibodies, and 100 ng/ml CXCL12 for the indicated times, phospho-ITK and Vav1 were determined using immunoblot. (C and D), Jurkat cells and primary CD4 T cells from human tonsillar tissue were treated with 1 µM of ITK inhibitors and them stimulated with 100 ng/ml CXCL12 for 2 h. The migrated cells were measured by using chemotaxis/migration assay as described under materials and methods. The experiments were performed in triplicate and the data reported as mean ± SEM, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ (two-tailed P value with unpaired t test).

3.2.8 ITK is required for activation of Rac1 and Cdc42 in CD4+ T cells and Jurkat cells

In this section we examined the role of ITK in RhoA GTPase activity. Secondly, we investigated whether the PLCγ pathway is involved in activation of Rho family GTPases.

3.2.8.1 The role of ITK inhibitors on activation of Rho family of small GTPases.

To determine the effects of CXCL12 on phosphorylation of ITK in Jurkat T cells. Cells were activated with CXCL12. The finding shows stimulation of Jurkat cells with CXCL12 led to the rapid activation of ITK (Figure 3.13B). Secondly, we examined the effect of ITK inhibitors on small GTPases family members; Rac1, Cdc42 and RhoA in purified CD4 T cells from human tonsils and Jurkat cell line. Treatment of T lymphocytes with ITK inhibitors significantly impaired chemokine-induced Rac1, Cdc42 and RhoA activation (Figure 3.14A and B). It can be noted that siRNA-mediated inhibition of ITK in Jurkat cells in response to CXCL12 was minimally affected in levels of RhoA activity (Figure 3.7). Similar results with respect to RhoA have been obtained with ITK inhibitors at the concentration of 3 µM, whereas activated RhoA was significantly reduced at 6 µM of the inhibitors in both Jurkat and CD4 T cells (figure 3.14A and B). It is worth mentioning that blank and positive control were used in this experiment in order to determine the linear range of the assay. In the linear range of the assay, the positive control reads 5 to 7 times/folds higher than the blank, a guide for acceptable backgrounds (cytoskeleton, Inc). This is important to avoid high background readings in the blank. According to G-LISA activation assays (cytoskeleton, Inc), positive control wells should read between 0.4-1.0 depending on type of assay kits and buffer-only wells (blank) should read between 0.10 – 0.4.

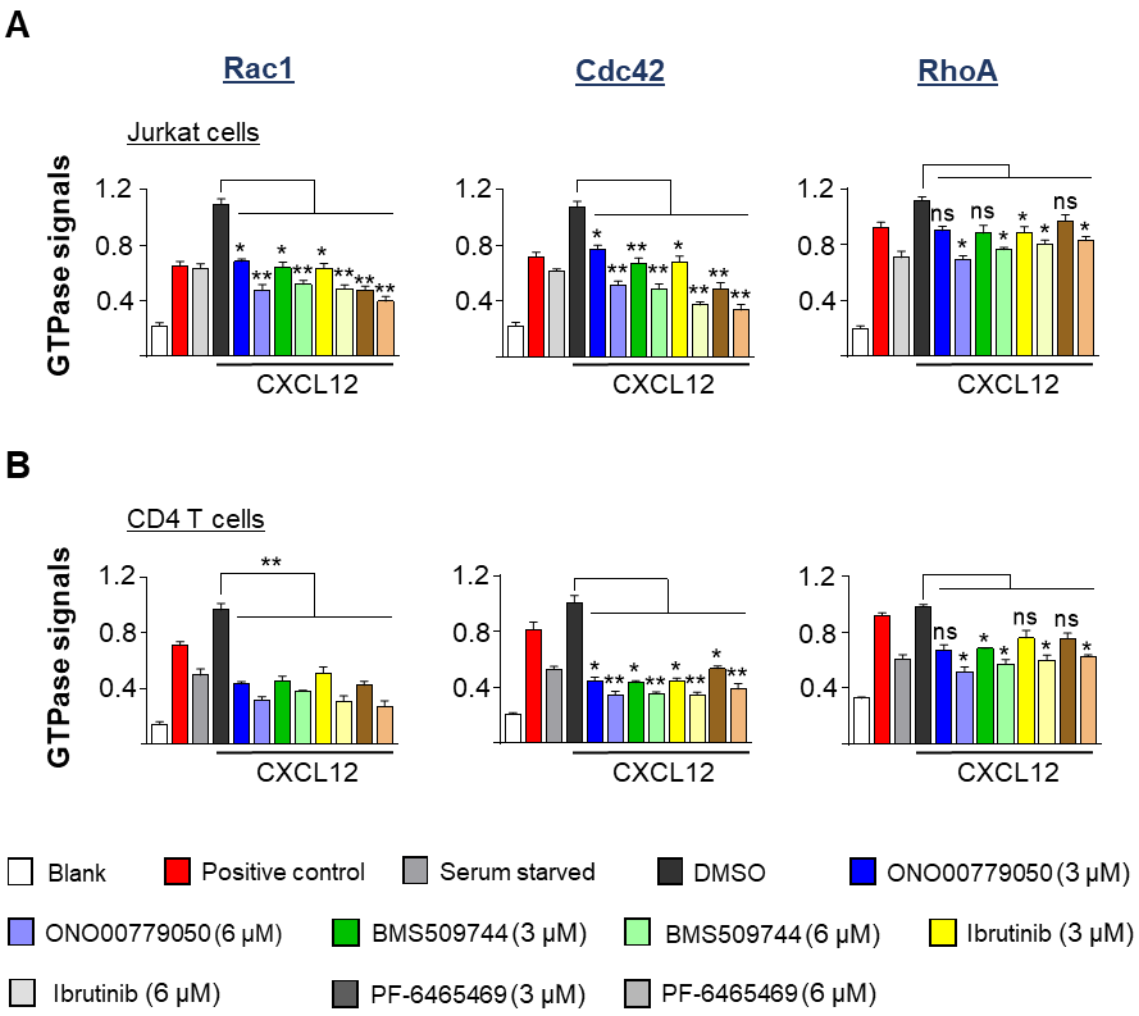


Figure 3.14. ITK inhibitors block activities of RhoA family GTPase in response to CXCL12. (A and B), freshly isolated CD4 T cells from human tonsils and Jurkat cells were serum starved (see section 2.8 in methods and materials for more details). Then cells were treated with vehicle (DMSO) or ITK inhibitors at the indicated concentrations for 1 h at 37°C in humidified air with 5% CO_2 in the presence of 100 ng/mL CXCL12 for 5 min. RhoA, Rac, and Cdc42 signals were measured using G-LISA activation assays. The data reported as mean ± SEM in duplicate, $*P < 0.05$, $**P < 0.01$ and NS represents Not Significant. (two-tailed P value with unpaired t test).

3.2.8.2 ITK activates Rho GTPases activity through a pathway independent of PLC-γ.

To identify the effects of the main substrate of ITK, PLC-γ1, on Rho family GTPases, and provide additional evidence of the signalling pathway involved in Rho GTPase activation, Jurkat cells were treated with indicated concentrations of phospholipase C (PLC) inhibitor. The results demonstrated that inhibition of PLC-γ1 did not have significant effects on Rho GTPases activities (Figure 3.15A and B), suggesting that ITK

has a role in activation of Rho proteins through other pathways, see section 3.2.9 for more information. Thus, the data demonstrate that while ITK is required for RAC1 and CDC42 activation the signalling pathway is not likely to use PLC-γ1.

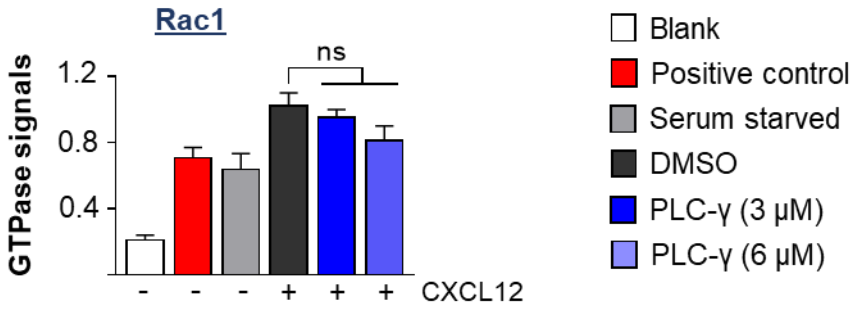


Figure 3.15. PLC-γ inhibitor inhibitor does not block activity of RhoA family GTPase, Rac1, in response to CXCL12. (A), Jurkat T cells were serum starved for 24 hours and treated with the indicated concentrations of PLC-γ inhibitor for 1 hour at 37°C in the presence of CXCL12 (100 ng). Rac signal was measured using G-LISA activation assays.

3.2.9 ITK inhibitors to investigate T-cell receptor signalling pathways in Jurkat cells.

Following the study of PLC-γ and in order to further understand the signalling machinery regulating chemokine and TCR signalling, studies were carried out to investigate the importance of PI3K and ZAP-70 for effects on ITK. Previous studies demonstrated that ZAP-70 plays an important role in CXCR4 signalling and migration, and ZAP-70 deficiency in the ZAP-70-deficient Jurkat T-cell line P116 results in reduced migration levels to CXCL12 (Ottoson *et al*., 2001). Additional research indicated that CXCR4 can lead to the activation of PI3K in Jurkat cells (Sotsios *et al*., 1999, Dutt *et al*., 1998). Fischer *et al*., (2004) pointed out that PI3K might be essential for CXCL12-induced chemotaxis by orienting the cell toward the chemokine source.

ITK is downstream of PI3K and ZAP-70 in the T cell receptor (TCR) signalling pathway (Shan and Wange, 1999; August *et al*., 1997; Lu *et al*., 1998). An additional study showed that ITK is engaged in the regulation of CXCL12 signalling and migration, and CXCL12 treatment leads to the activation of ITK (Fischer *et al*., 2004). However, there have been no previous studies determining the effect of PI3K and ZAP-70 on phosphorylation of ITK upon TCR and chemokine pathways side-by-side *in vitro*. To investigate this, ZAP-70 and PI3K were knocked down, then ITK and Vav1 phosphorylation in response to

either anti-CD3/CD28 or CXCL12 stimulation was determined using immunoblot analysis.

3.2.9.1 Activation of ITK in Jurkat cells required ZAP70 and PI3K pathway

To investigate the role of PI3K and ZAP-70 in ITK activation during anti-CD3/CD28 and CXCL12 stimulation, Jurkat cells were transfected with specific siRNA's targeting *PI3K* and *ZAP-70* using HiPerFect and DharmaFECT transfection reagents. Lysates from cells were immunoblotted with anti-ITK, anti-PI3K and anti-ZAP-70 antibodies.

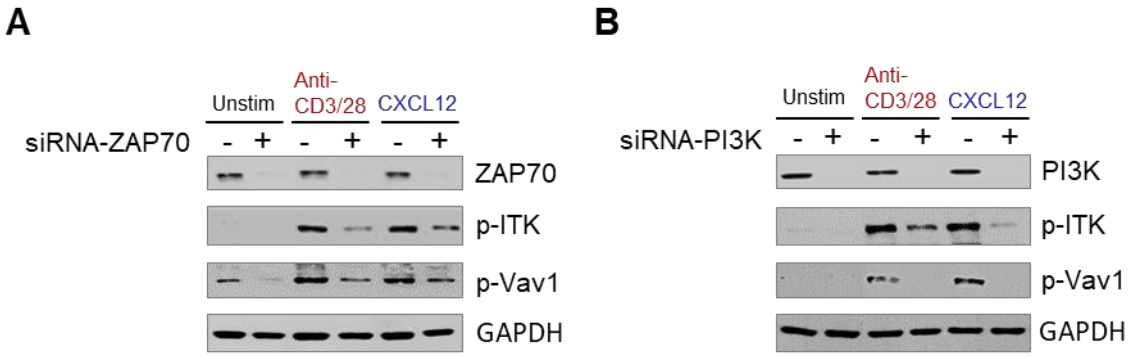


Figure 3.16. T-cell receptor (TCR) and chemokine (CXCR4) receptor signalling both activate ITK via ZAP-70 and PI3K. (A) and (B), siRNA specific ZAP-70 and PI3K were transfected into Jurkat cells at concentration of 100 nM by either by DharmaFECT 1 reagent or HiPerFect Transfection Reagent. Cells at the density of 3×10^4 cells per well were incubated at 37 °C for 48 h in 96-well plate. cells were activated with 5 µg/ml plate bound anti-CD3 and 2 µg/ml anti-CD28 at 45 min or with 100 ng/mL CXCL12 for 5 minutes. The depletion of phospho-ITK and Vav1 analysis was performed using western blotting.

There is reduction in the phosphorylation of ITK and VAV1 caused by ZAP70 knockdown following both TCR and chemokine signalling with a slightly greater relative reduction in p-ITK following anti-CD3/anti-CD28 cross-linking (Figure 3.16A, 3.17). ZAP70 might, therefore, be involved in both signalling routes. Knockdown of PI3K produced more pronounced p-ITK reductions in CXCL12 mediated signalling than in responses to anti-CD3/anti-CD28 (Figure 3.16B, 3.17). Taken together, these data demonstrate that ITK plays an essential role in both chemokine (CXCL12) and TCR signalling complexes, which both require PI3K and ZAP-70 for full activity (Figure 3.18).

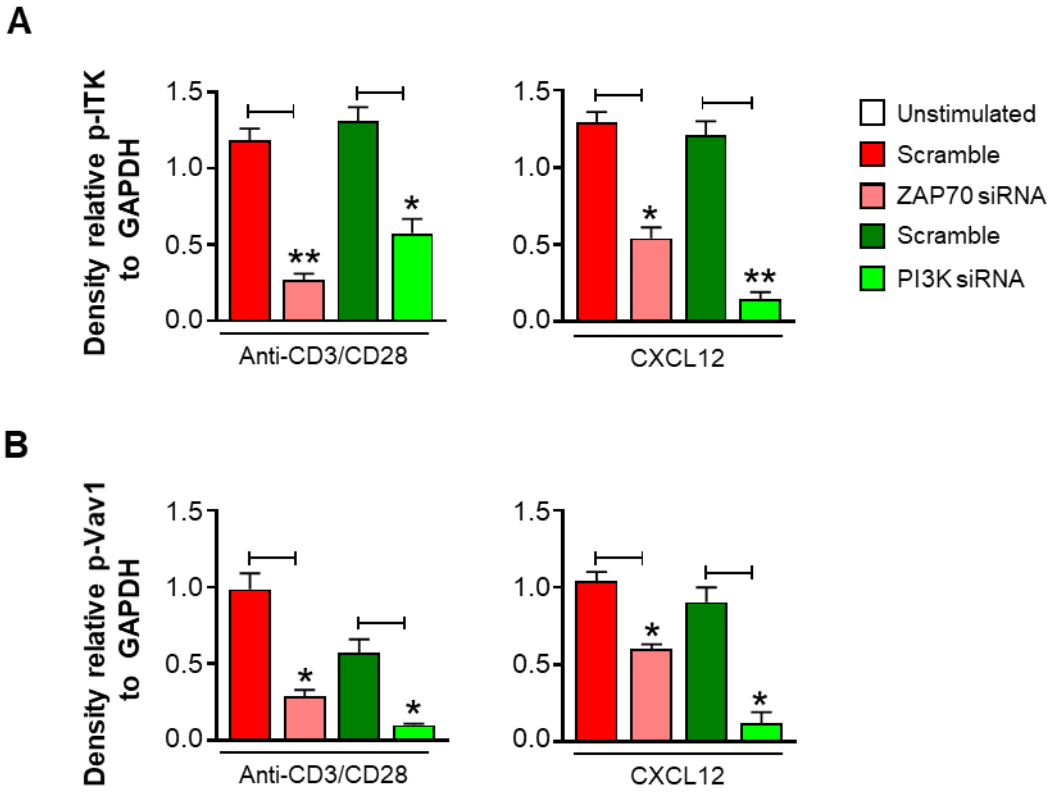


Figure 3.17. These bar charts showed density relative of p-ITK and p-Vav1 after ZAP70 or PI3K knockdown in Jurkat cells. Raw density was normalized to the intensity of GAPDH using the ImageJ analysis software. The figures suggest a model for involvement of ITK in T-cell receptor (TCR) and chemokine receptor signalling activating ITK via ZAP-70 and PI3K. The experiments were repeated in duplicate and the data represent as mean ± SEM, $*P < 0.05$, $**P < 0.01$ using two-tailed P value with unpaired t test.

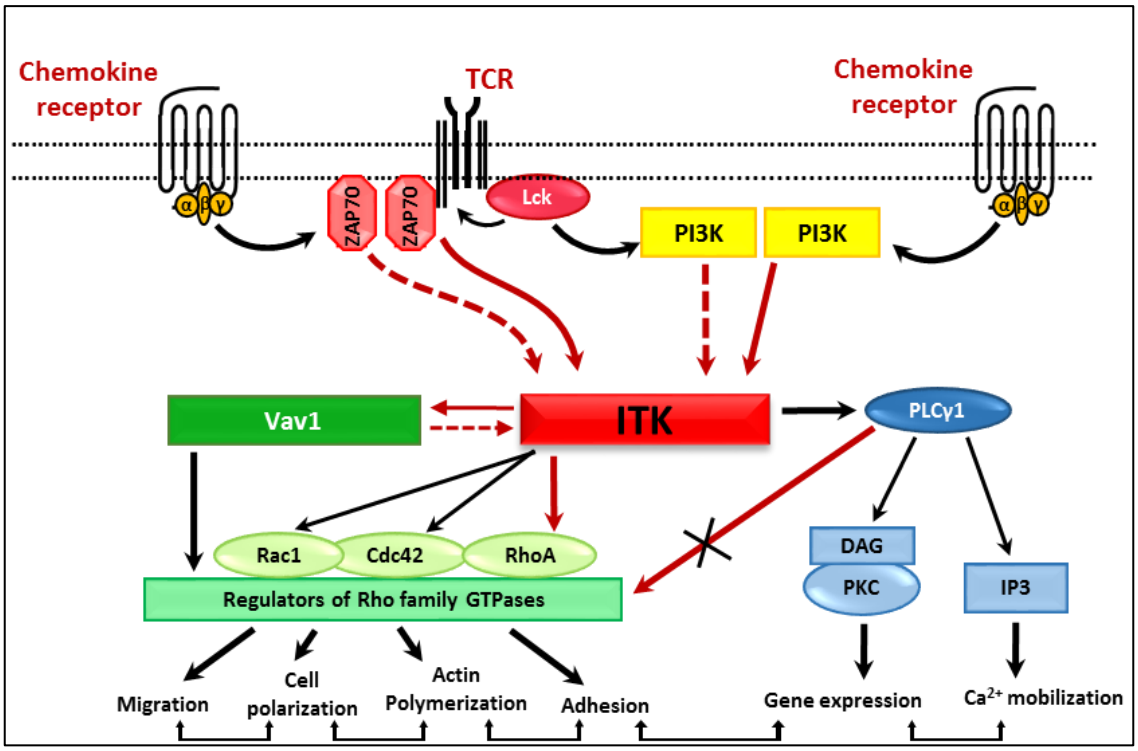


Figure 3.18. Model for involvement of ITK kinase in chemokine signalling. This figure shows T-cell receptors and chemokine receptors signal through ITK, and cross-regulation between ITK and Vav1 in these signalling complexes. The red arrows in the diagram display novel findings in signalling pathways in T cells. Dashed red lines represent the partial effects of the variable on the response that contribute through different pathways.

3.3 Discussion

Small molecule inhibitors are among the most exciting methods to assess ITK signalling activity (Sahu and August, 2009; Savig-Barfi *et al*., 2015). Homology between BTK and ITK suggest Ibrutinib can be used for both BTK and ITK inhibition and Dubovsky *et al*. (2013) demonstrated that it was actually capable of inhibiting ITK and its downstream activation of T-cells after TCR stimulation both *in vitro* and in pre-clinical mouse models for the treatment of parasitic infection and infectious diseases such as *Listeria monocytogenes*.

In addition, an ITK inhibitor, CTA056, has been reported to induce apoptosis in T-cell lymphoma (Guo *et al*., 2012). However, the studies of CTA056 on T-cell lymphoma are limited. Clinical use of Ibrutinib, which also represses ITK, for the treatment of B-cell lymphoproliferations, such as chronic lymphocytic leukaemia or mantle cell lymphoma, shows that this compound is well tolerated. Ibrutinib has potential for the treatment of patients with T-cell lymphoma and as far is known, there is no reported use of this agent in preclinical trials of T-cell lymphoma.

In this chapter, a systematic *in vitro* analysis was performed of four ITK inhibitors: PF-06465469, Ibrutinib, ONO-07790500 and BMS509744 in order to begin to assess the potential of ITK as a target for treatment of peripheral T-cell lymphomas. These compounds are all potent ITK inhibitors but likely differ in their specificity (ibrutinib clearly inhibits both BTK and ITK whereas ONO-07790500 is believed to be more specific for ITK) and pharmacokinetics in ways that have not been fully described. Analysis of signalling showed that ONO-07790500 and BMS509744 do not inhibit MEK or AKT phosphorylation (Figure 3.10), in line with their greater specificity for ITK. The other inhibitors, PF-06465469 and Ibrutinib, might produce some of their cellular effects though inhibition of these extra pathways either directly or indirectly. Another possibility is that the ITK inhibitors produce their effects through inhibition of the related TEC family kinase, RLK. Levels of *RLK* mRNA are lower than those of *ITK* in human T-cell lines and primary T-PLL (Figure 3.1) but this remains a formal possibility.

The approach taken in this chapter has been to compare the effects of small molecule inhibitors with knockdown of ITK by siRNA. Both siRNA and small molecule inhibitors

might have off-target effects but potentially this is a route to determine the specificity of effects of small molecule ITK inhibitors.

Knockdown of ITK showed that it plays an essential role in maintaining the viability of Jurkat cells, calcium mobilisation and production of IL-2. These effects were largely reproduced by the small molecule inhibitors but reduction of IL-2 production following knockdown appears less than in cells treated with ITK inhibitors. This may be either due to incomplete knockdown or because of off-target effects by the inhibitors.

Further evidence that the effects of inhibitors was in fact through inhibition of ITK is that repression of cell viability and induction of apoptosis occurred in an ITK-dependent manner i.e. cell lines expressing most ITK also had the greatest responses to inhibitors. Knockdown of ITK produced only modest levels of apoptosis and this is consistent with the effects of ITK inhibitors in this work and the literature. For example Dondorf *et al* (2015), suggest that BMS-509744 treatment does not cause apoptosis of Jurkat cells but they used very low inhibitor concentration. Cell death is obviously an objective of treatments for cancer and it is, therefore, likely that ITK inhibitors will need to be used alongside chemotherapy. Synergy with other agents including conventional chemotherapy is explored in Chapter 5.

Another area of interest in the field of treatment of lymphoma is the role of ITK in the control of cell migration. ITK is implicated in chemokine mediated signalling and is crucial for actin reorganisation following cell stimulation with chemokines, through G protein–coupled receptors (GPCRs) to initiate migration and cell polarisation (Schwartzberg *et al*., 2005). Treatment of many cell types with chemokines such as CXCL12 results in membrane recruitment of many TEC kinases and to ITK phosphorylation (Fischer *et al*., 2004; Takesono *et al*., 2004; Nore *et al*., 2000; Lachance *et al*., 2002). Exposure of Jurkat T cells to CXCL12 resulted in a rapid increase in phosphorylation of ITK (Takesono *et al*., 2004). Fischer and colleagues showed that overexpression of ITK enhanced migration in Jurkat cells, and absence of Itk in mice lacking ITK led to a significant reduction of migration in response to CXCL12 compared to wild type (Fischer *et al*., 2004), indicating that ITK is capable of controlling the ability of CXCR4 to induce migration.

We found that Jurkat cells migrated in response to CXCL12 and the ITK inhibitors were highly effective in reducing CXCL12 mediated migration. Takesono *et al*. (2002)

suggested that VAV1/2 are necessary for the transmission of ITK signals to the actin cytoskeleton. VAV family members are a small subset of guanine nucleotide-exchange factors (GEFs) that activate RHO GTPase family members such as RAC, CDC42 and RHOA, which contribute to reorganisation of the actin cytoskeleton (Bustelo, 2001). T cells from mice bearing homozygous disruptions of Vav1 suppress activation of the RHO family small GTPases RAC1 and CDC42 (Reynolds *et al*., 2002; Finkelstein *et al*., 2004). An additional study showed that Jurkat cells expressing mutant versions of ITK exhibit defective CXCL12-mediated activation of RAC and CDC42 (Takesono *et al*., 2004). Therefore, there is an established pathway from ITK through VAV1 to RHO family GTPases.

In this chapter, all four ITK inhibitors were found to block signalling to the RHO family GTPases-RAC1, CDC42 and, to a lesser extent, RHOA. Several studies have revealed that ITK regulates PLC-γ and calcium flux in T lymphocytes, and that most of the defects in ITK-deficient T cells are likely to be caused by defects in TCR-mediated signalling including PLC-γ1 and downstream transcription factors, such as NFAT proteins. However, a PLC- γ1 inhibitor did not reproduce the effects of ITK inhibitors on RAC1 activity (Figure 3.15) suggesting that ITK might act directly through VAV1 on the RHO GTPases.

We have carried out a systematic *in vitro* analysis to compare the effects of CXCR4 signalling with TCR signalling (Figure 3.16) on phosphorylation of ITK and VAV1. This is important in order to estimate the relative importance of ITK to the effects of these two pathways. While both chemokine and TCR signalling induced phophorylation of ITK and VAV1, the TCR effects appear slightly more dependent on ZAP70 than the chemokine effects. By contrast the chemokine effects are much more dependent on PI3K than are the TCR effects. This suggests a route to select drugs for synergy with ITK inhibitors. Previous studies show that ZAP-70 and PI3K are required for ITK activation in the TCR pathway (Shan and Wange, 1999; August *et al*., 1997; Lu *et al*., 1998). We discovered that ITK requires the activity of ZAP-70 and PI3K for its activation not only in the TCR pathway but also requires chemokine pathway for its efficient activation. There are many PI3K inhibitors and combining with an ITK inhibitor might cause a large reduction in both T-cell migration and TCR signalling effects.

In summary inhibition of ITK by small molecule inhibitors produces effects comparable to those seen following siRNA mediated knockdown of ITK. This study has demonstrated, for the first time, that there was a clear difference in effects on phosphorylation of MEK1/2 and AKT, with the more specific inhibitors sparing these pathways. ITK inhibitors supressed cell viability and induced cell death in an ITK-dependent manner. Overall the spectrum of effects on cell lines suggests that ITK inhibitors might be therapeutically useful in some T-cell lymphomas but this work does not support their use as single agents.

Chapter IV. Effects of ITK inhibitors on normal primary human T-cells

4.1 Introduction

The differentiation of functionally distinct subsets of helper T cells is required to confer immunological protection against cancers and pathogens (Pardoll *et al*., 1998; Topalian, 1994) and also contributes to chronic inflammation and autoimmune diseases (Szabo *et al*., 2003; Wang *et al*., 1994; Boehm *et al*., 1997). The development of diverse T cell subsets is directed by the nature of the cytokine milieu, the expression of trasncription factors and the engagement of other surface receptors (Tian and Zajac, 2016). CD4+ T cells are crucial for mediating the adaptive immune response to a variety of pathogens as well as tumour immunity (Zhu *et al*., 2010). Naïve CD4+ T cells differentiate into distinct lineages of T helper (Th) cells, such as T helper 1 (Th1), T helper 2 (Th2), IL-9-producing Th9, T helper 17 (Th17), regulatory T cells (Treg) and T follicular helper (Tfh) cells. Figure 4.1 shows $CD4^+$ T cells subsets and their plasticity.

In T lymphocytes, the interleukin-2-inducible T-cell kinase (ITK) is activated by T cell receptor (TCR) stimulation and plays a central role in the development of naïve CD4+ and CD8+ T cells (Liao and Littman., 1995; Schaeffer *et al*., 1999; Fowell *et al*., 1999; Hu *et al*., 2007). Many studies have demonstrated that Itk-deficient T cells results in defects in conventional T cell and innate T cell development and differentiation (Berg *et al*., 2007; Prince *et al*., 2009). ITK regulates T helper (Th) cell differentiation including Th1, Th2, Th17, Treg and Th9. Itk knockout mice show that deficiency of Itk affects CD4+ T cells (Liao and Littman., 1995; Hu and August, 2008) and displays reduced levels of differentiation of $CD4^+$ Th2, but the Th1-produced cytokine IFN-γ is little affected in Itk-deficient $CD4^+$ T cells (Fowell *et al*., 1999). In addition, ITK is needed to regulate IL-17 producing T helper cells and promote Th17 differentiation. Itk-deficient mice display reduced IL-17 production and defective differential regulation of Th17 cells *in vivo* in *Itk*−/− mice (Gomez-Rodriguez *et al*., 2009).

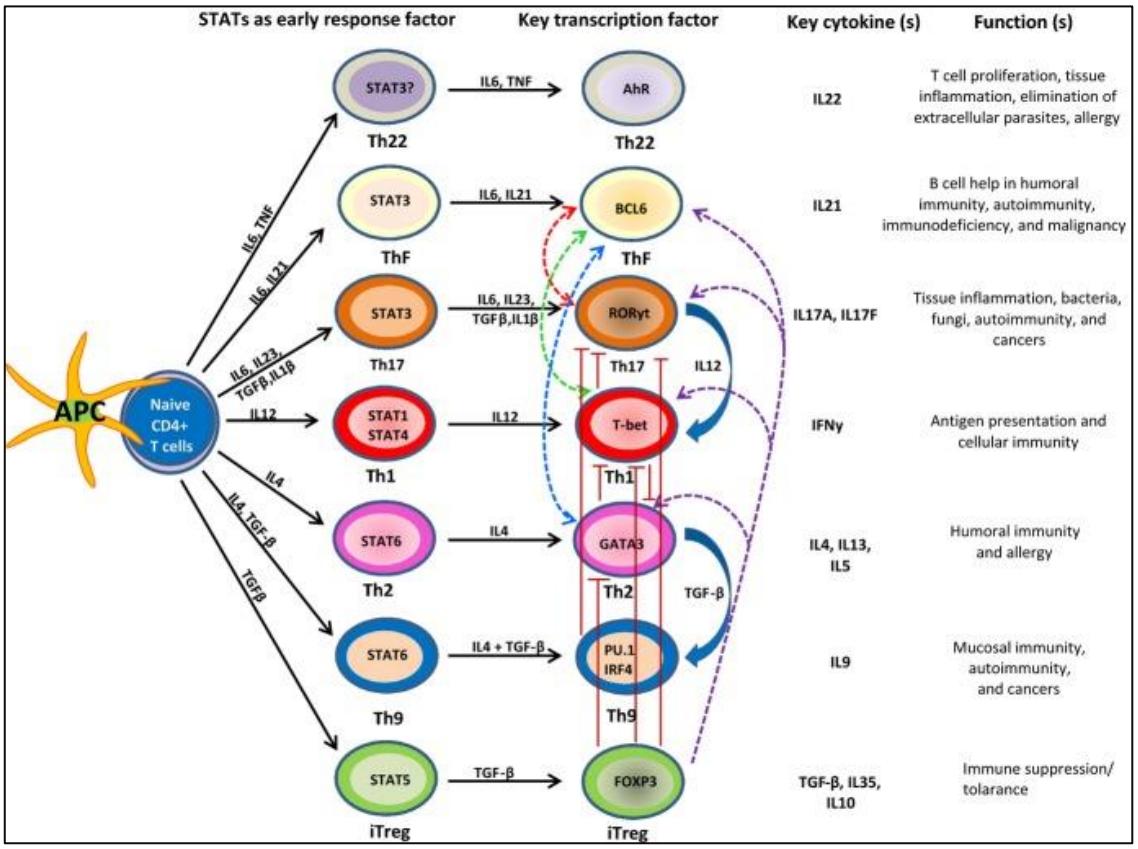


Figure 4.1. This diagram shows CD4+ T cell subset and their plasticity. Naive $CD4^+$ T helper cells can differentiate into various types of effector T helper cells and regulatory T-cell subsets under the influence of the secretion of different cytokines, the expression of cell surface markers and lineage-specific transcriptional regulators. These effector $CD4^+$ Th cells can convert from one subset into another. (Tripathi and Lahesmaa, 2014).

According to Gomez-Rodriguez *et al*., (2014), Itk contributes to the balance between Th17 and Treg cell differentiation under Th17 polarised conditions in which Itk-deficient cells preferentially develop into Treg cells. Another *in vivo* model analysis shows that Itk can alter the function and development of natural regulatory T cells, and negatively tunes the expansion of IL-2-induced Treg cells (Huang *et al*., 2014). Regarding the role of Itk in Th9 cells, a new study found that Itk is a key regulator of Th9 cells producing IL-9 and is required for its differentiation (Gomez-Rodriguez *et al*., 2016).

Due to the essential role of ITK in the development and differentiation of $CD4^+$ T cells, therefore, dysregulation in ITK function results in T cell related disorders, such as allergy and hypersensitivity (Mueller and August, 2003; von Bonin et a., 2011), infection (Hu *et al*., 2007; Atherly *et al*., 2006; Bachmann *et al*., 1997), autoimmune diseases (Jain *et al*., 2013) and T cell malignancies (Kaukonen *et al*., 1999; Shin *et al*., 2007; Zhao, 2010;

Dancey *et al*., 2009; Streubel *et al*., 2006). We, therefore, sought specific effects of ITK inhibitors on normal $CD4^+$ T cells in this chapter and tumour $CD4^+$ T cells in chapter V.

This chapter will describe the impact of ITK on the plasticity of certain subtypes of CD4+ T helper cells and intriguing implications of ITK inhibitors on functions and phenotype of $CD4^+$ Tfh, Treg and Th17, Th2 and Th1 cells from human tonsillar tissue. It will further focus on the effect of inhibition of ITK on IL-21 expression in CD4+ T cells upon TCR stimulation.

4.2 Result

4.2.1 Effects of ITK inhibitors on tonsillar T-cell proliferation and apoptosis

4.2.1.1 Effects on tonsillar T-cell proliferation.

To study the effects of ITK inhibitors on Tfh cell proliferation, CD4+ T-cells isolated from human tonsil and patients with T-cell lymphoma (n=2), and were stained with CellTrace violet (CTV) (5 µM). The cells were pretreated with all four ITK inhibitors or vehicle (DMSO) for 30 minutes, then stimulated with anti-CD3/CD28 with and without IL-12 or anti-CD3/ionomycin for 6 days. Before analysis, the cells were stained with CD4, CXCR5 and PD-1 to identify Tfh cells.

Without inhibitor, cells underwent about 6 divisions (as expected from the length of time in culture) (Figures 4.2, 4.3 and 4.4). After day 6, flow cytometry analysis showed that ITK inhibitors remarkably inhibited the anti-CD3/CD28 ± IL-12 induced proliferation of Tfh and non-Tfh subpopulations, see Figures 4.2 and 4.3. It can be noted that ONO-7790500 and BMS509744 were slightly less effective in reducing Tfh cell proliferation as they were in reducing Tfh differentiation. The data further demonstrated that proliferation of Tfh and non-Tfh was partially attenuated under anti-CD3/ionomycin conditions with the most specific ITK inhibitors, ONO-7790500 and BMS509744 (Figure 4.4).

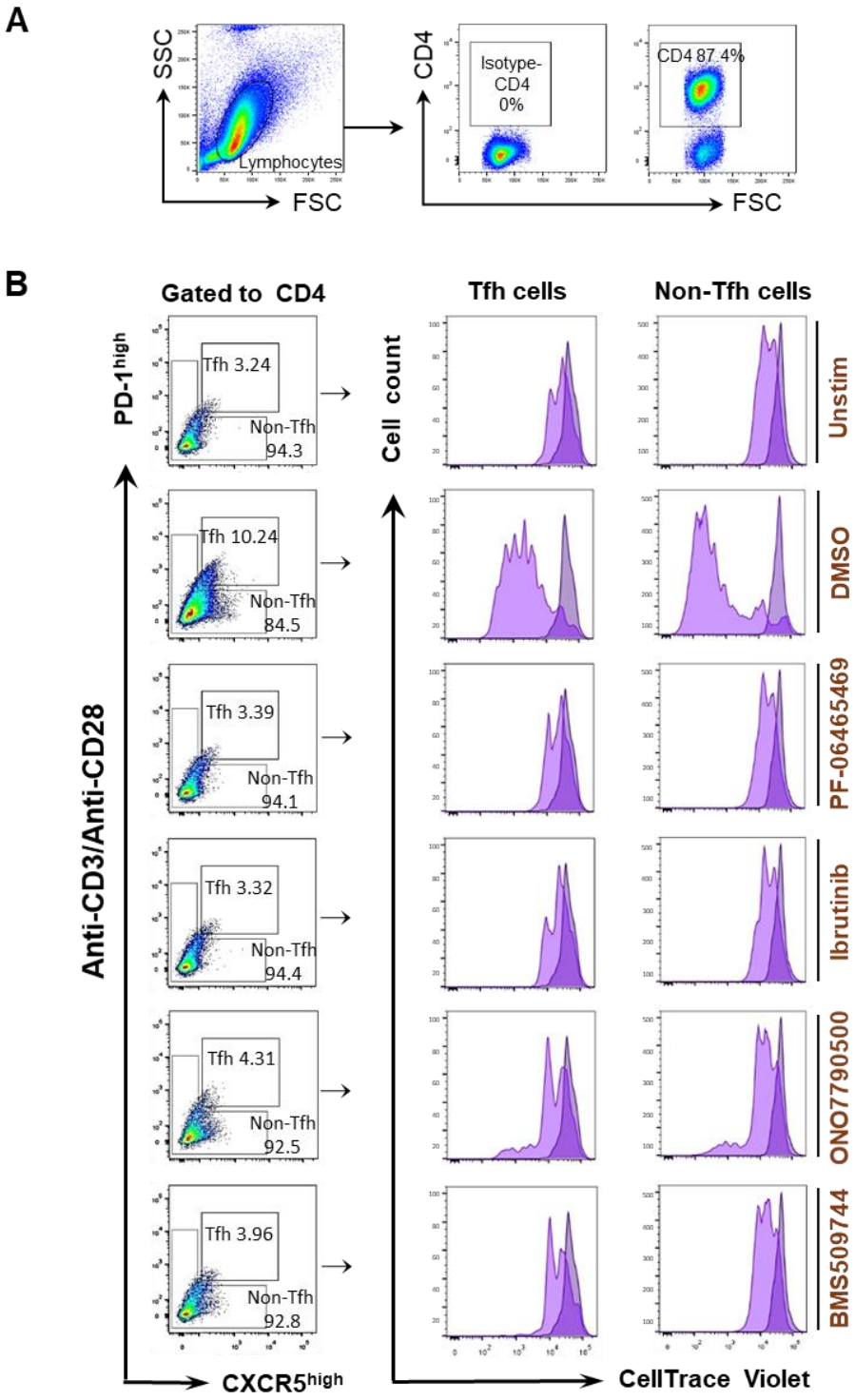


Figure 4.2. ITK inhibitors block TCR-induced primary Tfh cell proliferation. CD4+ T cells isolated from human tonsil were labelled with CellTrace Violet (CTV); having been pre-treated with ITK inhibitors (1 µM) or vehicle (DMSO) for 30 minutes. For each culture condition dot-plots show proportions of high-Tfh ($CD4^{+}CXCR5^{high}PD1^{high}$) and non-Tfh cells and histograms represent the CTV fluorescence just after loading with dye (dark purple) and after six days (light purple). (A) Example of described the early steps of gating strategy for the identification of CD4+ T-cells in control (DMSO) condition. (B) T-cells were stimulated with anti-CD3/anti-CD28.

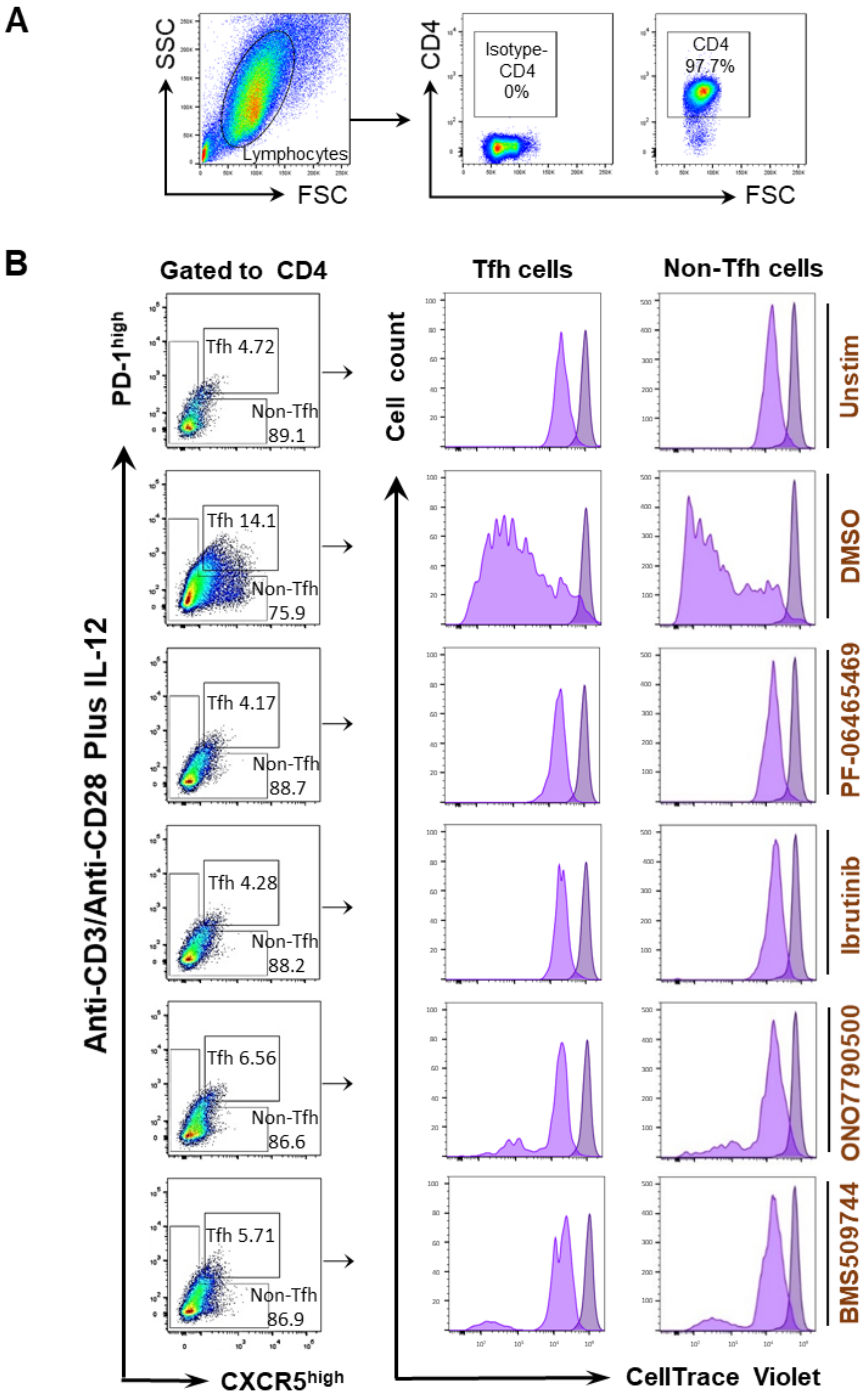


Figure 4.3. ITK inhibitors repessed TCR-induced primary Tfh cell proliferation under anti-CD3/CD28 and IL-12. CD4+ T cells were purified from human tonsil, then cells were labelled with CellTrace Violet (CTV); following pre-treated with ITK inhibitors (1 µM) or vehicle (DMSO) for 30 minutes. For each culture condition dot-plots demonstrates percentages of high-Tfh ($CD4^{+}CXCR5^{high}PD1^{high}$) and non-Tfh cells and histograms represent the CTV fluorescence. (A) Example of gating strategy for the identification of CD4+ T-cells is shown on control (DMSO) condition. (B) T-cells were stimulated with anti-CD3/anti-CD28 and IL-12. The figure shows expressing high levels of CD4+PD-1+CXCR5+ Tfh cells.

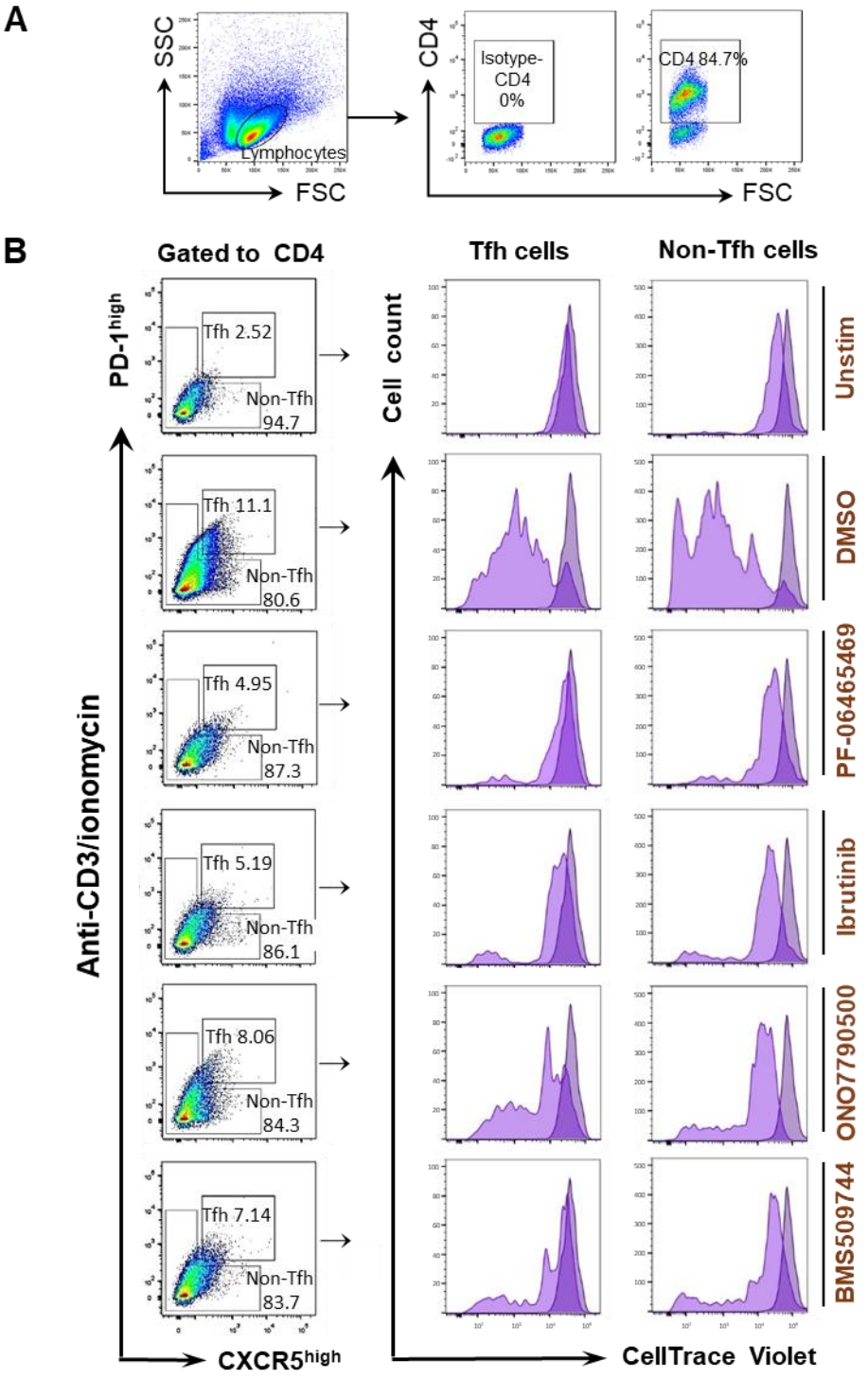


Figure 4.4. ITK inhibitors were not able to supress proliferation of Tfh cell proliferation under anti-CD3 and ionomycin condition. After isolation of CD4+ T cells from human tonsil and labelled with CellTrace Violet (CTV); cells were pre-treated with ITK inhibitors (1 µM) or vehicle (DMSO) for 30 minutes. Dot-plots show high proportions Tfh ($CD4^{+}CXCR5^{high}PD1^{high}$) and non-Tfh cells and histograms represent the CTV fluorescence. (A) Example of gating strategy to get access to CD4+ T-cells is demonstrated on DMSO condition. (B) T-cells were stimulated with PMA/ionomycin.

4.2.1.2 Inhibition of ITK induces apoptosis in follicular helper T-cell (Tfh) cell.

Apoptosis, or programmed cell death is an important target of many treatment strategies for cancer (Wong, 2011), and has provided the basis for novel targeted therapies (Ghobrial, *et al*., 2005). Thus, we next conducted an *in vitro* analysis to investigate responses of Tfh and non-Tfh apoptosis or cell death to ITK inhibitors. The data demonstrated that all four inhibitors induced apoptosis confirmed that inhibitors induced apoptosis. For both Tfh and non-Tfh cells, PF-6465469 and Ibrutinib induced more apoptosis than the other inhibitors. We also observed differences between Tfh and non-Tfh populations (Figure 4.5). The percentage of annexin/Draq7-apoptotic cells in the non-Tfh population was significantly lower (~10%) than those in Tfh cells (Figure 4.5).

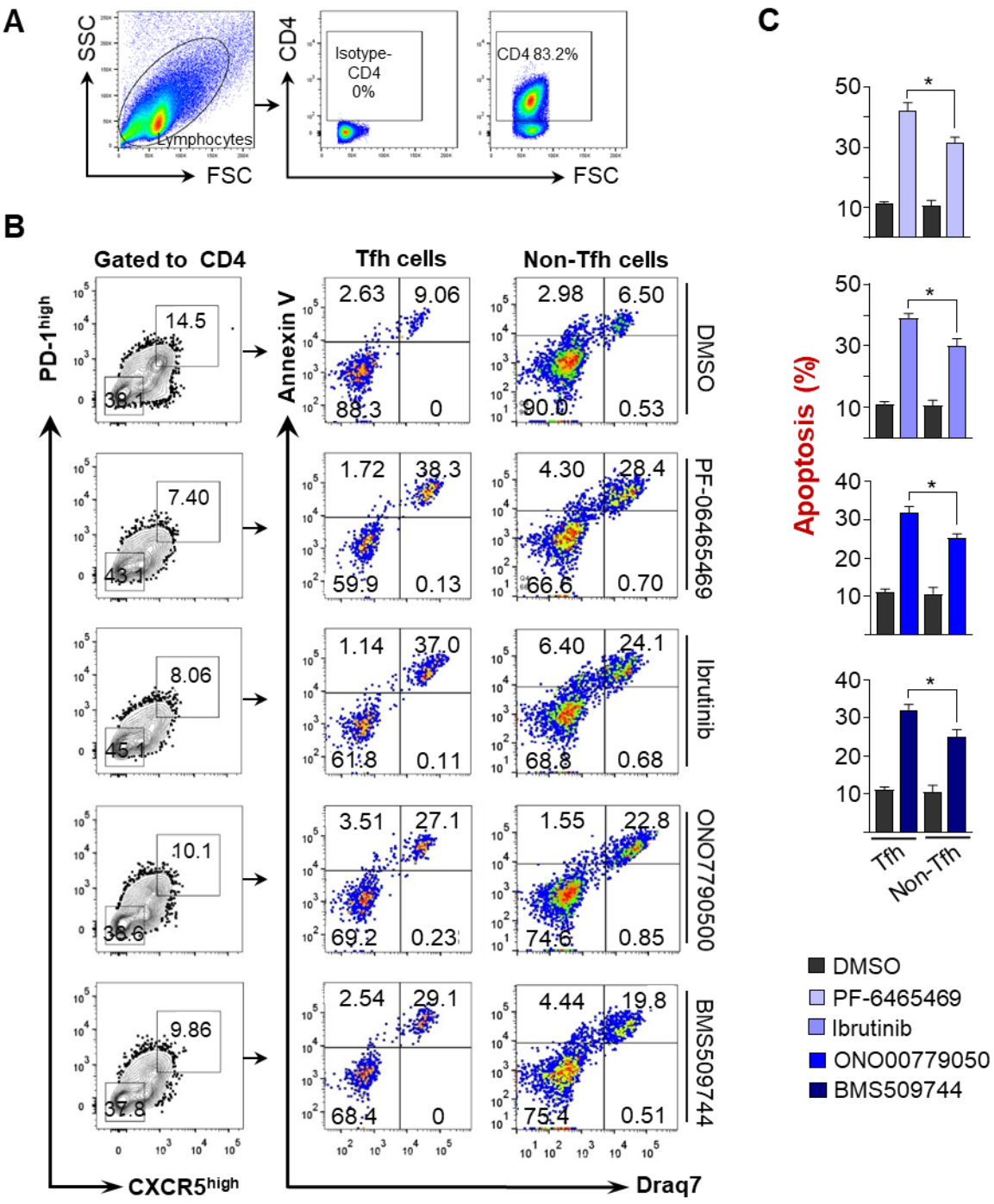


Figure 4.5. ITK inhibitors induce apoptosis. (A) Example of gating strategy for the identification of CD4+ T-cells after isolation is demonstrated on control (DMSO) condition. (B) Isolated CD4+ T cells from human tonsil were pre-treated with ITK inhibitors (5 µM) or vehicle (DMSO) for 30 minutes and then stimulated with anti-CD/anti-CD28 antibodies for 48 hours. The left-hand dot plots show the percentages of Tfh cells and the right-hand dot-plots show annexin V/Draq7 staining. Percentage in each quadrant is presented. (C) Data are shown as the percentage of apoptotic cells i.e. all cells except annexin V and Draq7 negative cells, in both Tfh and non-Tfh populations. Data are the mean ± SEM are representative of the experiments. *$P < 0.05$ (two-tailed P value with Unpaired t test). NS represents Not Significant.

Next, the effect of ITK inhibitors were examined on human tonsillar cells. After single cell separation from tonsil tissue, cells were treated with various concentrations of ITK inhibitors for 30 min as indicated in Figure 4.6. Then, cells were cultured in the presence of anti-CD3 and anti-CD28 antibodies at different time points 24 h, 72 h and 6 days. The result demonstrated that ITK inhibitors were not able to cause apoptosis during 24 h and 72 h, but these cells were undergone apoptosis with the inhibitors only at 6 days, suggesting that ONO-7790500 and Ibrutinib can induce apoptotic death over longer incubation periods following TCR stimulation.

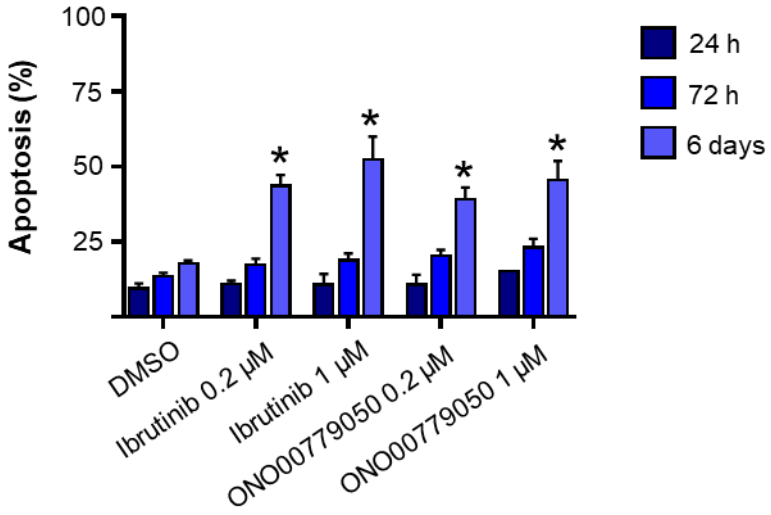


Figure 4.6. ONO-7790500 and Ibrutinib caused relatively little apoptosis in human tonsillar cells. CD4+ T cells were cultured at two different doses of ITK inhibitors (0.2 or 1 µM) or DMSO and then stimulated with anti-CD3/anti-CD28 antibodies at different time points: 24 h, 72 h and 6 days). (A) The graphs show the effect of ITK inhibitors on cell apoptosis (B) Apoptosis (%) is shown in bar chart, and both ONO-7790500 and Ibrutinib comounds significantly induced apoptosis at concentration of 1 µM at day 6 compared to DMSO control. The data were shown from two independent experiments and values are expressed as mean ± SEM. P-value was measure by two-tailed t test $P < 0.05$ *.

4.2.2 ITK inhibitors and tonsillar T-cell differentiation

4.2.2.1 ITK inhibitors repress Tfh differentiation

Isolated primary human tonsillar T-cells were cultured with immobilised anti-CD3 (Rasheed *et al*., 2006) plus anti-CD28 antibody (Walker *et al*., 1999). To characterise and isolate a Tfh subset, currently, two different phenotypic definitions can be used based on

the strong expression of CXCR5 with the high expression of either PD-1 or ICOS (Tangye *et al*., 2008; Rasheed *et al*., 2006).

Two culture systems were compared. Firstly, anti-CD3/anti-CD28 and secondly, anti-CD3/anti-CD28 with IL-12 (Figure 4.7). Published researches have suggested that IL-12 promotes the generation of Tfh cells in humans (Schmitt, *et al*., 2013, Schmitt *et al*., 2009), and deficient in IL-12 have diminished Tfh cell and germinal centre (GC) responses (Schmitt *et al*., 2013). Anti-CD3/anti-CD28 with IL-12 produced a larger Tfh population than anti-CD3/anti-CD28 alone (32.6% versus 21.1%). Ibrutinib and PF06465469 reduced differentiation to baseline levels whereas ONO07790500 and BMS509744 appeared slightly less effective especially in the anti-CD3/anti-CD28 with IL-12 culture conditions.

In order to demonstrate ITK dependence further, anti-CD3 and ionomycin were employed to stimulate tonsillar T-cells. Ionomycin bypasses the functional effects of ITK on calcium signalling and, therefore, would be expected to reduce the effects of ITK inhibitors if mediated through this pathway. ITK inhibitors were indeed less effective in reducing the proportion of Tfh cells supporting the idea that calcium signalling is important for *in vitro* Tfh differentiation (Figure 4.7).

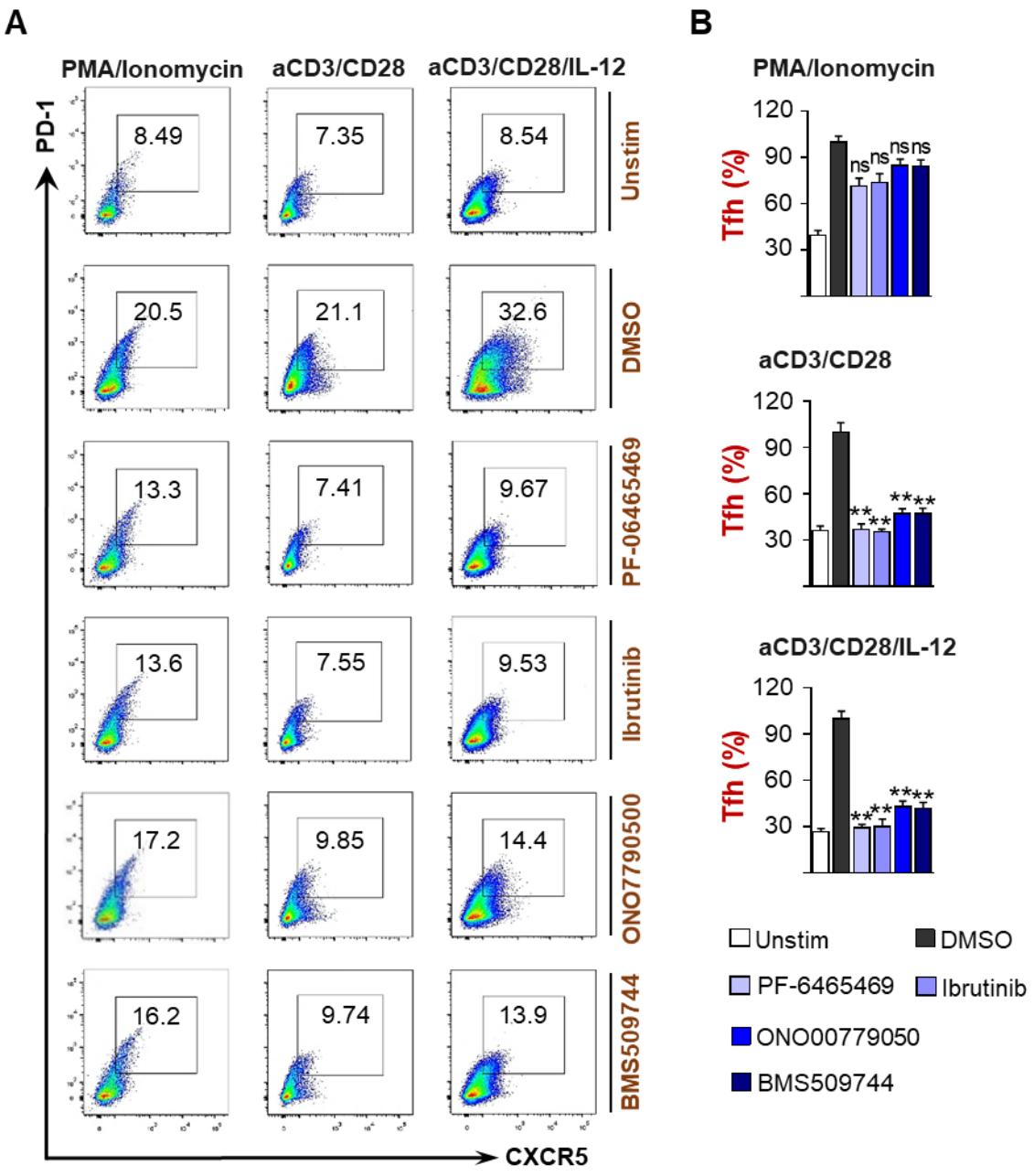


Figure 4.7. Identification and isolation of the expression of Tfh cell-related molecules in human tonsil. (A) Flow cytometry identified the percentage of Tfh cells within purified CD4+ T cells under different culture conditions i.e. with anti-CD3/CD28 in the absence and presence of IL-12 or with PMA/ionomycin. (B) Bar charts (right) show the percentages of CD4+PD-1+CXCR5+ Tfh cells in the tonsils using the gating strategy as described in panel A. Error bars represent mean ± SEM from two independent experiments. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (two-tailed P value with Unpaired t test). NS represents Not Significant.

4.2.2.2 ITK inhibitors enhance regulatory T-cell (Treg) differentiation.

Schwartzberg and her group observed an increased proportion of FOXP3+ cells in *Itk*−/− cells at various concentrations of anti-CD3 stimulation (Gomez-Rodriguez *et al*., 2014). Therefore, we sought to identify Tregs in normal human tonsil under certain conditions, and examine the specific effects of ITK inhibitors on $CD4^+$ Treg subsets.

We evaluated the differentiation of naïve CD4 T cells under Treg cell conditions by stimulating the cells with anti-CD3/CD28 plus TGF-β1 (Gomez-Rodriguez *et al*., 2014) in the presence or absence of IL-12. Small but significant increases in the percentage of Treg cells were observed under Treg polarising conditions compared to the unstimulated condition. After treatment of cells with ITK inhibitors, we observed increased Treg differentiation, approximately two-fold more than the level observed in controls (DMSO) without inhibitor (Figure 4.8). PMA/ionomycin rescued both the percentage and number of Treg cells in the tonsil (Figure 4.8). The data suggest that ITK inhibitors can alter T-cell differentiation such that Treg differentiation is promoted.

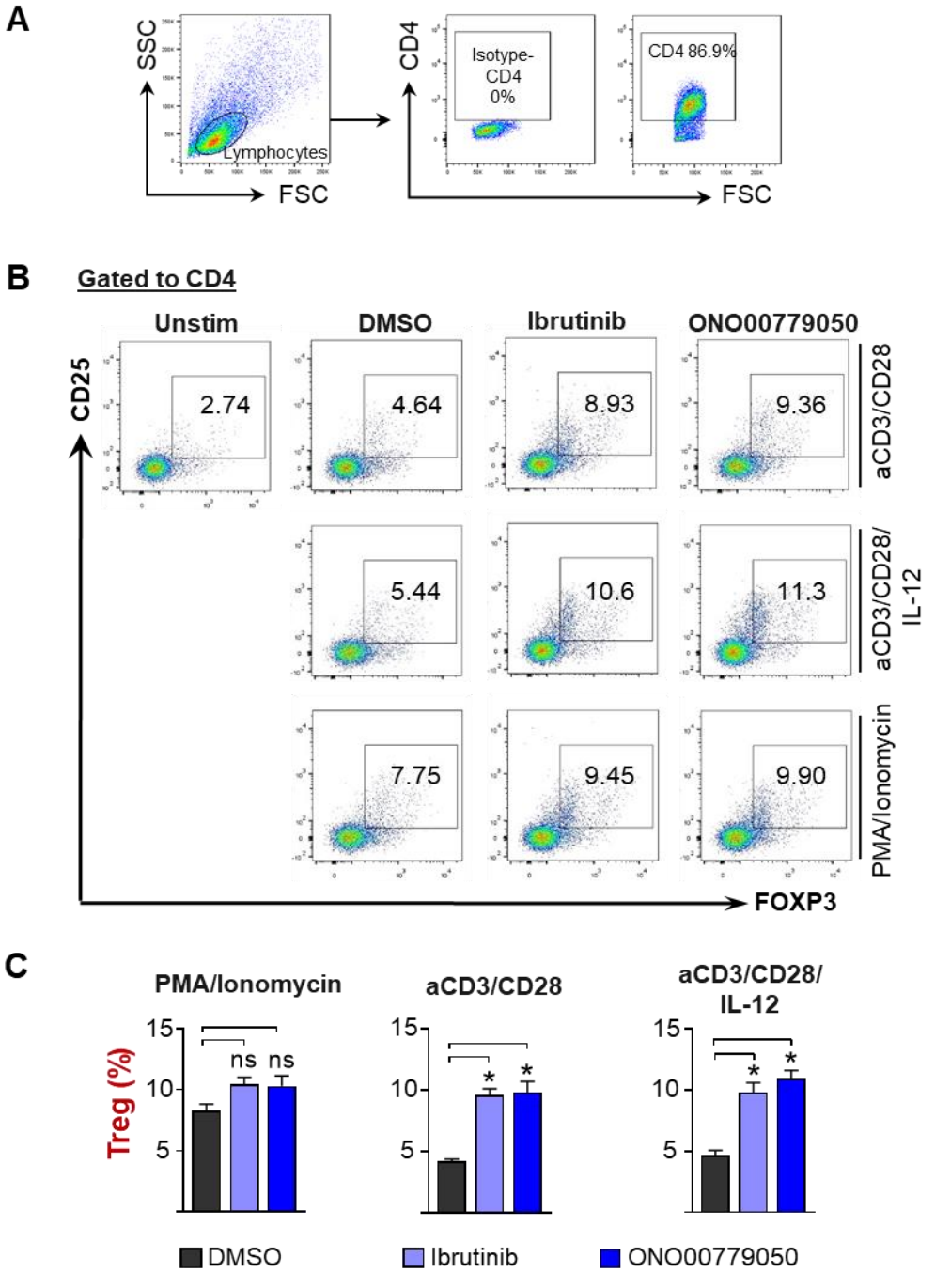


Figure 4.8. ITK inhibitors increase Treg differentiation in human tonsil. (A) Example of flow cytometric plots showing CD4+ T-cells on vehicle control (DMSO) condition. (B) CD4+ T cells from tonsil were treated with DMSO or ITK inhibitors and differentiated for CD25+FOXP3 Treg expression under different culture conditions 1) PMA/ionomycin, 2) anti-CD3/CD28 antibodies with TGF-β1 3) anti-CD3/CD28 antibodies with TGF-β1 with IL-12. CD25+FOXP3 Tregs were analysed by intracellular staining. (C) The bar graphs show the percentages of Tregs expression and data are representative of two independent experiments. Data are the mean ± SEM and are representative of two experiments. *$P < 0.05$ (Unpaired t test, two-tailed P value). NS represents Not Significant.

4.2.3 IL-21 blockade on CD4+ effector T cells *in vitro*.

4.2.3.1 IL21R–Fc suppresses follicular helper T-cell (Tfh) differentiation.

High- affinity antibody production depends on the germinal centre (GC) reaction during which B cells undergo class switch recombination, somatic hypermutation and affinity-based selection (Victor and Nussenzweig, 2012). Tfh cells are crucial for the maintenance and formation of GCs and their differentiation depending on the major transcriptional regulators, signal transducer and activator of transcription 3 (STAT3) and B cell lymphoma 6 (BCL6), which are both induced by the cytokine interleukin-21 (IL-21) (Crotty, 2014, Vogelzang *et al*., 2008; Nurieva *et al*., 2008).

IL-21 itself is induced by TCR signalling through NFATc (Mehta *et al*., 2005). It has already been shown (Chapter 3, section 3.2.6) that ITK inhibitors repress IL-21 expression in the Jurkat T-cell line. Therefore, we hypothesised that IL-21 has a role in driving Tfh differentiation. To determine the effect of IL-21 on differentiation, naïve CD4+ T cells were isolated from human tonsil. As an initial experiment, we examined the expression of IL-21 in purified CD4 T cells from tonsil. As demonstrated in Figure 4.9, the expression of this cytokine is significantly increased from less than 100 ng/ml to over 400 ng/ml upon anti-CD3/CD28 stimulation. Next, we investigated the impact of human IL-21R-Fc protein, a chimeric fusion protein between the IL-21 receptor and the Fc portion of an antibody heavy chain, which binds to and prevents the effects of IL-21 on differentiation of naive tonsillar T-cells to Tfh cells.

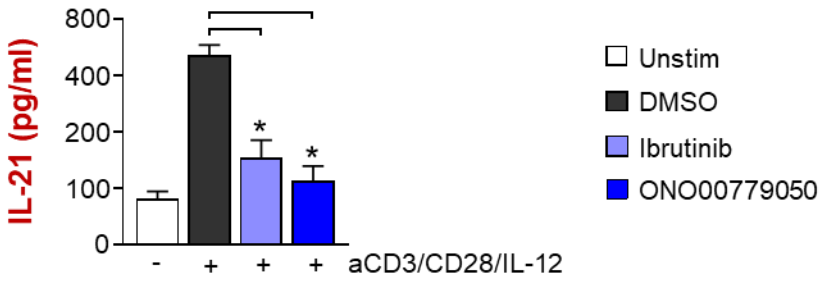


Figure 4.9. Impaired IL-21 expression in ITK-deficient cells. Purified human tonsillar CD4+ T cells were cultured in the presence of ITK inhibitors (1 µM) and vehicle (DMSO), then the cells were stimulated with anti-CD3/CD28 antibodies plus IL-12 for 24 h. The secretion of IL-21 was determined by ELISA. Error bars represent from two independent experiments (mean ± SEM). *$P < 0.05$ (two-tailed P value with Unpaired t test).

CD4+ T cells were cultured and induced by anti-CD3/CD28 antibodies in the presence and absence of IL-21R-Fc (15 μg/ml) for 6 days. Blockade of IL-21 significantly reduced the percentage of Tfh ~ from 21% to 13% (Figure 4.10B and D). Next, the effects of IL-21R-Fc on differentiation with anti-CD3/anti-CD28/IL-12 were investigated. The percentage of Tfh cells was elevated from 13% to 21% under these conditions, but IL-21R-Fc was still capable of suppressing the Tfh expansion (Figure 4.10C and D), indicating that IL-21R-Fc regulates the function and differentiation of the Tfh subset and suppression of IL-21 production might be a major mechanism of action of ITK inhibitors.

Next, the role of ITK inhibitors in combination with IL-21R-Fc was evaluated. If IL-21 was the only route by which ITK inhibitors exert their effects, then the addition of these small molecule inhibitors should have no extra effect in the presence of IL-21R-Fc. The findings indicate that both ONO00779050 and Ibrutinib significantly attenuated the percentage of Tfh cells, but it can be noted that the ITK inhibitors have a more profound suppression of Tfh expansion than IL-21R-Fc alone (Figure 4.10). These data suggest that ITK inhibitors do exert their effects through suppression of IL-21 secretion but also through other mechanisms.

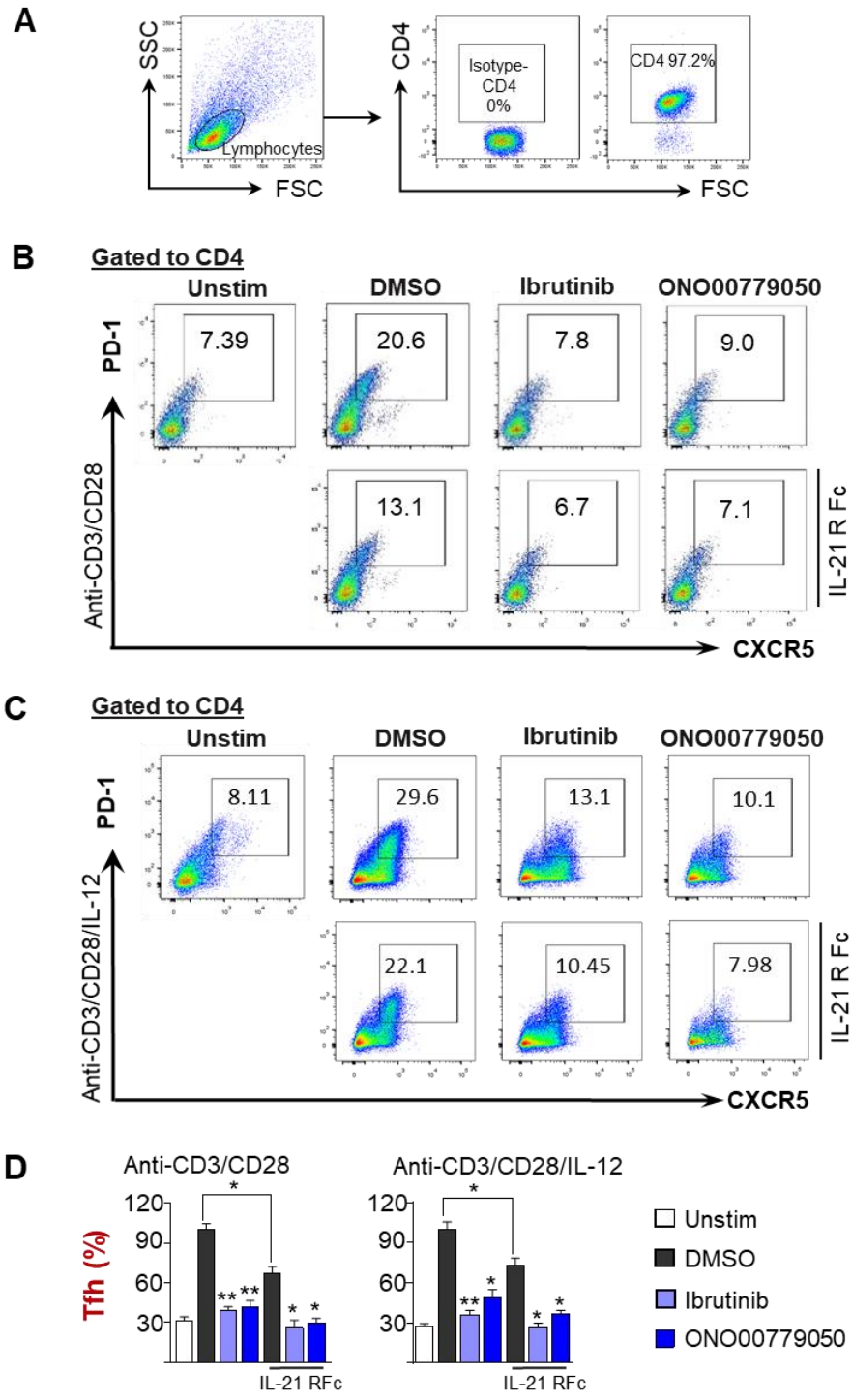


Figure 4.10. IL-21 blockade partially inhibits Tfh differentiation. (A) Example of gating strategy for the identification of CD4+ T-cells on DMSO condition. (B) Purified CD4+ T cells were pre-treated with IL-21R-Fc (15 µM) and then activated with anti-CD3/CD28 antibodies. (C) anti-CD3/CD28 antibodies plus IL-12 in the presence and absence of ITK inhibitors for 6 days. (D) Bar chart shows that IL21R-Fc significantly represses the percentage of Tfh cells and ITK inhibitors reduce this further.. Data are representative as the mean ± SEM for two independent experiments. *$P < 0.05$ and **$P < 0.01$ (two-tailed P value with Unpaired t test).

4.2.3.2 IL-21 blockage has little or no effect on regulatory T-cell (Treg) differentiation.

It has been shown that the cooperation of IL-21 and transforming growth factor beta (TGF-β) restricts the differentiation of Tregs, while accentuating the development of Th17 cells (Nurieva *et al*., 2007; Korn *et al*., 2007; Zhou *et al*., 2007). As mentioned earlier (4.2.3.1), IL-21 expression was detected in purified $CD4^+$ T cells, and IL-21 production was significantly impaired in the absence of ITK. We again wondered whether the effects of ITK inhibitors on Treg differentiation was mediated by IL-21. To determine whether IL-21 blockage can promote Treg expansion, cells were treated with IL-21R-Fc in the presence and absence of ITK inhibitors, and differentiated under Treg differentiation conditions for 2 days. Intracellular staining revealed that a higher proportion of CD25+FOXP3+ Treg cells were generated from IL-21 blockade than the condition without IL-21R-Fc (Figure 4.11A and B). However, blockade of IL-21 exhibited only a mild impact, from 5.9% to 7.15%, on cell expansion as demonstrated in Figure 4.9C. This might be due to the length of incubation with IL-21R-Fc being insufficient, as it is shown that its effect on Tfh differentiation is at its greatest after 6 days (Figure 4.10).

Next, we determined the effects of blockade of IL-21 in combination with ITK inhibitors on Treg expansion. Cells were cultured with ITK inhibitors in the presence and absence of IL-21R-Fc (Figure 4.11B). The percentage of CD25+FOXP3+ was slightly increased in the presence of both IL-21R-Fc and ITK inhibitors compared to ITK inhibitors alone (Figure 4.11), suggesting that ITK inhibitors induce the development of Treg cells and that their mechanism of action is only partly by suppression of IL-21 activity.

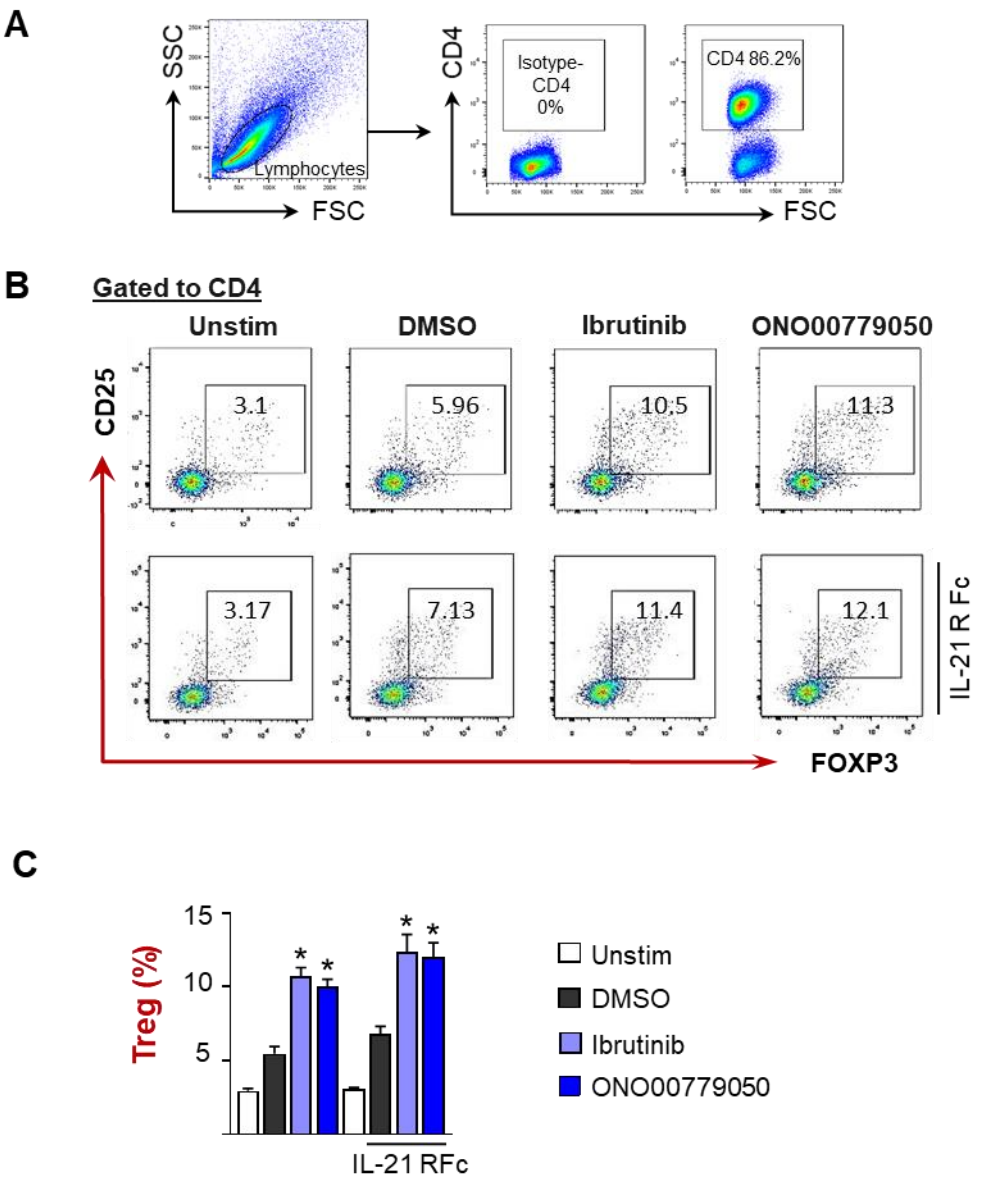


Figure 4.11. Blockade of IL-21 produces a trend towards increased Treg differentiation. (A) Example of density plots representation of CD4+ helper T-cells after isolation from human tonsil cells on DMSO condition. (B) CD4+ T cells from tonsil were pretreated with IL-21R-Fc (15 µM) protein with and without ITK inhibitors. CD4+ T cells were stimulated under Treg differentiation conditions (anti-CD3/CD28 antibodies plus IL-12 in the presence of TGF-β1) for 48 hours. CD25+FOXP3 Tregs were analysed by intracellular staining. Percentage of Treg cells are presented. (C) Bar chart shows the percentages of Treg cells and data are representative of two independent experiments. Error bars represent mean ± SEM. *P < 0.05 (Unpaired t test, two-tailed P value).

4.2.4 ITK inhibitor effects on differentiation to other CD4+ T-cell lineages

4.2.4.1 Th17 differentiation and IL-17 expression.

Itk-deficient mice show diminished IL-17 production under Th17 differentiation conditions (Gomez-Rodriguez *et al*., 2009). These conditions are transforming growth

factor beta (TGF-β) and interleukin 6 (IL-6) (Korn *et al*. 2009) and cause naive T-cells to express pro-inflammatory cytokines including IL-17A, IL-21, IL-22 and IL-17F. Th17 cells play a crucial role in antimicrobial activity and the inflammatory response against fungi and bacteria (Gomez-Rodriguez *et al*., 2014) especially in the gastrointestinal system.

To investigate the role of ITK inhibitors on the Th17 subset, naïve CD4+ T cells were purified from human tonsil. As an initial approach, we examined the effects of ITK inhibitors on CD4+ T cells from human tonsil on *IL17A* expression. Cells were treated with Ibrutinib and ONO00779050 and qRT PCR was performed to evaluate *IL17A* gene production. The data was normalised to unstimulated conditions in all cases. As illustrated (Figure 4.12), the level of *IL17A* mRNA expression under Th17 differentiation condition was two-fold higher than the expression of *IL17A* in naïve CD4 T cells, supporting that CD4+ T cells were differentiated effectively in the presence of anti-CD3/anti-CD28/TGF-β/IL-6. The data further shows that inhibition of ITK significantly reduced *IL17A* expression, except in the presence of PMA/ionomycin, which bypasses ITK activity (Figure 4.12).

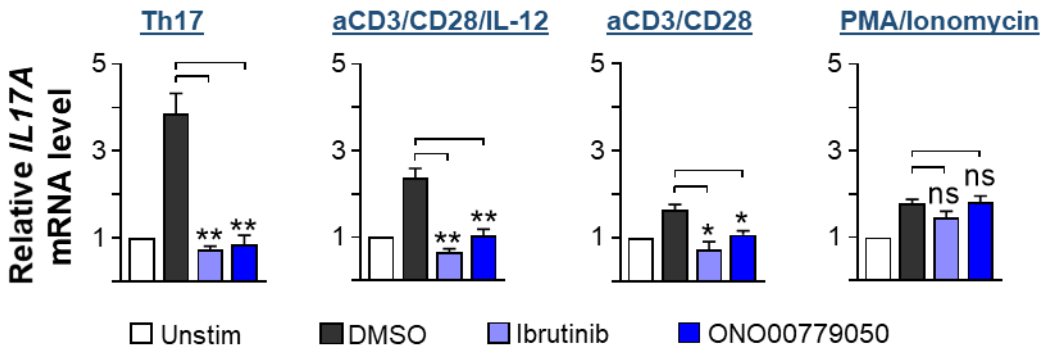


Figure 4.12. Inhibition of ITK in CD4+ T cells suppresses the expression of IL-17A. Isolated CD4+ T cells were pretreated with ITK inhibitors (1 μM) for 30 minutes, then the cells were stimulated for 2 days under four different conditions: 1) under Th17 differentiation conditions (anti-CD3/CD28 antibodies plus IL-6 and TGF-β1); 2) anti-CD3/CD28 antibodies plus IL-12; 3) anti-CD3/CD28 antibodies; 4) PMA/ionomycin. The expression level of mRNA IL-17 was measured by RT-PCR, and the data were normalised to unstimulated conditions. Data are the mean ± SEM from 2 independent experiments. *$P < 0.05$ and **$P < 0.01$ by two-tailed P value with t test. NS represents Not Significant.

By employing Itk-deficient mice (Gomez-Rodriguez *et al*., 2014), it has been suggested that Itk signalling specifically regulates the balance between Th17 and regulatory T cells

(Treg cells). To evaluate the role of ITK on the balance between Th17 and Treg cells, human tonsillar CD4+ T cells were stimulated with anti-CD3/CD28 plus IL-2 and TGF-β1 in the presence or absence of ITK inhibitors. The small molecule inhibitors, ONO-7790500 and Ibrutinib, were able to reverse the effects of Th17 differentiation conditions and convert a high percentage of IL17A expressing cells to FOXP3 expressing cells (Figure 4.13). The data suggest that ITK has a similar role in the differentiation of human tonsil CD4+ T-cells to that previously demonstrated in mice, and that small molecule ITK inhibitors can simulate effects observed by others with $Itk^{-/-}$ animals.

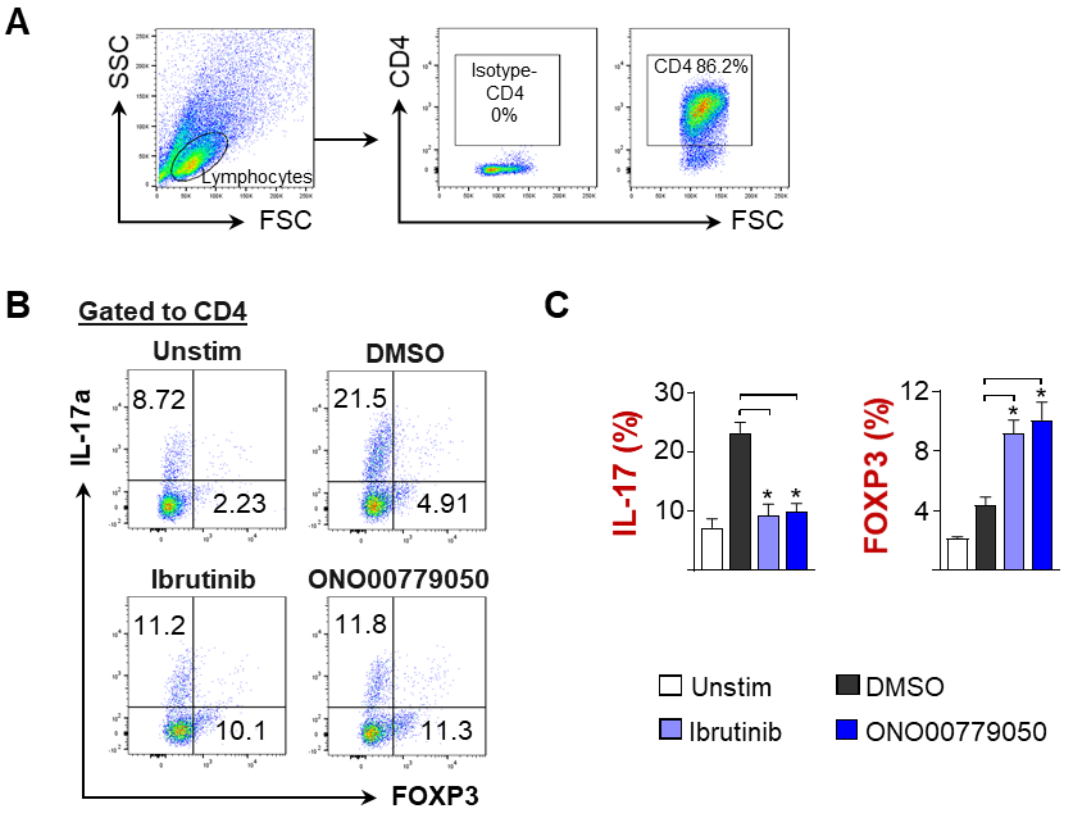


Figure 4.13. ITK inhibitors repress Th17 and induce Treg. Isolated CD4+ T cells from human tonsil were treated with ITK inhibitors under Th17 cell conditions (anti-CD3/CD28 plus IL-6 and TGF-β1) for two days. (A) Example of gating strategy to define CD4+ helper T-cells on DMSO condition. (B) IL-17 and FOXP3 expression were analysed by intracellular staining. (C) the bar charts demonstrate the percentages of both IL-17 and FOXP3 in Th17 cells. The data represent the mean ± SEM. Results of two independent experiments. *$P < 0.05$ by two-tailed with unpaired t test.

4.2.4.2 Th2 differentiation.

ITK is required for Th2 differentiation (Readinger *et al*., 2009; Fowell *et al*. 1999). To determine the role of ITK in Th2 cell differentiation, we used a small molecule inhibitor highly specific for ITK, ONO-7790500, and a non-specific inhibitor, Ibrutinib. Based on *in vitro* analysis, ONO-7790500 is two-fold more potent in inhibiting ITK than Ibrutinib and demonstrates less potency toward the Tec kinase BTK in B cells (Figure 3.8 in section 3.3.31 and Supplemental Fgure C). CD4+ T cells from human tonsil were again isolated, and treated with anti-CD3/CD28 plus IL-4, then analysed for cytokine production (IL-4) by intracellular staining (Miller *et al*. 2004). The expression level of IL-4 was increased (>20%) upon anti-CD3/CD28/IL-4 stimulation (Figure 4.14B). Addition of ONO-7790500 and Ibrutinib significantly diminished IL-4 production from 22.6% (vehicle) to 6.19% and 6.73%, respectively (Figure 4.14C), indicating that Ibrutinib and the selective ITK inhibitor ONO suppress the TCR-mediated production of IL-4 cytokine by Th2 cells. This analysis confirms that Ibrutinib and the more selective ITK inhibitor ONO-7790500 target ITK kinase activity in Th2 CD4+ T cells, to impair IL-4 production and inhibit activation of Th2 cells.

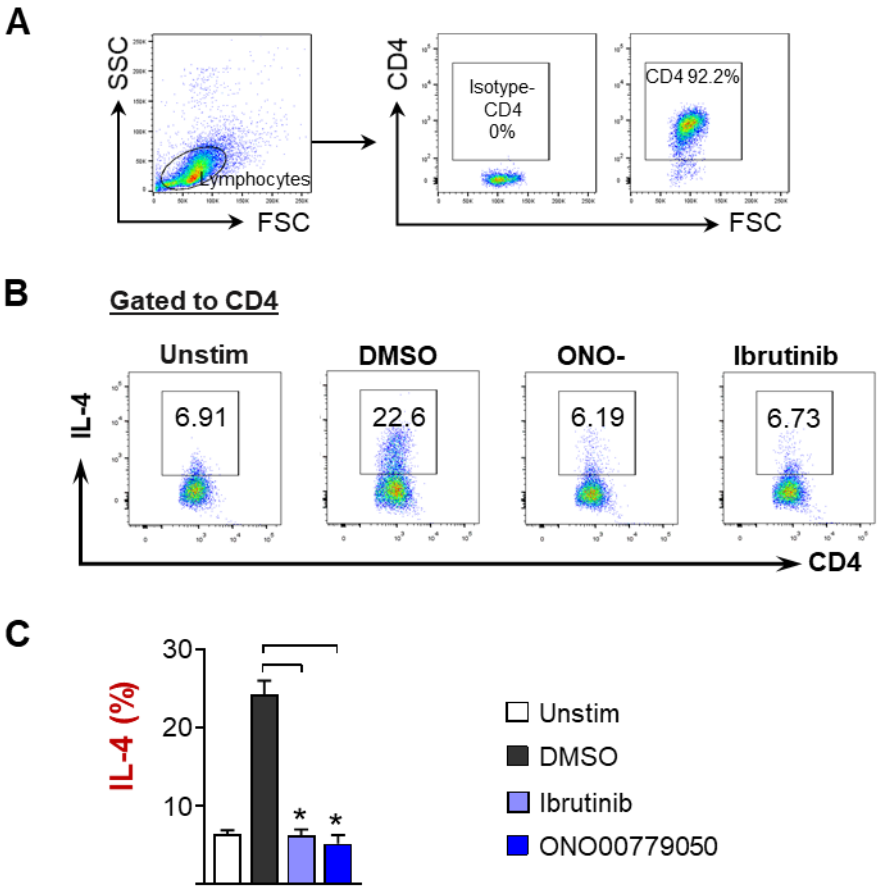


Figure 4.14. ITK inhibitors block CD4+ Th2 differentiation. (A) Example of gating strategy for CD4+ helper T-cells in patient samples on DMSO condition. (B) Purified human tonsillar CD4+ T cells were stimulated in Th2 polarised conditions in the presence of ONO00779050 and Ibrutinib at the concentration of 1 µM for 48 h. The cells were analysed by intracellular staining. DMSO was used as a untreated control. (C) The inhibitory effect of the compounds on IL-4 are shown from two independent experiments. Data are the mean ± SEM and are representative of two experiments. *$P < 0.05$ (two-tailed P value with Unpaired t test)..

4.2.4.3 ITK inhibitors do not inhibit Th1 cell differentiation.

Effector Th1 cell populations are important in producing interferon γ (IFN-γ) and interleukin 2 (IL-2) and evoking cytotoxic effects, which are essential for promoting cellular responses to intercellular pathogens (Readinger *et al*., 2009). Previous *in vitro* studies demonstrate that naïve Itk-deficient CD4+ T cells were defective in their capability to differentiate into Th2, but instead differentiate into Th1 cells (Miller *et al*., 2004). To determine effects of ITK inhibitors (ONO-7790500 and Ibrutinib) under Th1 polarising conditions CD4+ T cells were isolated from human tonsil and stimulated to obtain enriched cultures of IFN-γ–producing Th1 cells. Intracellular staining showed that IFN-γ expression increased ~2-fold under Th1 differentiation conditions (Figure 4.15). Deficiency of ITK did not impair Th1 differentiation.

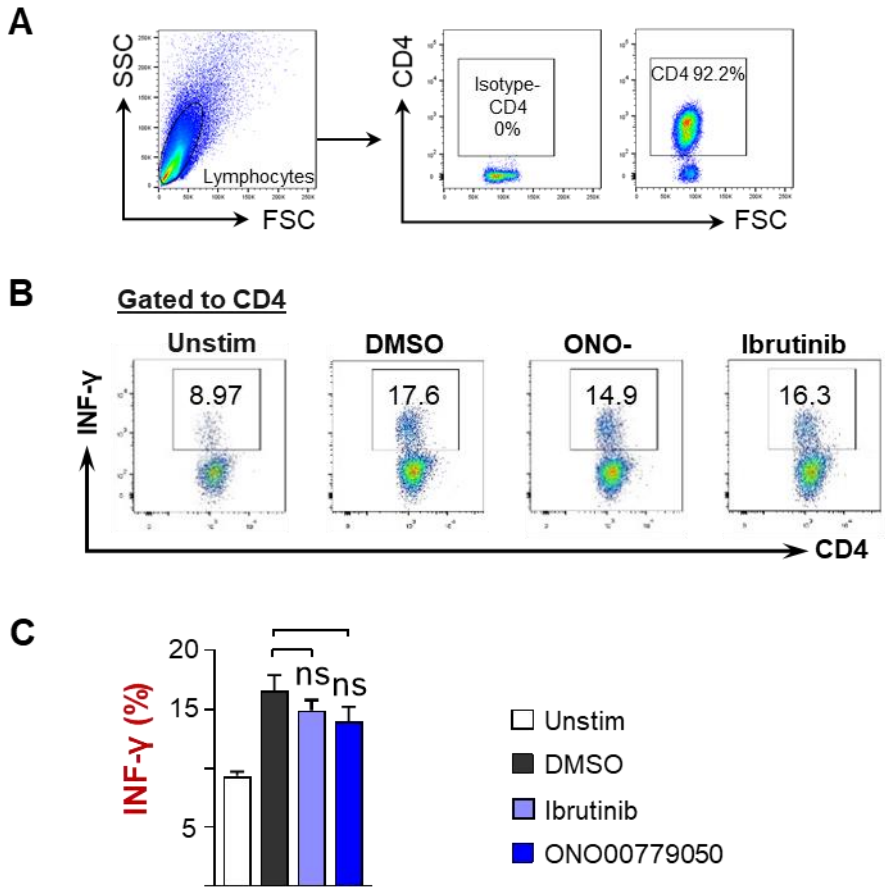


Figure 4.15. ITK inhibitors were not able to repress CD4+ Th1 differentiation. (A) Example of described the early steps of gating strategy for the identification of CD4+ T-cells in control (DMSO) condition. (B) Purified human tonsillar CD4+ T cells were stimulated in Th1 polarised conditions in the presence of ONO00779050 and Ibrutinib at the concentration of 1 µM for 48 h. The cells were analysed by intracellular staining. DMSO was used as a untreated control. (C) The inhibitory effect of the compounds on IFN-γ are shown from two independent experiments. Data are the mean ± SEM and are representative of two experiments. *$P < 0.05$ (two-tailed P value with Unpaired t test). NS represents Not Significant.

4.3 Discussion

The discoveries of Mosman and Coffman (Coffman, 2006; Mosmann and Coffman, 1989) showed that differentiated effector T-cell subsets develop from naive and undifferentiated T-cells. Since then many more polarised T-cell subsets have been described including Th9, Tfh and Th17 activating T-cell subsets and repressive Tregs (DuPage and Bluestone, 2016). ITK mediates signals from the T-cell receptor (TCR) and chemokines, and characterisation of animal models (Gomez-Rodriguez *et al*., 2014) suggest that it has roles in regulating the balance between Th17 and Treg cells in normal immunity and that this balance is functionally important. Indeed, ITK has been shown to mediate specific TCR signals to IL-17a through the transcription factor NFATc1 in order to drive Th17 differentiation (Gomez-Rodriguez *et al*., 2009).

During their lifetimes, T-cells can show both polarized functions and also phenotypic plasticity (Crotty, 2014; O'Shea and Paul, 2010; Oestreich and Weinmann, 2012). Some immunologists use the original description polarized T-cell subset because of the implication that effector status is the result of an active process that has a number of possible outcomes, and potentially there is flexibility in phenotypes over time (DuPage and Bluestone, 2016). Collectively, the more recent view is that T-cells can transition between phenotypes, not as a consequence of failed maintenance of a 'specified' programme, as implied by loss of lineage stability, but owing to a programmed retention of plasticity or the capacity of T cells to dynamically control their function in response to changing contexts.

Many studies have revealed that ITK signalling has a central role in the regulation of T-cell effector functions and T-cell differentiation (Fowell *et al*., 1999; Gomez-Rodriguez *et al*.,2014). Surprisingly however, it seems that the role of ITK in Tfh differentiation has not been investigated in mice deficient for Itk.

In this chapter the ability of ITK inhibitors to mimic the known results on Th17/Treg differentiation was initially investigated and it was apparent that the small molecules could indeed promote Treg differentiation of tonsillar T-cells at the expense of Th17 cell numbers. Next, the first studies of ITK inhibitors on tonsillar T-cell differentiation to Tfh cells were carried out and demonstrated that all four compounds tested reduced differentiation. This suggests that ITK has roles in polarisation to Tfh cells as well as its

known functions in Th17 and Th2 differentiation. This finding needs confirmation in Itk deficient mice.

Small molecule inhibitors also produced profound reductions in proliferation and increased levels of apoptosis. All the biological processes investigated i.e. apoptosis, proliferation and differentiation, must be occurring simultaneously in cell culture. It is possible that the increased proportion of Tregs plays some role in reducing proliferation and increasing apoptosis. However, apoptosis, which was significantly greater in Tfh rather than non-Tfh subsets only occurred up to ~ 40% and, therefore, it is possible that apoptosis occurs as a consequence of failed differentiation. ITK inhibitors also profoundly reduced proliferation. This effect might be responsible for the reduction in the proportions of differentiated Tfh cells but it is not likely to be the only explanation because inhibitors increased the proportion of Tregs. Overall, the work reported here demonstrates that ITK inhibitors can modify T-cell differentiation, possibly to produce a repressive environment with more Tregs, and also increase apoptosis and reduce proliferation.

IL-21 is known to have roles in the differentiation of Tfh cells (Vogelzang 2008; Nurieva 2008) as well as being required for B-cell differentiation. We found that ITK is required for TCR induced IL-21 production in CD4+ T cells at protein levels (Figure 4.6), and its expression in Jurkat T cells at the mRNA and protein levels (Figure in chapter 3.6 and 3.13A in chapter 3).

In order to investigate the mechanism by which ITK inhibitors repressed differentiation, we hypothesized that reduction of IL-21 secretion by the small molecule inhibitors had a role. In order to test this, we employed IL-21R-Fc, a chimeric fusion protein between IL-21R and the Fc portion of an antibody heavy chain. IL-21R-Fc will bind to and antagonise the effects of IL-21 in the culture medium. The chimeric protein partially inhibited Tfh differentiation suggesting that it plays some role but it is not sufficient to cause complete inhibition. This might be due to the effects of other cytokines, which are required in addition to IL-21. For example, Crotty (2014) states that IL-6 might compensate for the function of IL-21, and therefore, future experiments could explore the effects of IL-6. Overall, the data suggest that ITK in the plasticity of CD4+ T cells can be benefits for host immunity, and can play an important role in cancer and autoimmune diseases.

Chapter V. *In vitro* differentiation of primary human T-cell lymphoma cells and effects of ITK inhibitors.

5.1 Introduction

There has been very little work on primary human T-cell lymphoma because of the difficulties in obtaining material and because systems to culture these cells over long periods *in vitro* have not been developed. During the course of the work reported in this thesis, two patients with peripheral T-cell lymphoma, who had large numbers of accessible lymphoma cells presented at the Leicester Royal Infirmary. One patient had lymphoma cells circulating in the peripheral blood while the other had a highly cellular pleural effusion.

It was possible to isolate lymphoma cells and determine differentiation responses to combinations of growth factors as described for tonsil CD4+ T-cells in the previous chapter. In addition, in order to understand the dependence of these primary lymphoma cells on ITK as a possible therapeutic target, the effects of small molecule ITK inhibitors on proliferation, apoptosis and differentiation were determined.

5.2 Results

5.2.1 Characterisation of lymphomas

5.2.1.1 Tumours from CD4+ T cell patients exhibit T-cell clonality

The detection of clonal lymphoproliferations by polymerase chain reaction-based analysis of rearranged T-cell receptor (TCR) genes is an important technique in suspected lymphoproliferative disorders (Boone *et al*., 2013). The aim of clonality analysis is to examine the significant monoclonal expansion of T cells and correlate this information with histological and clinical data, helping to discriminate between malignant and reactive lymphoproliferation (Diss *et al*., 2011). The general rule is that malignant/tumour proliferations are monoclonal, and reactive proliferations are polyclonal (Levy *et al*., 1977). Analysis of T cell clonality targets T cell receptor (TCR) genes including TCR beta (TCRβ), TCR gamma (TCRG) or TCR delta (TCRD). During T lymphocyte development, variable (V), diversity (D), and joining (J) genes of antigen receptor gene complexes rearrange to yield diverse, functional coding sequences (Van Dongen *et al*., 2003). Random coupling between one of many variable (V), diversity (D), and joining (J) genes results in the formation of a V(D)J exon that encodes the actual antigen-binding moiety of the TCR chain (Langerak *et al*., 2012), see figure 2.2. The process of V(D)J recombination is specific to a particular clone which yields V(D)J exons of various size in different clones (Diss *et al*., 2011).

In this study, the rearrangement of V(D)J exons were amplified by using PCR and agarose gel electrophoresis (size analysis) as well as GeneScan (capillary analysis of fluorescently labelled products). A TCRβ gene clonality assay was performed on primary lymphoma $CD4^+$ T cells in patient #1 and patient #2. According to Neerad *et al*. (2012), approximately 95% (or >90%) of T lymphocytes express the αβ heterodimer, whereas the γδ heterodimer is expressed in the minority (or <10%) of T cells. Figure 5.1 demonstrates that the expanded clones are shown by dominant bands in the presence of clonal control DNA-IVS-0009 (Lane 2 and 4), representing monoclonal/malignant proliferations. Lane 1 and 3 represent polyclonal populations because they have more than three bands.

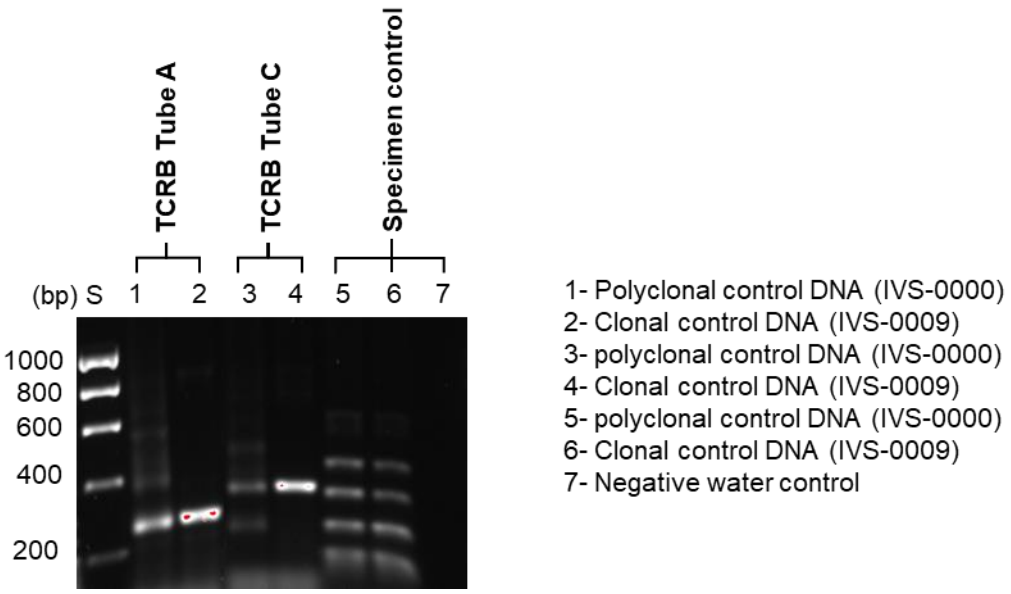


Figure 5.1. T cell receptor beta chain gene rearrangement assay for TCRB positive, negative and no template controls (TCRB T-cell clonality assay gel detection).
This figure shows agarose gel (1%) of TCRB PCR products. (S) is DNA size marker. Lane 1 and 2 are PCR products from TCRB tube A (Vβ + Jβ1/2). Lane 3 and 4 are PCR products from tube C (Dβ + Jβ1/2). Lane 5 and 6 represent Specimen control size ladder (Multi Genes). Lane 2 and 4 showing evidence of monoclonal T-cell expansion (reproducible dominant bands), whereas in lane 1 and 3 no clonal rearrangements are present (products with no dominant bands). According to Invivoscribe Technologies, valid size range for TCRB tube A is between 240-285 bp and for tube C ranges from 170-210 (Dβ2), or 285-325 (Dβ1). Lane 7 shows no template controls (water). IVS-0009 clonal control DNA displays a positive control DNA and IVS-0000 polyclonal control DNA displays a negative (normal) control DNA.

On a GeneScan analysis, polyclonal control for Vβ + Jβ1/2, Vβ + Jβ2, and Dβ + Jβ1/2 (primers-fluorescently labelled) were performed as negative controls (Table 5.1). Additionally, clonal Control DNA (IVS-0009) for target Vβ + Jβ1/2 (TCRβ tube A) and Dβ + Jβ1/2 (TCRβ tube C), and IVS-0004 for target Vβ + Jβ2 (TCRβ tube B) were shown as positive controls, see figure 5.2A, B and C. Finally, polyclonal Control DNA (IVS-0000) and clonal Control DNA (IVS-0009) were used for specimen control size ladder (Multiple Genes). Multiple Genes control ladder is essential because it ensures that sufficient quality and quantity of DNA was present to yield a valid result (Figure 5.2D). Notably, the product size (nt) is represented for all master mixed in Table 5.1.

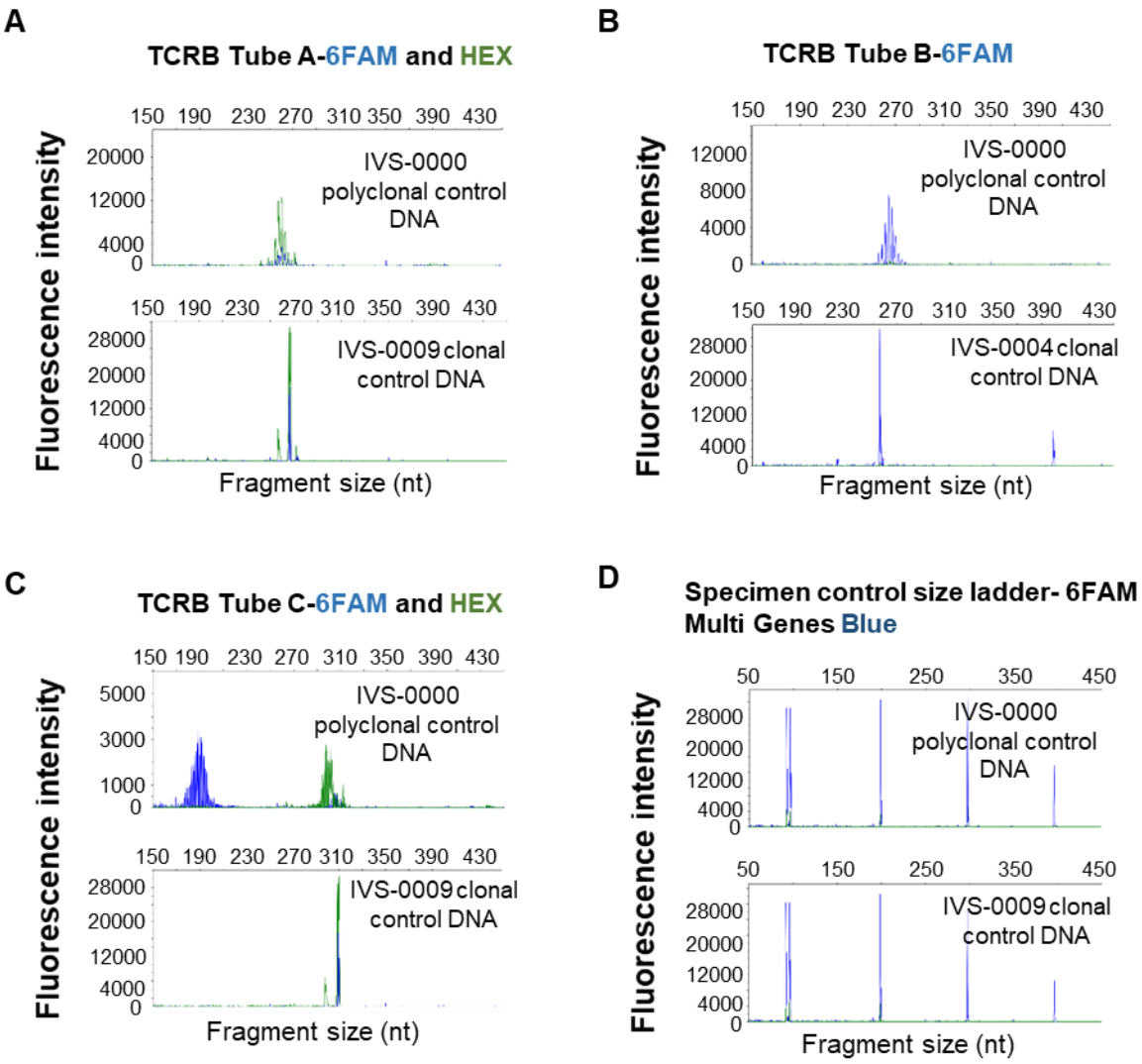


Figure 5.2. T cell receptor beta chain gene rearrangement assay for TCRB positive, negative and no template controls using GeneScan. The data demonstrated in this figure were generated by using the TCRB master mixes and TCRB positive (clonal control DNA), negative (plyclonal control DNA) and Specimen control size ladder (Multiple Genes). Amplified PCR products were run on an ABI3730 instrument. (A and B) A size range in nucleotides (nt) is around 250-270 bp for both tube A (**Vβ + Jβ1/2**) and tube B (**Vβ + Jβ2**) including positive and negative control. (C) In tube C (**Dβ + Jβ1/2)**, ABI fluorescence detection was detected two peaks, first peak represented in blue colour is around 190, whereas the second peak (green) is around 300. (D) The specimen control size ladder (Template amplification control master mix) targets multiple genes (housekeeping genes) and generates a series of amplicons of about 100, 200, 300 and 400 basepairs. Fluorescence detection the 600nt peak did not appear during normal run times, this is more likely due to a maximum DNA fragment in DNA size standard (carboxy-x-rhodamine, Rox dye) used in this study is 500 bp.

Master Mix	Target	Colour	Control DNA	Cat#	Product Size in Nucleotides
TCRβ Tube A	Vβ + Jβ1/2	**Blue (Jβ2.X)** + **Green (Jβ1.X)**	IVS-0009 Clonal Control DNA	4-088-0490	**240-285** 264
TCRβ Tube B	Vβ + Jβ2	**Blue (Jβ2.X)**	IVS-0004 Clonal Control DNA	4-088-0190	**240-285** 253
TCRβ Tube C	Dβ + Jβ1/2	**Blue (Jβ2.X)** + **Green (Jβ1.X)**	IVS-0009 Clonal Control DNA	4-088-0490	**170-210 (Dβ2), 285-325 (Dβ1)** 309
Specimen Control Size Ladder	Multiple Genes	**Blue**	IVS-0000 Polyclonal Control DNA	4-092-0010	**100, 200, 300, 400, 600**

Table 5.1. The amplicon sizes (product size in nucleotides) listed were determined using an ABI 3100 platform**.** Amplicon sizes on other specific capillary electrophoresis instruments are likely to differ 1 to 4 nucleotides (nt) from those listed in this table, depending on the version of the analysis software used and the platform of detection. (Invivoscribe Technologies, San Diego, USA).

To determine clonal T cell populations in our patients (patient #1 and patient #2), genomic DNA from patient specimens was extracted in purified CD4+ T cells. Clonality of tumour CD4+ T cells was examined by PCR amplification of TCRβ genes using GeneScan. Clonal rearrangements were found in both patients (patient #1 and patient #2). A clonal peak of TCRβ genes was only found in Vβ + Jβ2 genes in patient #1, whereas the clonal TCRβ genes were observed at Dβ + Jβ1/2 rearrangements in CD4+ T cells from patient #2 (Figure 5.3B and C). Clonal samples appear as dominant peaks, and peaks around 300 bp and 270 bp in patient #1 and patient #2 CD4+ T cells, respectively. Together, these data suggest that expanded cell clones in our patients may precede tumour development. Moreover, the GeneScan analysis also shows that polyclonal CD4+ T cell populations at Vβ + Jβ1/2 gene rearrangement in both patients, and peaks are between 230-270 bp, as seen in Figure 5.3A. The data further demonstrates that TCRβ which represents Vβ + Jβ2 in patient #1 Dβ + Jβ1/2 in patient #2 failed to detect any clonality expansions in lymphoma CD4+ T cells. No template controls (water) were also performed for all three master mixes as indicated in Figure 5.3D. No template controls displayed no peaks in any of the master mixes, indicating there is not any possible contamination problem with the master mixes used in this study.

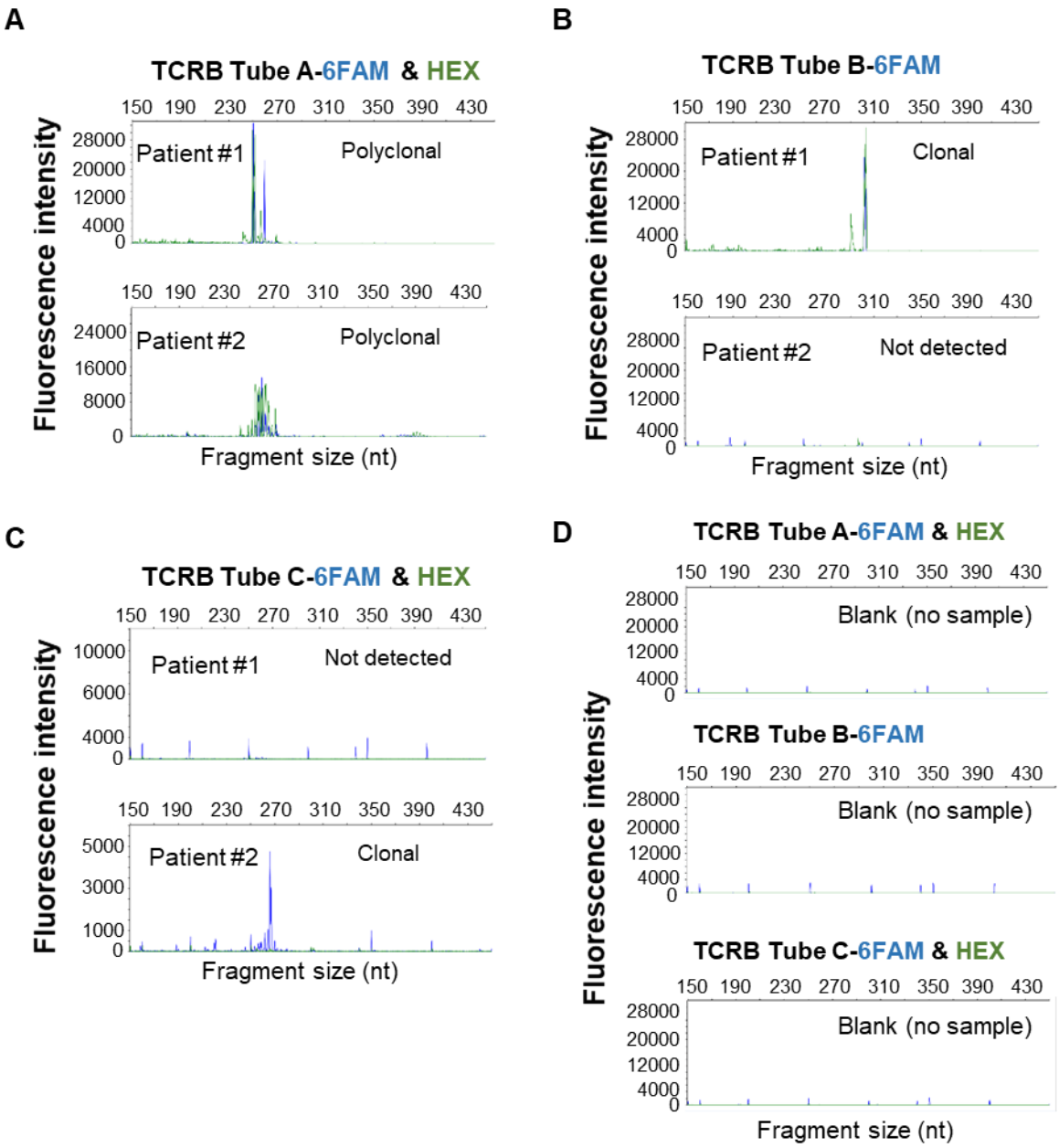


Figure 5.3. GeneScan analysis of TCRB PCR gene rearrangement for genomic DNA in primary T cell lymphoma cells (GeneScan fragment analysis).
Three TCRB primers were used in this assay, tube A (**Vβ + Jβ1/2**), tube B (**Vβ + Jβ2**) and tube C (**Dβ + Jβ1/2**), see table X. After amplified the samples on a thermocycler using PCR (see M and M, section X), ABI capillary electrophoresis instrument (ABI3730) was performed to detect T-cell clonality in patients. (A) A size range for tube A in nucleotides (nt) is around 250-270 bp. A polyclonal peak for TCRB-Vβ + Jβ1/2 is identified in both patient #1 and patient #2. (B) A dominant single peak (clonal) is detected for tube B in patient #1 and a range size is ~ 300 bp, whereas no peaks have been detected in patient #2. (C) Opposite to tube B master mix, no peak is seen for tube C in patient #1, whereas the clonal peak is identified for lymphoma DNA in patient #2. (D) No template control (water) was tested for all master mixes (tube A, B and C) to ensure the proper sterile technique, and GeneScan analysis didn't identify any peaks, evidence of no cross contamination/contamination in the master mixes and buffers.

5.2.1.2 Patients' clinical histories

Patient #1

The patient presented at 36 years old in December 2015 with lymphadenopathy and systemic symptoms including weight loss and night sweats. Lymph node biopsy showed a diagnosis of PTCL-NOS. She received 3 cycles of CHOP (cyclophosphamide, hydroxydaunorubicin, vincristine, prednisolone) combination chemotherapy followed by two cycles of IVE (ifosphamide, etoposide, epirubicin) chemotherapy and then proceeded to LEAM (lomustine, etoposide, cytosine arabinoside, melphalan) conditioned autologous stem cell transplant in June 2016. Following this PET-CT scan was reported as showing complete metabolic remission. However, in February 2017 she relapsed with oedema, ascites and pleural effusions and lymphoma cells were present in the peripheral blood. The PI3Kdelta inhibitor, idelalisib, was tried as was further chemotherapy with GDP (gemcitabine, dexamethasone, cisplatin) but without success. The patient did not wish to have further treatment and she was treated with supportive care only and died after a few weeks.

Patient #2

The patient was a 72-year old woman who presented in October 2014 to the respiratory physicians with eosinophilia. She was found to have lymphadenopathy and a peripheral blood population of monoclonal T-cells. A lymph node biopsy showed AITL. The patient had several comorbidities including chronic obstructive pulmonary disease and bronchiectasis. She was treated with 6 cycles of CHOP combination chemotherapy and achieved an almost complete remission (CRu) on CT scan. The remission lasted about 18 months but she then re-presented with rising peripheral blood lymphocyte count, pruritus and skin lesions. Lymph node biopsy confirmed relapsed AITL. She was treated with steroids followed by palliative chemotherapy (gemcitabine and dexamethasone) but without response. The patient was then treated supportively only and died a few weeks later.

5.2.1.3 ITK inhibitors inhibit ITK signalling.

To confirm that TCR-induced activation of ITK purified CD4+ T cells from T cell lymphoma patients (n=2) were stimulated with anti-CD3 and anti-CD28 antibodies. Whole cell-lysates were analysed by western blot. The blots showed that anti-CD3/anti-CD28 significantly increased ITK expression compared to unstimulated cells in CD4+ T lymphoma cells (Figure 5.4A and B). To confirm that ITK inhibitors prevent ITK phosphorylation, purified cells were treated with four ITK inhibitors following anti-CD3/anti-CD28 stimulation. All four inhibitors block ITK activation, but ONO-7790500 and BMS509744 appear slightly less effective than PF-6465469 and Ibrutinib, as seen in Figure 5.4C.

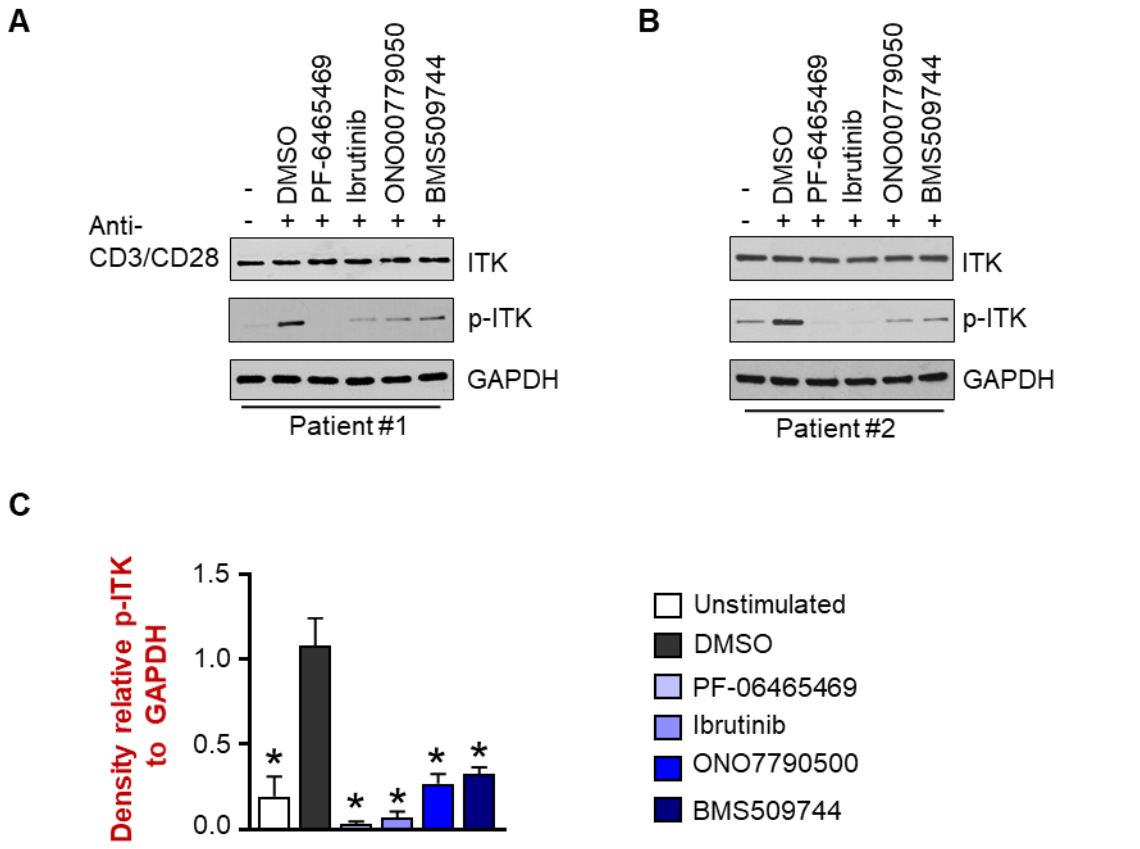


Figure 5.4. In primary T-cells, ITK inhibitors (PF-06465469, Ibrutinib, ONO7790500 and BMS509744) target ITK. (A and B) Western blot analysis of freshly purified ITK inhibitors pre-treated primary CD4+ T-cells from patients with T-cell lymphoma, unstimulated and anti-CD3/CD28 stimulated, whole-cell lysates. Blot probed for ITK, phospho-ITK in patient #1 and patient #2. (C) Densitometric ratios between phospho-ITK and GAPDH are provided in both patients using ImageJ. Error bas represent SEM, (significance was considered for *,$P < 0.05$ by using unpaired two-tailed t test).

5.2.2 Primary T-cell lymphoma differentiation

After purification of naïve CD4+ T cells from patients, the cells were cultured and activated by plate-bound anti-CD3 and anti-CD28 antibodies. Purity of CD4+ T cells was over 90% (Figure 5.5).

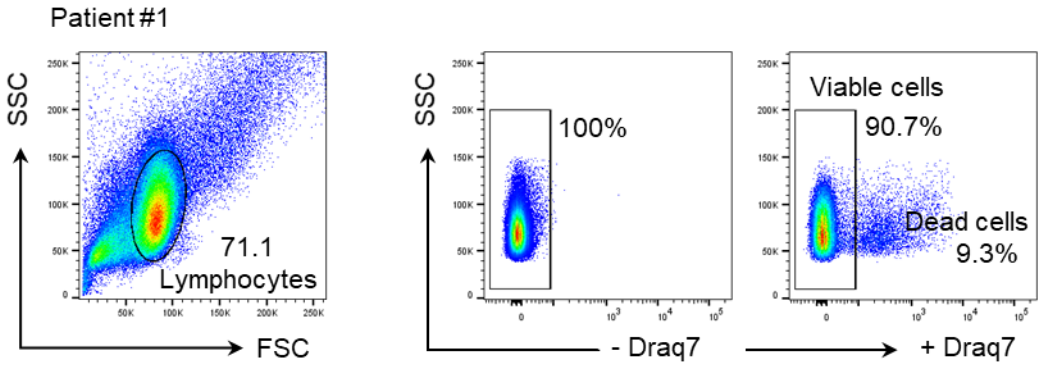


Figure 5.5. Flow cytometric analysis demonstrating purification of CD4+ cells. (A) CD4+ cells were purified from pleural effusion and (B) CD4+ cells were purified from peripheral blood. After isolation, cell populations were >90% pure, and the data was analysed by FlowJo.

Isolated cells were incubated in the presence of the following conditions as previously described in details in chapter 2, Section 2.3.2: Tfh (5 ng/mL IL-12), Th17 (20 ng/mL IL-6, 5.0 ng/mL TGF-β) and Treg (5.0 ng/mL TGF-β). To determine the Tfh phenotype, naïve CD4+ T cells were stained with CXCR5 and PD-1. CD4+ T cells were stained for CD25, FOXP3, and IL-17A for determination of regulatory T cells (Treg) and Th17 cells, respectively. In all conditions, viable cells were gated on CD4+ lymphocytes (Figure 5.6). For proliferation assay, cells were stained with CellTrace violet (CTV), washed, and then were plated for five days to investigate Tfh proliferation. All CD4+ T helper cells (Tfh, Th17 and Treg) were significantly stimulated under their polarised conditions, as is seen in the next sections of this chapter.

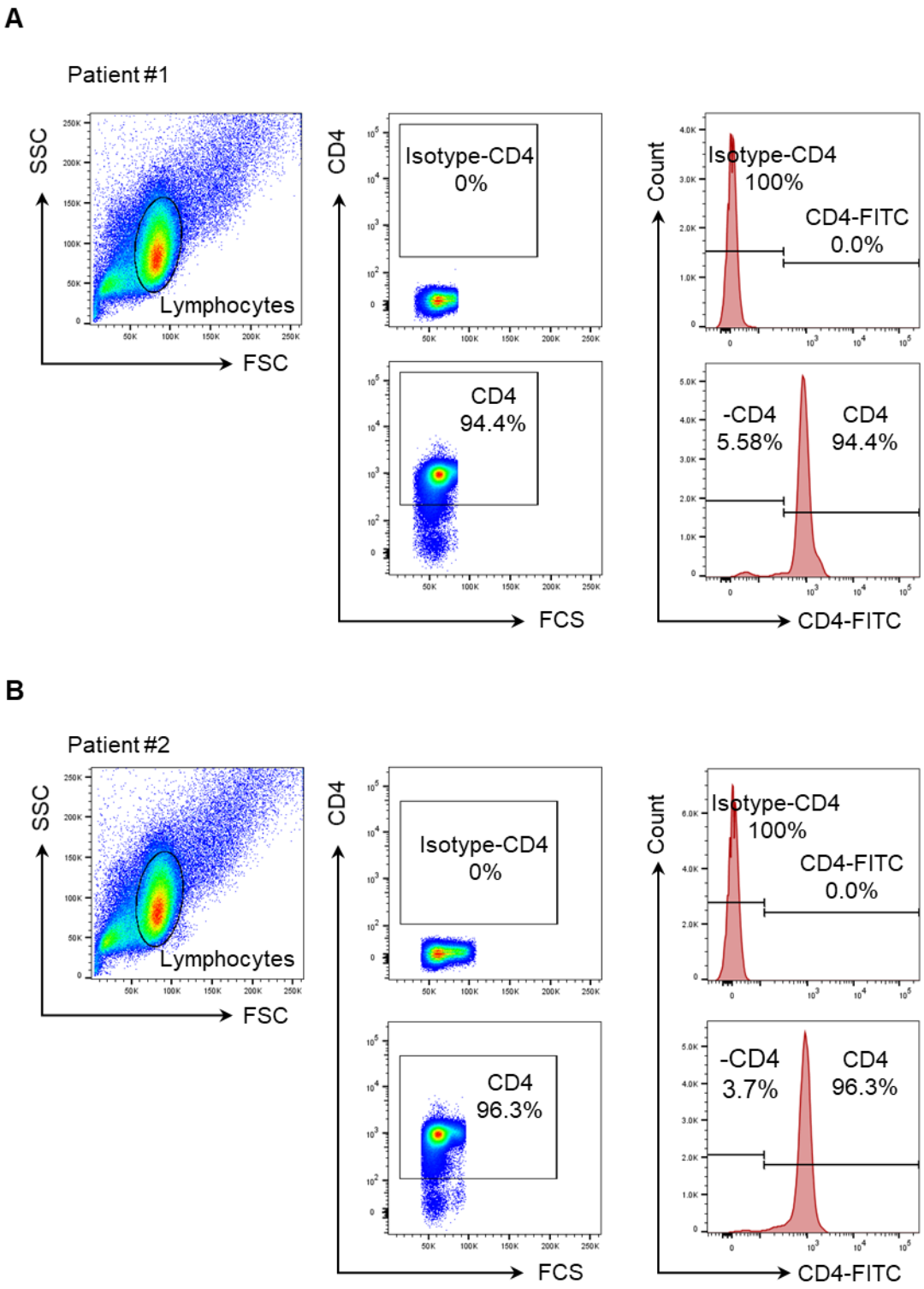


Figure 5.6. Example of selection of viable cells prior to analysis of proliferation.
Selection of cells based on the forward scatter (FSC) and side scatter (SSC). To exclude dead cells, Draq7 was added to cell suspension before acquisition on a FACSAria. Viable cells, which are negative for Draq7 dye, were selected for Tfh cells gating strategy in lymphoma $CD4^+$ T cells.

5.2.3 ITK inhibitor effects

5.2.3.1 T-cell Differentiation

Tfh Differentiation

To examine the effect of ITK inhibitors on Tfh cell differentiation *in vitro*, purified CD4+ T cells were cultured under Tfh differentiation conditions (anti-CD3/CD28 plus IL-12) for 5 days. For identification of Tfh cells, cells were stained with anti-CD4, anti-CXCR5 and anti-PD-1 antibodies prior to analysis. In this experiment, dead cells (defined as Draq7+) were excluded. After 5 days, cells were harvested and flow cytometry analysis showed that the percentage of tumour Tfh cells ($CD4^+PD1^+CXCR5^+$) accounted for 41% of cultured cells and ITK inhibitors (Ibrutinib or ONO-7790500) significantly reduced this fraction to ~ 25% in response to anti-CD3/CD28 + IL-12 (Figure 5.7), suggesting that ITK as a key regulator of Tfh induction.

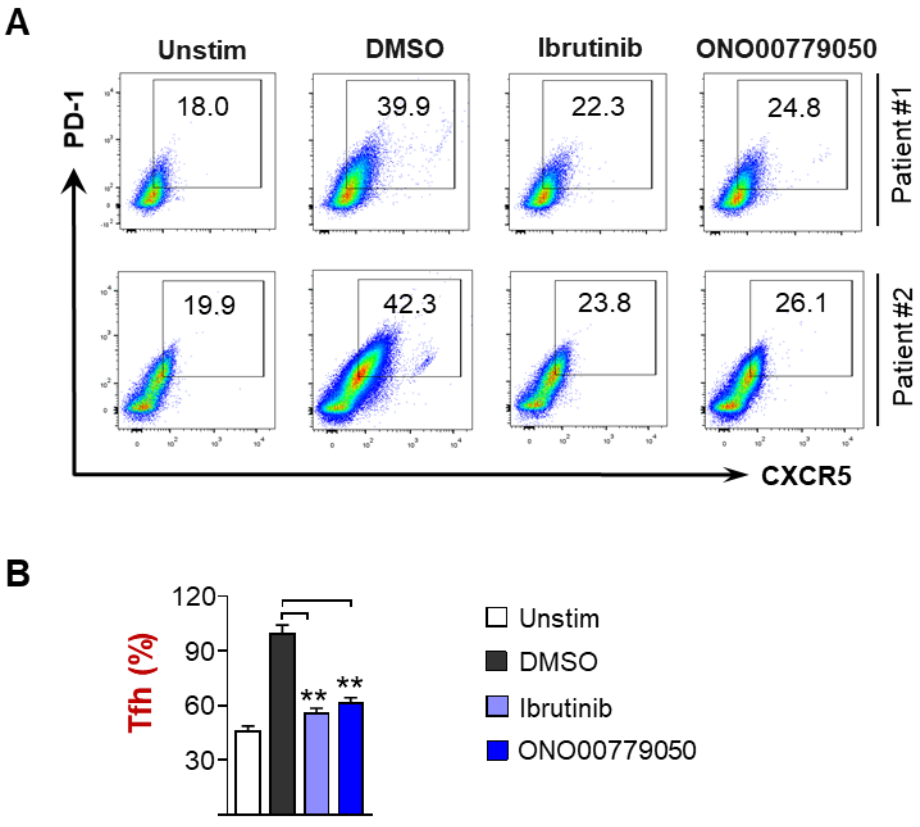


Figure 5.7. Primary human lymphoma cells have the capacity to differentiate to Tfh cells, which is reversed by ITK inhibitors. (A) Purified CD4+ T cells from patients with T cell lymphoma were stimulated in Tfh polarising conditions (anti-CD3/anti-CD28 antibodies plus IL-12) in the presence of ITK inhibitors or vehicle (DMSO) for 6 days. Dot plots show CD4 and CXCR5 expression (B) Bar chart showing CD4+PD-1+CXCR5+ Tfh cells (%). Error bars result from two independent experiments show mean ± SEM. $P < 0.01$** (Unpaired t-test).

Treg Differentiation

To investigate the effect of ITK inhibitors in the differentiation of Treg cells *in vitro*, CD4+ T cells were purified from T cell lymphoma (TCL) patients (n=2). Differentiation of naïve CD4+ T cells under Treg cell conditions (anti-CD3/CD28 plus TGF-β1 in the presence of IL-12) was evaluated by intracellular staining. As seen in Figure 5.9, a significant increase in Treg cells was observed in ITK inhibitor treated cells compared to DMSO (vehicle). After treatment of cells with ITK inhibitors, we observed that Treg cells gave rise to a significantly increased proportion of FOXP3 + CD25 + CD4 Treg cells from 7% (DMSO) to 11.5% with Ibrutinib and 12.9% with ONO-7790500 (Figure 5.8). The data suggest that ITK is essential in regulation of Treg differentiation, and it contributes to the functional stability of Tregs in T cell lymphomas.

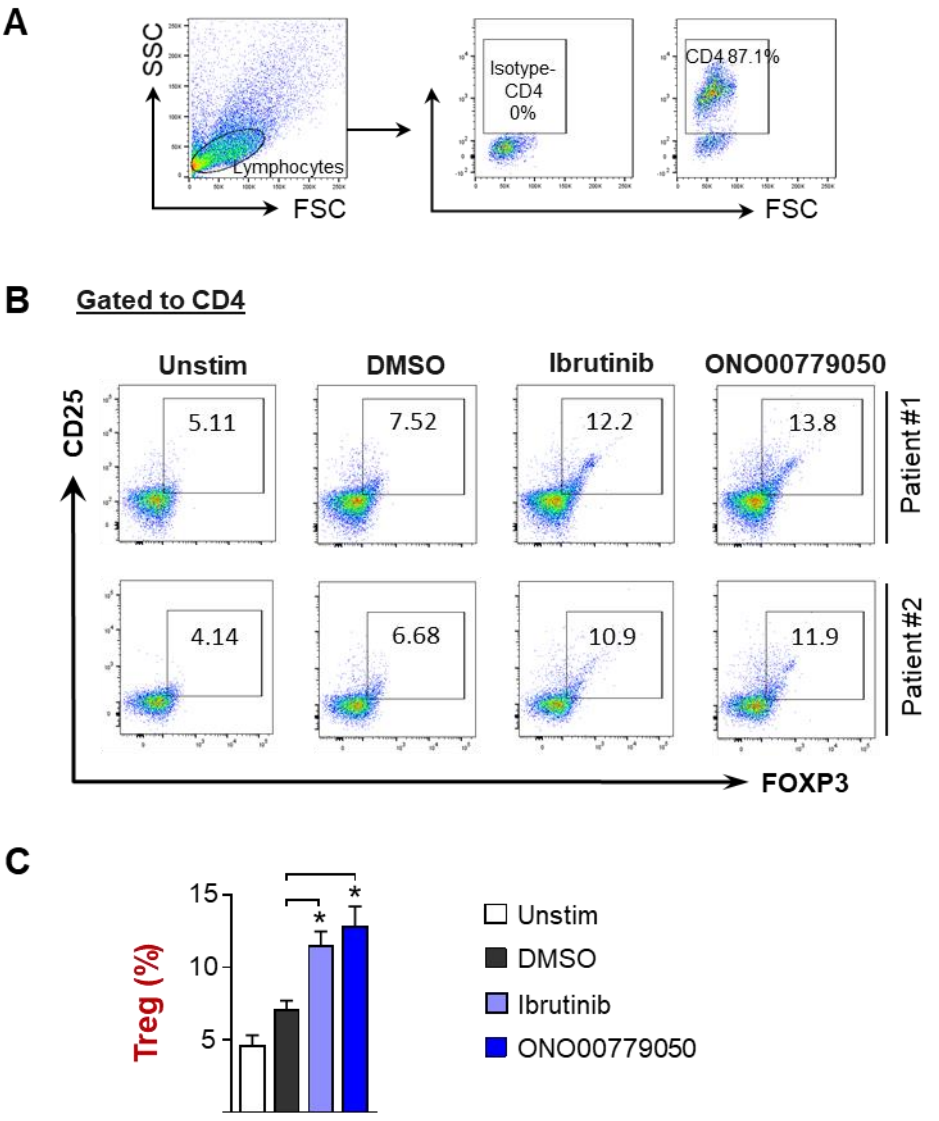


Figure 5.8. ITK inhibitors promote Treg differentiation of primary human lymphoma cells. (A) Example of gating strategy for the identification of CD4+ T-cells is shown on control (DMSO) condition. (B) CD4+ T cells were cultured under Treg differentiating conditions (anti-CD3/CD28 antibodies, IL-12 plus TGF-β1) for 2 days in the presence of ITK inhibitors or vehicle (DMSO) and expression of CD25+FOXP3+ CD4+ cells was determined. (C) The bar chart shows Treg cells (%) from two independent experiments. Data are mean ± SEM. $P < 0.05$ * (Unpaired t test).

Th17 Differentiation

Th17 cells are a particular subset of T helper cells which are characterised by high expression of IL-17. This subset accumulates specifically in many various tumours compared to healthy tissues (Kryczek *et al*., 2009; Su *et al*., 2010; Chen *et al*., 2012; Li *et al*., 2012), although the outcome might differ from tumour type to another. So far the role of ITK in lymphoma/tumour Th17 cells is unknown. To explore the role of our ITK

inhibitors in tumour Th17 cells, small molecule inhibitors; Ibrutinib and ONO-7790500 were employed on purified CD4+ T cells from patients with T cell lymphoma. The potential effect of both inhibitors was assessed on polarised Th17 cells after systematic stimulation of cells with anti-CD3/CD28 plus IL-6 and TGF-β1 (Gomez-Rodriguez *et al*., 2014). Analysis of IL-17 cytokine production was performed by intracellular staining. Figure 5.9 demonstrates that the expression of IL-17 levels was increased over two-fold upon anti-CD3/CD28 stimulation compared to unstimulated cells. Figure 5.9 also showed a significant reduction of IL-17 production in ITK-deficient Th17 cells from 21.8% and 27.8% (DMSO) to 8.74% and 14% in patient #2 and patient #1 cells, respectively. We observed that ONO07790500 appears slightly less effective than Ibrutinib on Th17 differentiation. Our phenotypic analysis confirms that Ibrutinib and ONO-7790500 impair IL-17 production and inhibit activation of Th17 cells.

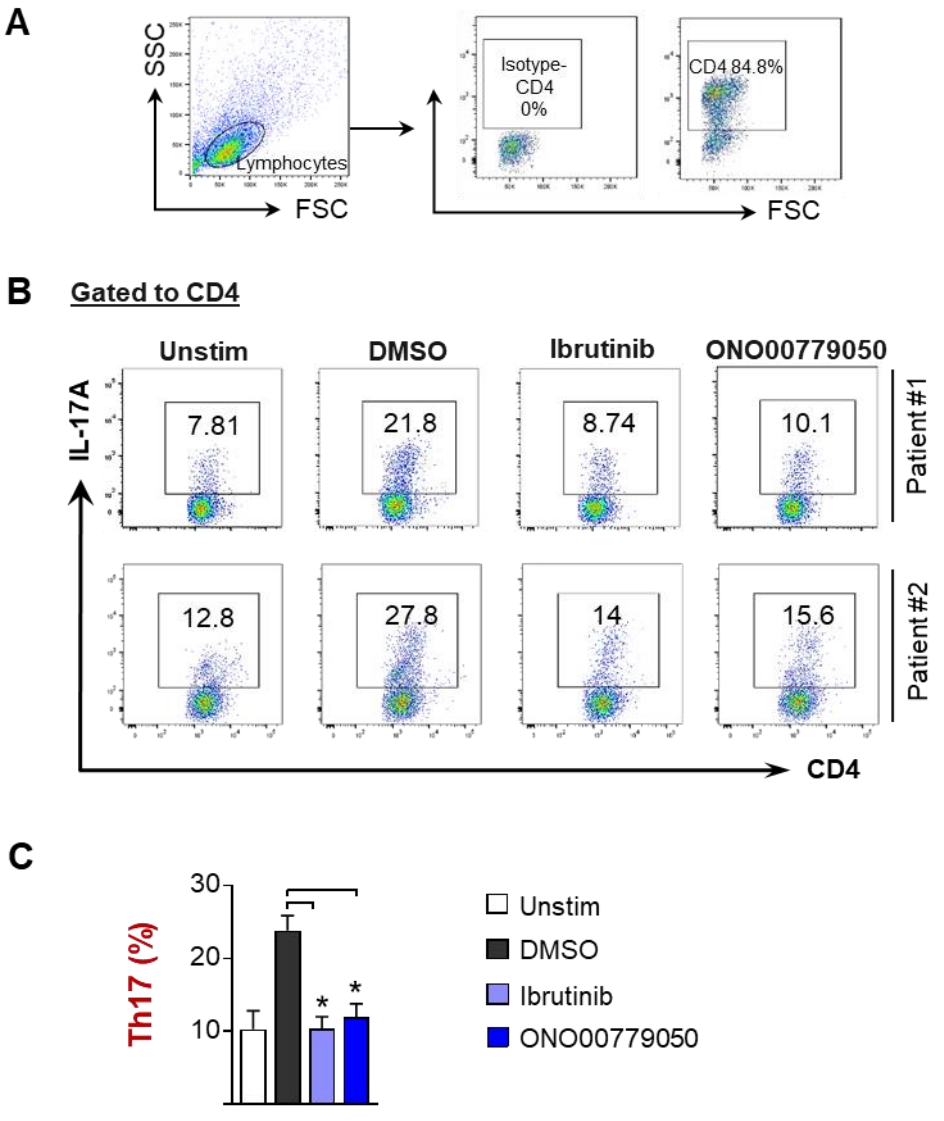


Figure 5.9. Primary human lymphoma cells have the capacity to differentiate to Th17 cells, which is reversed by ITK inhibitors.
(A) Example of gating strategy to get access to CD4+ T-cells is demonstrated on control (DMSO) condition. (B) CD4+ T cells were stimulated under Th17-polarising conditions (anti-CD3/CD28 antibodies, IL-6 and TGF-β1) in the presence of ITK inhibitors, ONO00779050 or Ibrutinib (1 μM), or vehicle control (DMSO) for 2 days. IL-17A and CD4 expression was determined. (C) Bar chart shows Th17 cells (%) from two independent experiments mean ± SEM. $P < 0.01$ ** (Unpaired t test).

5.2.3.2 Effects on apoptosis

To investigate the effect of ITK inhibitors on the induction of apoptosis, primary T-cell lymphoma cells from patients were treated with ONO-7790500 and Ibrutinib upon anti-CD3 and anti-CD28 antibodies at different concentrations (0.2 and 1 μM). Cells were harvested at the indicated time points, as seen in Figure 5.11. The percentage of induction of apoptosis was determined by flow cytometry after staining with Annexin-V and Draq7. Figure 5.10 indicated that there is not any significant level of apoptosis induction in T cell lymphoma cells upon treatment with either ONO-7790500 or Ibrutinib at 24 and 72 h. Results from flow cytometry analysis also shows that cells were undergoing apoptosis with these agents under *in vitro* culture conditions at 6 days, and we did not observe differences between the effect of ONO-7790500 and Ibrutinib. The results indicated that lymphoma T cells were not sensitive to ITK inhibitors after 24 and 72 h incubation in this study, albeit ITK inhibitors induced apoptotic cells after prolonged incubation time.

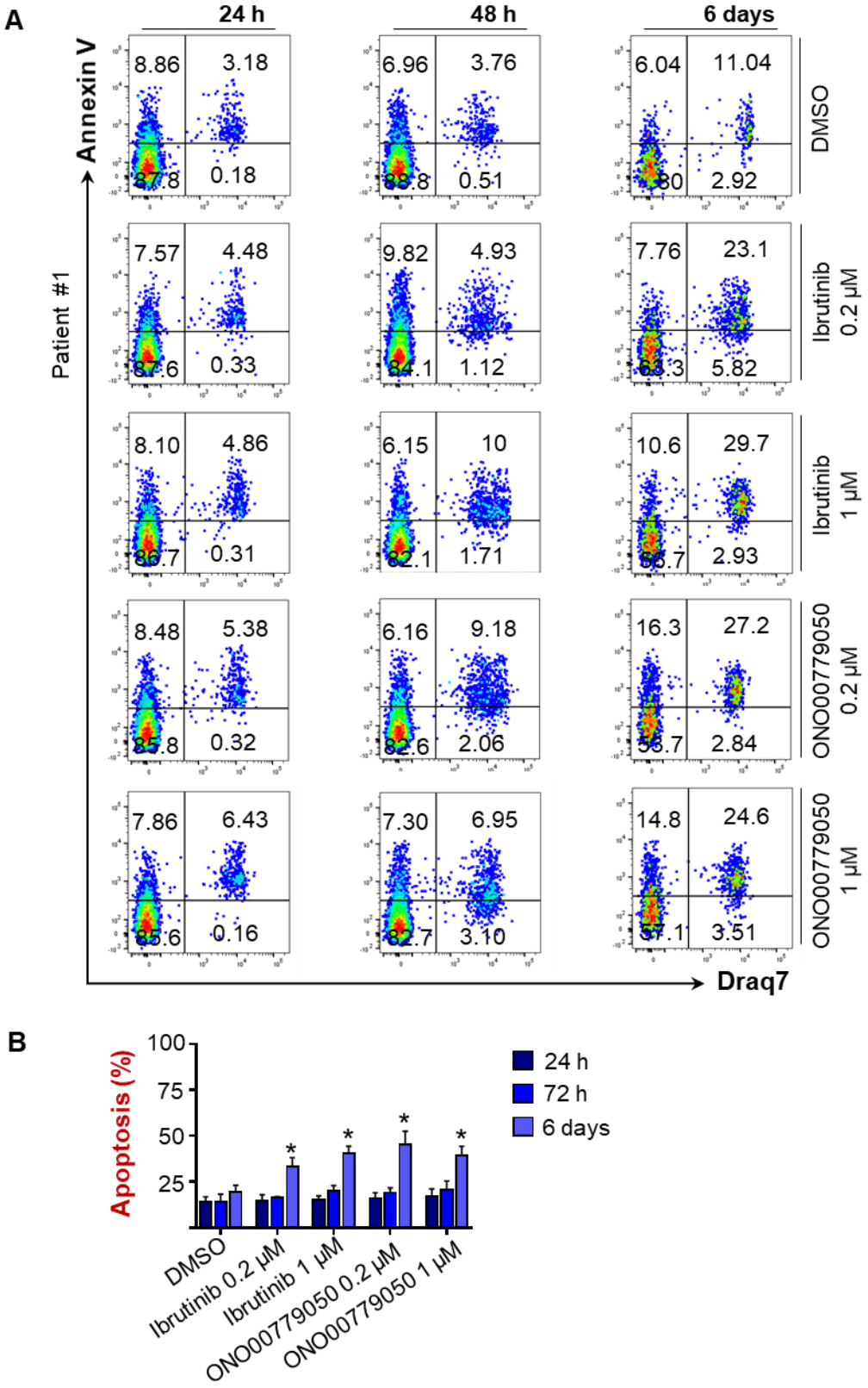


Figure 5.10. ITK inhibitors cause relatively little apoptosis.
CD4+ T cells were cultured in the presence of ITK inhibitors (0.2 or 1 µM) or vehicle (DMSO) and stimulated with anti-CD3/anti-CD28 antibodies at different time points (1, 3 and 6 days). (A) The graphs show the effect of ITK inhibitors on cell apoptosis (B) Apoptosis (%) is shown in bar chart. The data were analysed from two independent experiments and values are expressed as mean ± SEM. P value calculated by two-tailed t test $P < 0.05$ *.

5.2.3.3 Effects on proliferation

Given the inhibitory effect on TCR signalling, we sought to determine the effect of ONO-7790500 and Ibrutinib on tumour CD4+ T cell proliferation *in vitro*. Lymphoma CD4+ T-cells isolated from patients with T-cell lymphoma (n=2). Purified CD4+ T cells were labelled with CellTrace Violet (CTV), then were pretreated with ITK inhibitors or DMSO for 5 days to determine cell proliferation. After harvesting cells, CD4+ T cells were stained with anti-CD4, anti-CXCR5 and anti-PD1 antibodies to identify the CD4+ Tfh subset, and the effect of ITK inhibitors on cell growth was analysed by flow cytometry. Without addition anti-CD3/CD28 antibodies plus IL-12, we could not detect CD4+ T cell proliferation (Figure 5.11). However, the addition of anti-CD3/CD28 antibodies and IL-12 resulted in cell proliferation, with obvious the proliferative capacity of CD4+ T cells into 5 divisions and peaks. As seen in Figure 5.11, both Ibrutinib and ONO-7790500 effectively reduced cell proliferation to basal level (unstimulated cells) after day 5, with slightly less effect with ONO-7790500. The data suggest that ITK is required to maintain lymphoma/tumour T cell.

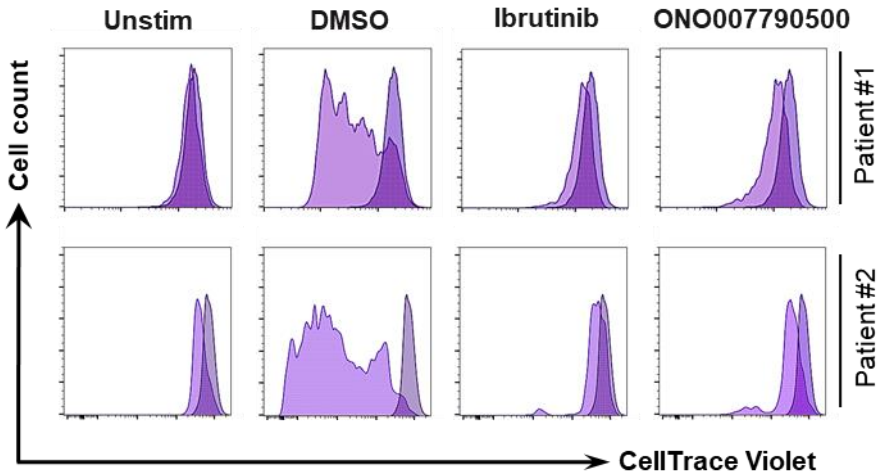


Figure 5.11. ITK inhibitors Ibrutinib and ONO-007790500 prevent proliferation due to anti-CD3/anti-CD28. Isolated CD4+ T cells from were stained with CellTrace Violet (CTV). The cells were cultured in the presence of ITK inhibitors (1 µM) or vehicle (DMSO) and stimulated with anti-CD3/anti-CD28 antibodies plus IL-12 for 5 days. Histograms show CTV of CD4+PD-1+CXCR5+ cells.

5.3 Discussion

There is interest in the use of small molecule inhibitors for the treatment of cancer. There are relatively few diseases in which a protein known to have a causal role in a disease can be targeted by a small molecule. An example is chronic myeloid leukaemia in which the BCR-ABL fusion protein, which is only expressed in leukaemic cells and is also fundamental to leukaemic cell survival and proliferation, can be targeted by tyrosine kinase inhibitors of which the first example was imatinib.

Structural rearrangements of ITK are rare in T-cell lymphoma but a translocation between chromosomes 5 and 9 producing an ITK-SYK fusion protein occurs in about 20% of a rare entity follicular Tfh lymphoma (Swerdlow *et al*, 2016). The ITK-SYK fusion protein appears to have an important role in lymphoma development (Pechloff *et al*, 2010) and in theory could be targeted by a small molecule inhibitor. However, ITK is expressed in AITL and probably all of the new designation of Tfh lymphoma (Agostinelli *et al*, 2014) from an un-rearranged locus.

ITK has fundamental roles in both TCR signalling and chemokine signalling (see Introduction pages 21-26 Section 1.8.3) in normal T-cells. It is also essential for normal T-cell differentiation and has been shown to be required for Th17 and Th2 differentiation. Itk deficient mice show skewed T-cell differentiation such that Th17 differentiation is inhibited and Treg differentiation is promoted (Gomez-Rodriguez *et al*, 2014).

The role of ITK in Tfh cell differentiation have not been investigated previously but in this thesis it is demonstrated that ITK inhibitors repress the Tfh differentiation of tonsillar CD4+ T-cells while promoting Treg differentiation. This raises the question of whether ITK is able to slow or prevent the development of autoimmune disease by repressing Tfh cells as this subset is required for autoimmunity (Linterman *et al*, 2009) or whether it has a role as a potential therapeutic in T-cell lymphoma.

There is very little literature investigating human primary peripheral T-cell lymphoma, which adds some importance to the patients studied here. Both were unusual in that relatively large numbers of lymphoma T-cells could be isolated for *in vitro* experiments. In addition, both patients expressed ITK. Interestingly ITK was not noticeably phosphorylated at baseline but only in anti-CD3/anti-CD28/IL-12 culture conditions. This suggests that in patients TCR signalling might also be required to activate ITK and

drive its functions in modifying gene expression and promoting proliferation. Also, surprisingly, the lymphoma cells appeared to show some capacity to differentiate both towards Tfh cells and to Th17 cells. A greater proportion of cells showed Tfh characteristics under anti-CD3/anti-CD28/IL-12 culture conditions than showed Th17 features under anti-CD3/anti-CD28/IL-6/TGF-β1 conditions. It is clear that the lymphoma cells demonstrate some plasticity in differentiation. It is difficult to interpret the proportions of cells that differentiate towards specific subsets but possibly the lymphoma cells show a preference towards Tfh differentiation.

ITK inhibitors appear to reproduce the effects seen in $Itk^{-/-}$ mice (in which Tfh differentiation was inhibited while that of Tregs was promoted) in tonsillar CD4+ T-cells suggesting that this is an on-target effect of the small molecules. The inhibitors demonstrated similar effects in lymphoma cells in inhibiting Tfh differentiation while allowing Treg differentiation. The intriguing possibility generated by this data is that *in vivo*, ITK inhibitors will change the differentiation potential from activating Tfh to repressive Treg. Ibrutinib, for which there is extensive safety data from the treatment of patients with chronic lymphocytic leukaemia, is being trialled in the USA in T-cell lymphoma (A Multicenter Phase I Study of Ibrutinib in Relapsed and Refractory T-cell Lymphoma, NCT02309580) with recruitment set to end in December 2018.

ITK inhibitors caused only modest apoptosis and prevented anti-CD3/anti-CD28 mediated proliferation of lymphoma cells. Previously published data demonstrates the low efficacy of ITK inhibitor (BMS-509744) and dual inhibitor ITK and RLK inhibitor (PRN694) on T cell apoptosis (Dondorf *et al*., 2015; Zhong *et al*., 2015). ITK inhibitors might, therefore, have multiple modes of action in T-cell lymphoma being anti-proliferative and pro-apoptotic agents but they might also alter the lymphoma environment from activating to repressive by modifying differentiation.

Chapter VI. Synergistic interactions between ITK inhibitors and chemotherapeutic agents in T-cell lymphoma/leukaemia cell lines.

6.1 Introduction

Patients with peripheral T-cell lymphomas (PTCL) have a poor prognosis and conventional chemotherapy does not produce durable remissions in the majority of cases. Even if the patients respond to intensive chemotherapy regimens, these agents can cause significant short-term and potentially long-term side effects. Therefore, new agents and approaches to treatment are needed to improve effectiveness and reduce toxicity (Abdul-Hay and Zain, 2012; Guo *et al*., 2012). Standardized approaches to introducing synergistic combinations of agents are being developed but there continues to be a large element of trial and error.

As mentioned in previous chapters, ITK plays a central role in T cell receptor (TCR) signalling, and regulates different aspects of T-cell development and differentiation. It has been suggested that ITK inhibitors show potential efficacy in other non-PTCL therapeutic areas requiring the modulation of Th1 and Th2 subsets in some animal models of infectious disease (Dubovsky *et al*., 2013; Cho *et al*., 2015; Sagiv-Barfi *et al*., 2015). Thus, the combination of ITK inhibitors and conventional chemotherapy is required to be determined for the treatment of PTCL.

Upon TCR activation, ITK is recruited to the immunological synapse by its pleckstrin homology (PH) domain and activated there through its association with phosphatidylinositol (3,4,5) trisphosphate (PIP3) generated via PI3K kinase (August *et al*., 1997; Ching *et al*., 1999; Woods *et al*., 2001), SLP-76/LAT complex (Bogin *et al*., 2007) and SRC family kinases such as LCK (Heyeck *et al*., 1997). The PI3Kδ inhibitor, Idelalisib is a selective inhibitor approved for the treatment of chronic lymphocytic leukaemia (Yang *et al*., 2015). The combined effects of PI3K inhibitors and ITK inhibitors have not been investigated but may affect survival and proliferation pathways in PTCL.

Small molecular compounds and specific antibodies against CXCR4 can efficiently inhibit the downstream signalling of the CXCR4/SDF-1 axis, and suppress cancer dissemination and improve outcomes in cancer patients (Lombardi *et al*., 2013; Debnath

et al., 2013; Mishan *et al*., 2016). A study showed that inhibition of the CXCL12/CXCR4 axis by a small-molecule antagonist of CXCR4 (AMD3100) blocks the migration of cells from adult T-cell leukaemia/lymphoma (ATLL) patients (Kawaguchi *et al*., 2009). ITK has been shown to play an important role in regulating T cell migration, and *Itk*-deficient mice exhibited defective T-cell migration (Takesono *et al*., 2004). Therefore, combining an ITK inhibitor and CXCR4 inhibitor may be a successful combination in disrupting T-cell lymphoma cell migration, and may be clinically useful.

In this chapter, we will evaluate the efficacy of several agents, including small molecule inhibitors or current chemotherapeutic agents, in combination with ITK inhibitors. CalcuSyn software is performed to determine whether a combination is synergistic, antagonistic or additive. The drug interactions are tested with a fixed ratio when the drugs have relatively the same type of growth inhibition curves with a comparable range of concentrations around IC50, and a non-fixed ratio is applied for drug combination when one drug is more active and has an action in a shorter time than the second drug by which it needs longer time to exert cytotoxicity (Bijnsdorp *et al*., 2011).

6.2 Results

6.2.1 ITK inhibitors synergistically enhance Doxorubicin activity in human T cell leukaemia/lymphoma cells.

Doxorubicin is a chemotherapeutic agent used for the treatment of several cancers, including lymphoma. It is a component of the CHOP regimen (cyclophosphamide, doxorubicin, vincristine, and prednisone) which is a standard frontline treatment for peripheral T cell lymphomas in the UK. Therefore, we tested the efficacy of Doxorubicin and ITK inhibitors to determine whether the combination therapy results in synergy.

In this experiment, two T cell leukaemia/lymphoma cell lines (Jurkat and MOLT-4), known to be ITK sensitive (Chapter 3, section 3.3.31), were chosen. The cell lines showed that Doxorubicin effectively suppressed the growth of tumour cells in a dose-dependent manner for 48 hours (Figure 6.1). The result in the present study demonstrate that the IC50 of Doxorubicin is about 100 nM based on a cell viability assay (CellTiter-Glo luminescent cell viability assay). As seen (Figure 6.1), the proliferation inhibition was remarkably increased when the dose of Doxorubicin was elevated. The data display that a low dose of ITK inhibitors-ONO7790500 and Ibrutinib (1 µM) employed in this work substantially enhanced the suppression of proliferation by Doxorubicin, see Figure 6.1. When the maximum concentration (C_{max}, 8 µM) of ITK inhibitors was added in combination with Doxorubicin, the IC50 of Doxorubicin shifted from 100 nM to ~ 15 nM in both Jurkat and MOLT-4 T cells (Figure 6.1A and B). Although this is not a realistic concentration to aim for therapeutically, it shows that the combination might be synergistic.

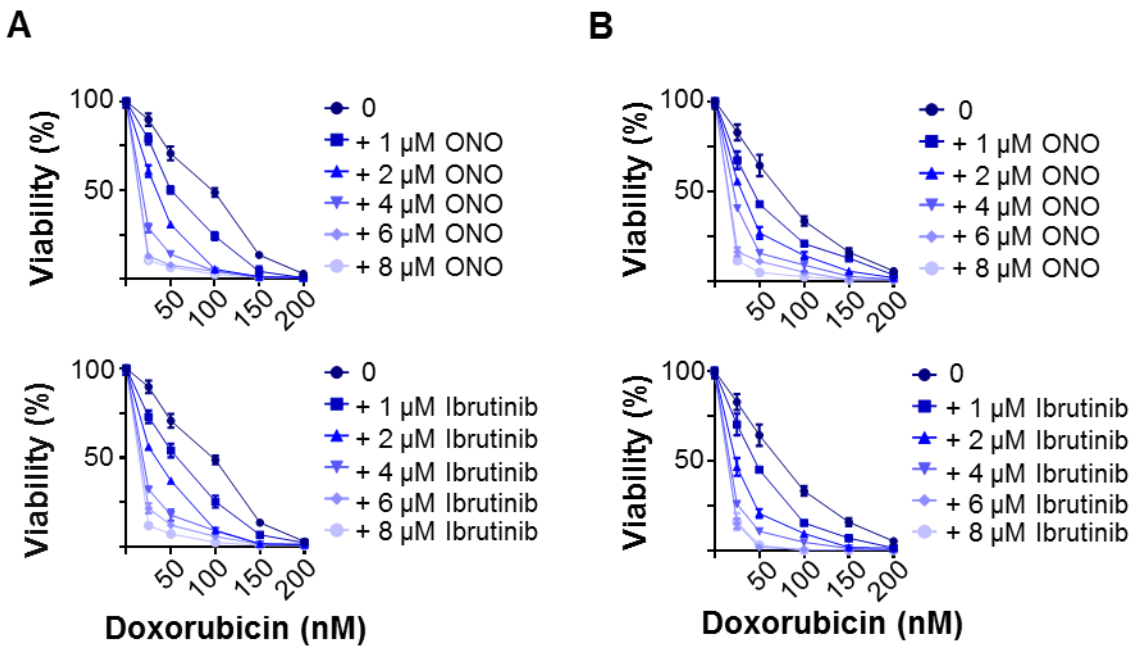


Figure 6.1. ITK inhibitors cooperate with doxorubicin to promote killing of Jurkat T and MOLT-4 cell lines. A) Viability (CTG assay) of Jurkat T cells, B) of MOLT-4 cells after 48 h treatment with a dose range of both ITK inhibitors (ONO7790500 and Ibrutinib) and doxorubicin or doxorubicin alone at the concentration (25, 50, 100, 150 and 200 nM) indicated in the figures. Data were normalized to vehicle (DMSO) and data indicate mean ± SEM of triplicate. ONO: ONO7790500.

To explore the synergy between Doxorubicin and ITK inhibitors, Jurkat and MOLT-4 T cell lines were treated with ONO-7790500 or Ibrutinib in combination with Doxorubicin as indicated (Figure 6.2) for 48 hours to determine the combination effect. CalcuSyn software was used to quantify the synergistic effect of drugs employing fractional effect (Fa) and combination index (CI). The fractional affected (Fa) is calculated from the percentage growth inhibition by the formula Fa = 1- (% growth inhibition/ 100) (Bijnsdorp *et al*., 2011). The program produces a variety of graphs, including a Fa-CI plot, a median-effect plot, and a dose-effect curve to formally assess synergy. One of the most important measures of synergy is the combination index (CI): a CI <1 demonstrates synergy, CI=1 demonstrates additive effects and a CI >1 demonstrates antagonism (Bijnsdorp *et al*., 2011).

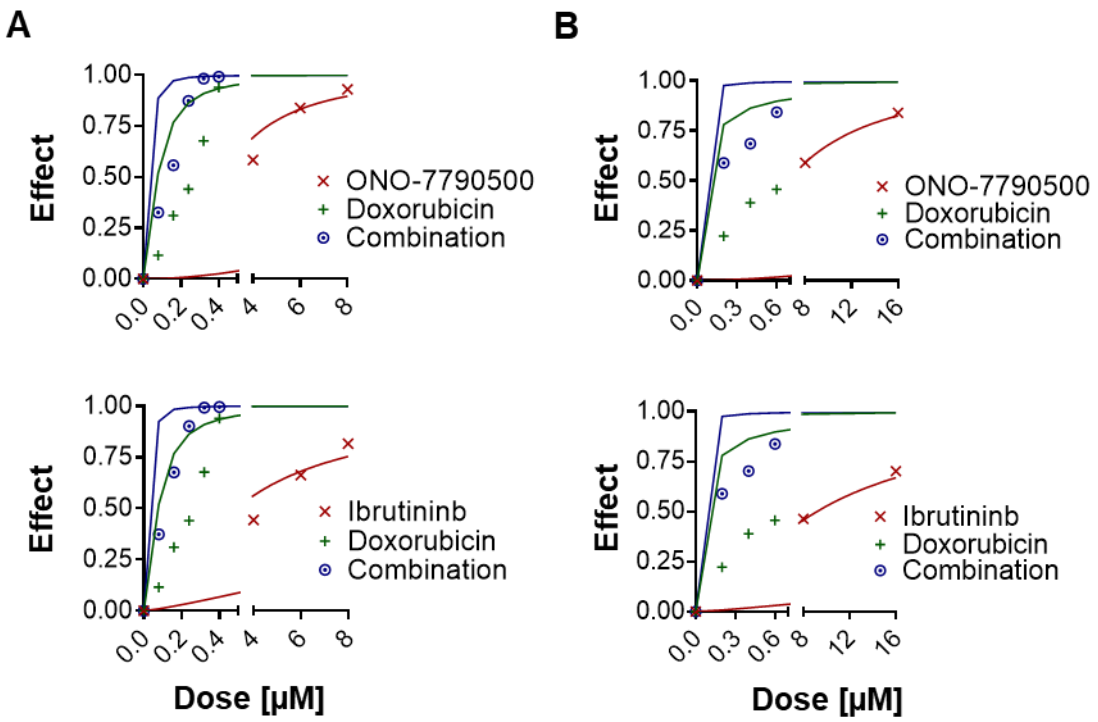


Figure 6.2. ITK inhibition synergises with doxorubicin in a model of T-cell leukaemia/lymphoma cell lines. The effect of ITK inhibitors and doxorubicin on A) Jurkat cells and B) MOLT-4) accessed by the CalcuSyn software on the IC50 values of the single agents. Data represent three independent experiments.

Using isobolograms to evaluate drug combinations (supplemental table A and B) shows that the combination of both the ITK inhibitors with Doxorubicin the CI was 0.3-0.4 in Jurkat and 0.2 in MOLT-4 (Figure 6.3), suggesting a synergistic effect. Isobolograms contain classical plots of each drug alone in certain dose ranges with variable concentrations of the other drug. Together, the data suggest that the combination therapy of ITK inhibitor with conventional chemotherapy might be a useful approach for treatment of T cell lymphomas.

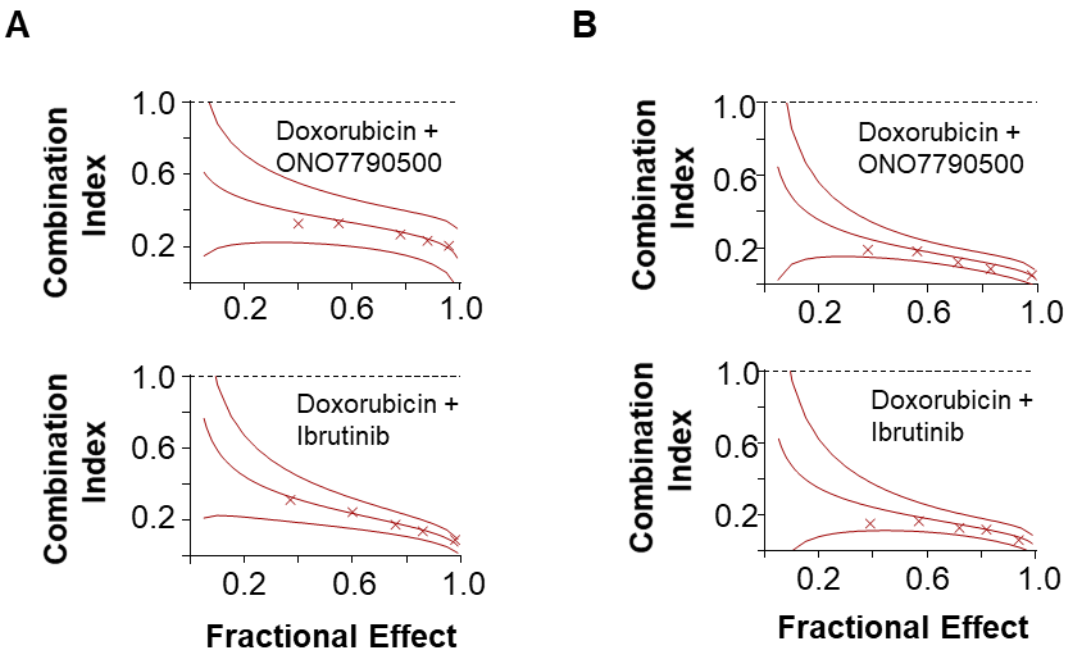


Figure 6.3. Combination index (CI) plots obtained from median-effect analysis in figure 6.2 (dose-effect curves). A) Jurkat and B) MOLT-4 cells were exposed to ITK inhibitors and doxorubicin for 48 h. The drugs were diluted at the ratio of their IC50 values as a series of two-fold dilution. The data show CI < 1. indicating synergism. CI plots were generated by using CalcuSyn software.

6.2.2 Antagonistic effects of ITK inhibitors and chemotherapeutic drugs on Jurkat T cell line.

In this section, we investigate the histone deacetylase inhibitor, romidepsin (Coiffier *et al*., 2012) and the dihydrofolate reductase inhibitor, pralatrexate (O'Connor *et al*., 2011), which are both licensed for the treatment of refractory/relapsed PTCL in the United States but not in Europe. Gemcitabine, a pyrimidine nucleoside analogue is currently being evaluated as part of a combination chemotherapy for PTCL (Evens *et al*., 2008; Rupoli *et al*., 2008). Another agent, cyclosporin A, is approved by FDA in the US for the treatment of many diseases (Lichtiger *et al*., 1994; Pilotstudie E., 2000) and was investigated here because its mechanism of action involves inhibition of calcium signalling in T-cells.

6.2.2.1 Drug interactions between ITK inhibitors and Cyclosporin A.

In this present study, the isobologram graphs showed the combination of ITK inhibitors and Cyclosporine A does not have synergistic effects, with a CI > 1 (Figure 6.4), suggesting this combination of drugs are antagonistic.

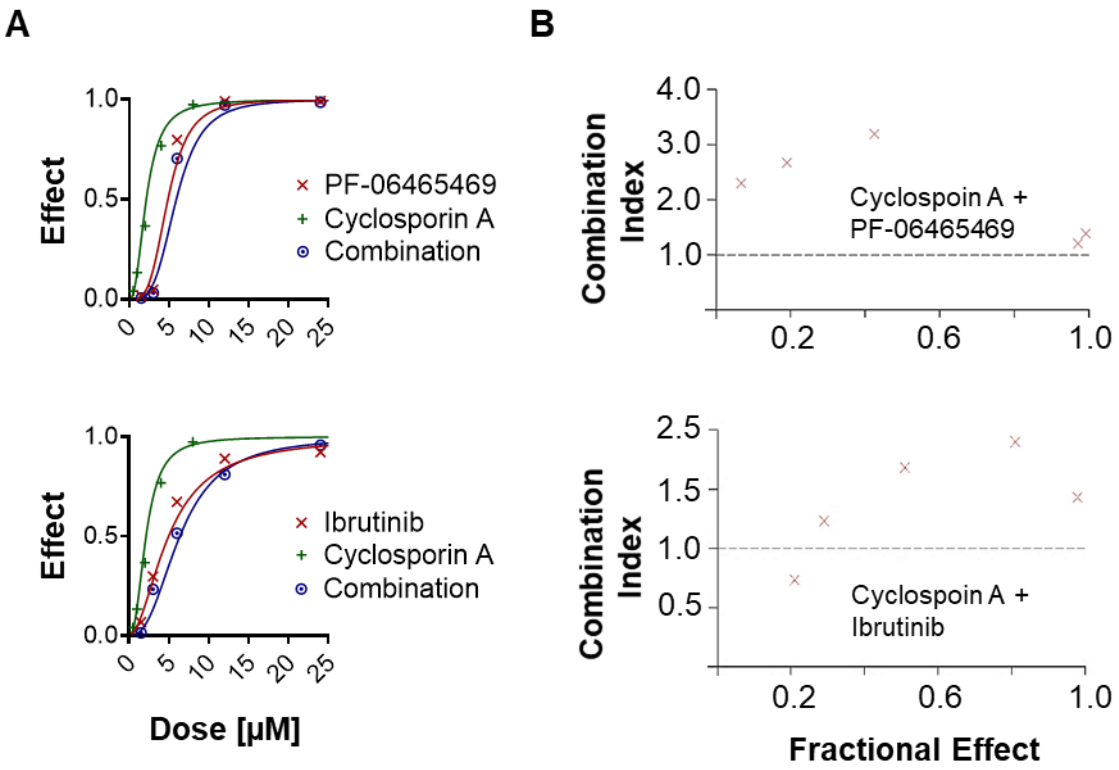


Figure 6.4. Generation of isobolograms and combination index plots by CalcuSyn approach demonstrate antagonism across range of ratios. A) Jurkat cells were exposed to ITK inhibitors and cyclosporin A for 48 h. PF-06465469 or Ibrutinib and cyclosporin A were diluted 4:1 and 2:1 (combination ratios), respectively. B) CI/Fa plots show CI > 1.

6.2.2.2 Evaluation of the combination of ITK inhibitors and Romidepsin

Romidepsin (IC50 8 nM) is much more active than ITK inhibitors (IC50 6.2 μM PF-06465469 and IC50 5.2 μM Ibrutinib) in suppressing cell proliferation (Figure X), and this makes the evaluation of the combination difficult. We did not observe synergy of Romidepsin with ITK inhibitors because Romidepsin is much more potent than the ITK inhibitors (Figure 6.5). Isobologram analysis confirmed that Romidepsin and ibrutinib were not synergistic in suppressing growth in Jurkat cell line (Figure 6.6).

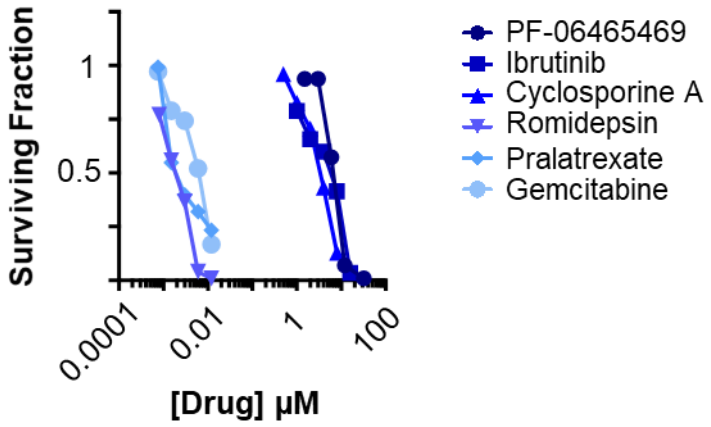


Figure 6.5. Effect of ITK inhibitors, cyclosporin A, romidepsin, pralatrexate and gemcitabine on viability of Jurkat T cells. Cell viability was analysed by CTG viability assay

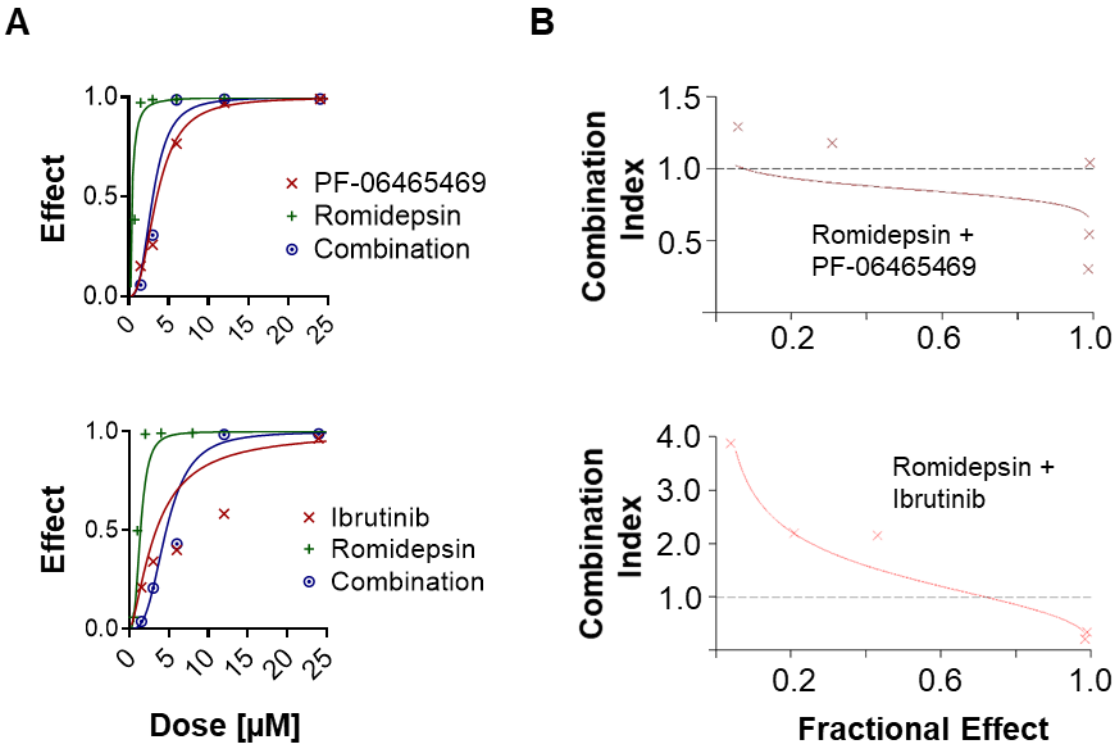


Figure 6.6. Isobolograms and combination index plots show antonyms on Jurkat T cells. A) Jurkat cells were treated with ITK inhibitors and Romidepsin for 48 h. PF-06465469 or Ibrutinib and Romidepsin were diluted 3000:1 and 2000:1 (combination ratios), respectively. B) CI/Fa plots show CI > 1 at lower doses of drug combination between ITK inhibitors and Romidepsin. The figures also show slight synergy between both compounds at higher doses.

6.2.2.3 Analysis of drug interactions between ITK inhibitors and Pralatrexate

Next, the combination of ITK inhibitor and pralatrexate was tested. Jurkat cells were treated with pralatrexate in combination with PF-06465469 and Ibrutinib using a non-fixed ratio or as a single agent for 48 hours. Cells were harvested and viability was determined by CTG viability assay. The results show that combination of pralatrexate and ITK inhibitors does not reduce viability in Jurkat cells in the dose range used in this study (Figure 6.7). Combined effects of pralatrexate and ITK inhibitors on Jurkat cell survival were less pronounced than that of pralatrexate alone. To define the combination effects of these agents more clearly, CI values of the data from combined treatment of Jurkat cells were determined using the Calcusyn software. Based on the CI values, the *in vitro* combination of pralatrexate and ITK inhibitors showed antagonistic effects throughout the dosing range of the drugs.

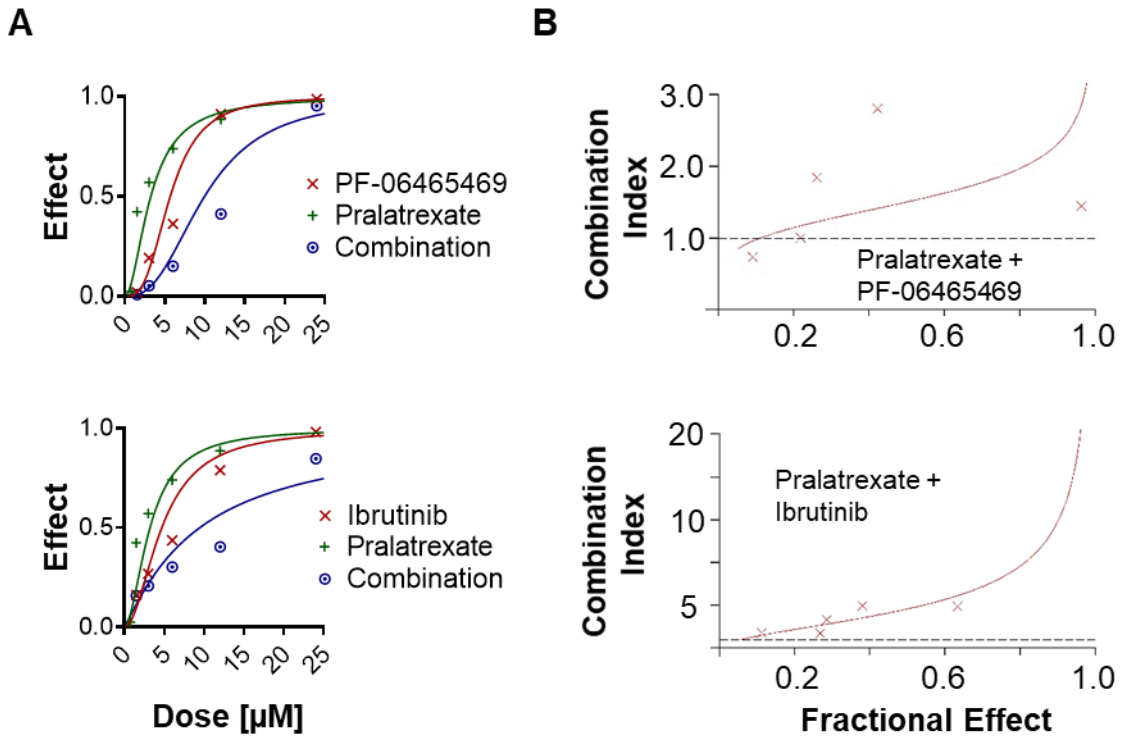


Figure 6.7. Dose-effect curve and combination index plots demonstrate antonyms on Jurkat T cells. A) Jurkat cells were exposed to ITK inhibitors and Pralatrexate for 48 h. PF-06465469 or Ibrutinib and Pralatrexate were diluted 2000:1 and 1333:1 (combination ratios), respectively. B) CI/Fa plots show CI > 1.

6.2.2.4 Drug interactions studies between ITK inhibitors and Gemcitabine

Growth inhibition studies showed that gemcitabine was more potent than ITK inhibitors in Jurkat cells (Figure 6.5) and, therefore, combination studies used non-fixed ratios of drug concentrations. Combination treatments yielded lower growth inhibition than either agent alone in Jurkat cell line tested (Figure 6.8). The results indicated that ITK inhibitors and gemcitabine were antagonistic with CI values > 1.

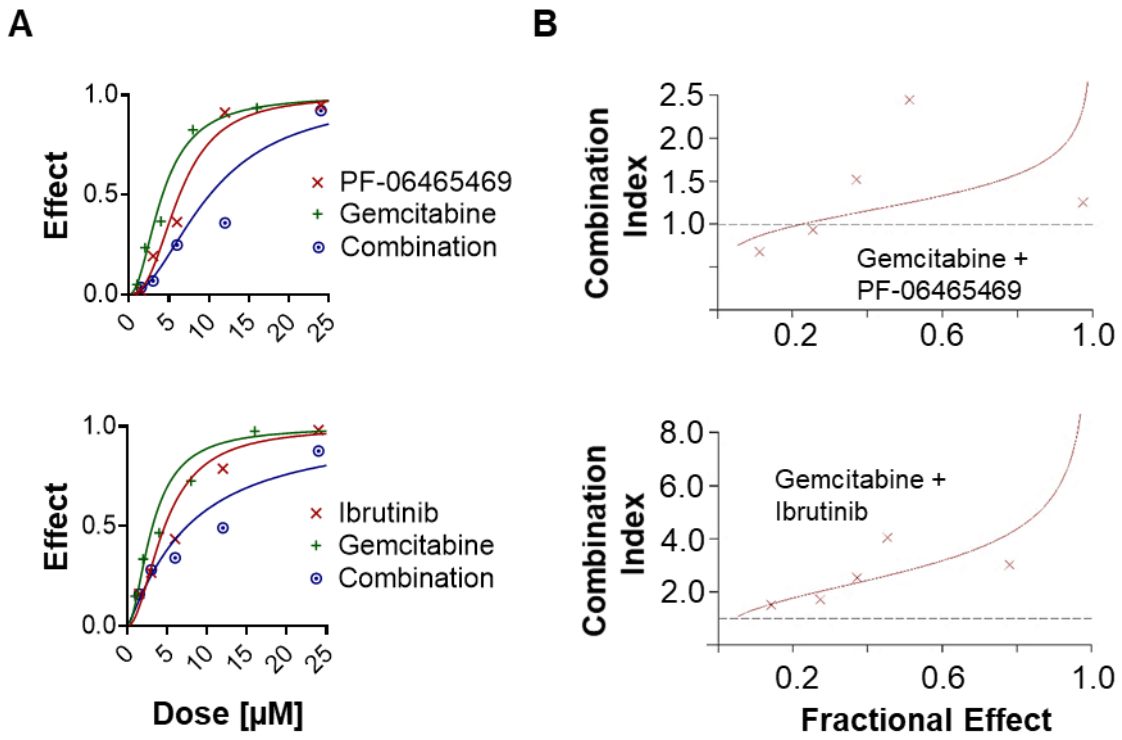


Figure 6.8. Interaction between ITK inhibitors and gemcitabine is not synergistic across range of ratio. A) Median-effect analysis demonstrates antagonistic effect of PF-06465469/Ibrutinib and gemcitabine in Jurkat T cells. B) The plots show CIs from both drug combinations are more than one, CI > 1. The combination ratio of PF-06465469 or Ibrutinib and gemcitabine were 2000:1 and 133:1, respectively.

6.2.3 The combination of ITK inhibitors and PI3K inhibitors led to impressive therapeutic effects in T cell leukaemia/lymphoma cell lines.

Phosphatidylinositol-3-kinase (PI3K) is known to be stimulated by various growth factor receptors and oncogenes, and its deregulation is considered a hallmark of many malignancies, including haematological malignancies (Courtney *et al.*, 2010). Many PI3K pathway inhibitors have been tested in oncology trials, such as the PI3K p110δ isoform inhibitor, Idelalisib, which is approved by the US Food and Drug Administration for the treatment of chronic lymphocytic leukaemia. In T cells, PI3K signalling plays an

important role in many cellular functions and regulates numerous biological processes including cell proliferation, differentiation and migration (Wullschleger *et al*., 2006; Lee *et al*., 2010; Okkenhaug and Vanhaesebroeck, 2003; Finlay, 2012; Klaus *et al*., 2014). According to Hussain *et al*., (2009), the activation of IL-2-inducible kinase-spleen tyrosine kinase (ITK-SYK) fusion protein shares similarities with ITK activation, which is also dependent on PI3K signalling, indicating that inhibition of PI3K may be an effective approach for the treatment of ITK-SYK initiated T cell lymphomas (Herman and Johnson, 2012; Zhong *et al*., 2014). We, therefore, sought to explore the synergy between ITK and PI3K inhibitors *in vitro*.

Jurkat and MOLT-4 T cell lines were screened for their sensitivity to ITK (ONO-7790500 and Ibrutinib) and PI3K inhibitors as assessed by change in cell viability. Before combination analysis, the single agent activities of drugs were determined to generate a sigmoid-shaped growth inhibition curve (Figures 6.9, 6.10, 6.11 and 6.12). The single agent responses of all four drugs were generated using CellTiter-Glo (CTG) luminescent cell viability assay.

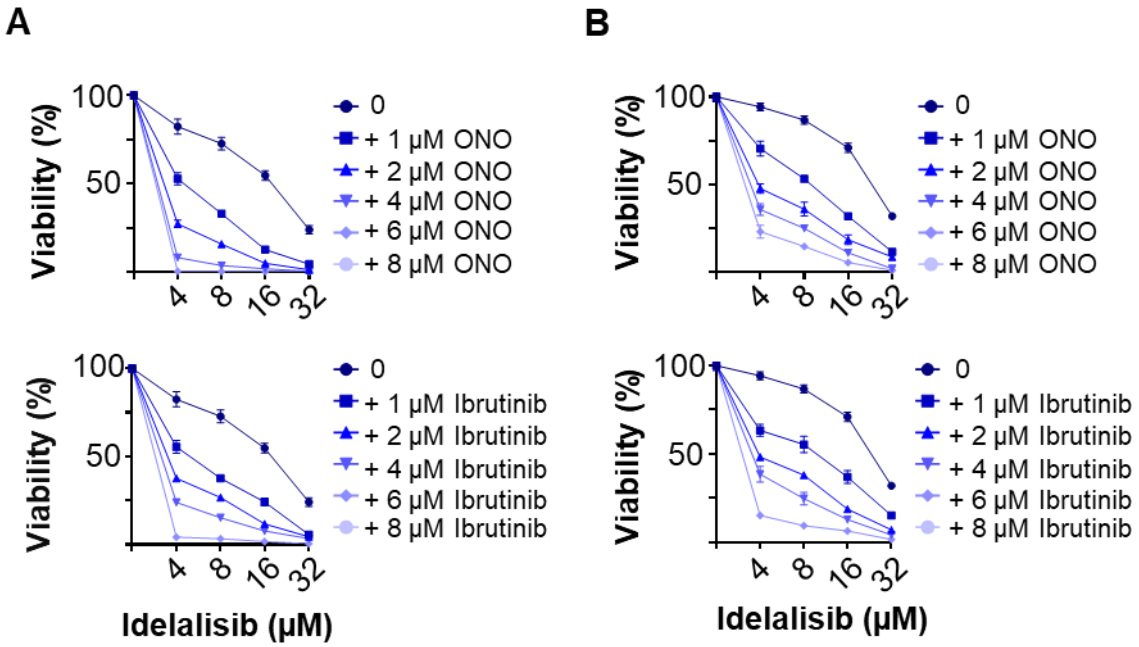


Figure 6.9. Effect of drug interaction on Jurkat and MOLT-4 cell viability. As shown by CTG assays, A) Concentration of ONO7790500 and Ibrutinib augmented the effect of idelalisib (PI3K inhibitor) on Jurkat T cell viability after 2 days' treatment with the concentration of both compounds in 96-well plates. Similarly, in B) both ITK inhibitors enhanced the effect idelalisib on MOLT-4 cell viability. The data represent the mean ± SEM of three independent experiments. ONO: ONO7790500.

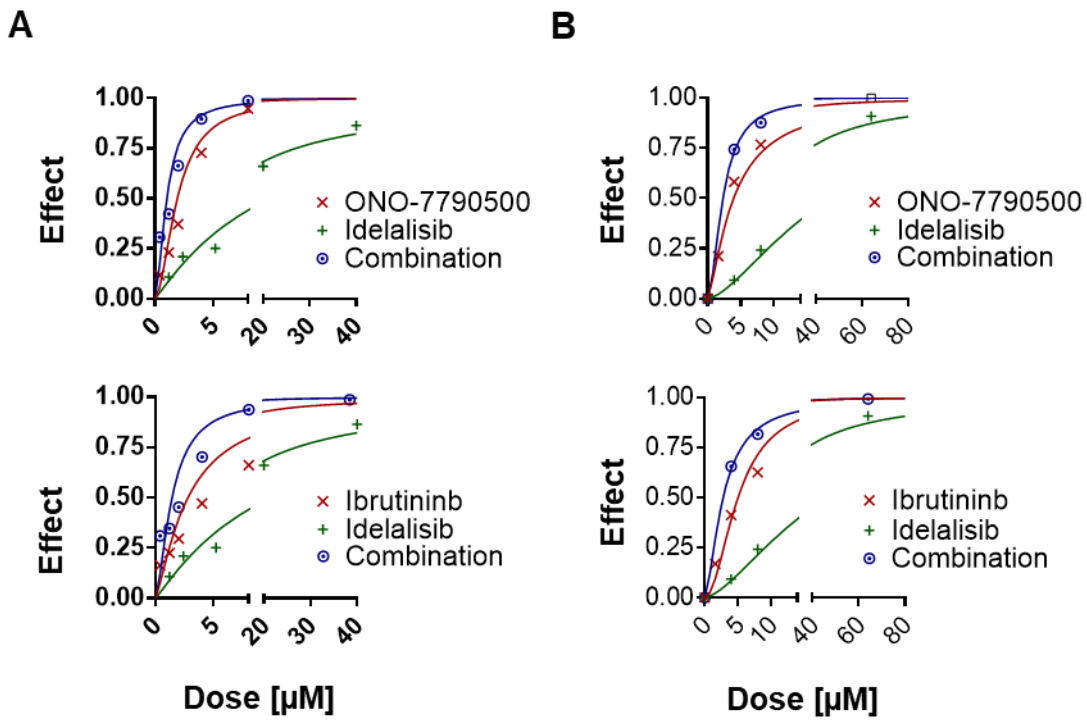


Figure 6.10. ONO7790500 and Ibrutinib synergy with idelalisib was generated by the CalcuSyn software program by using the fractional affected (Fa) and concentration for individual drug alone and their combinations. After treatment of A) Jurkat and B) MOLT-4 cell lines for 48 h, dose response curves obtained from ONO7790500 or Ibrutinib and their combination with idelalisib using a fixed concentration. The experiments were performed in triplicate.

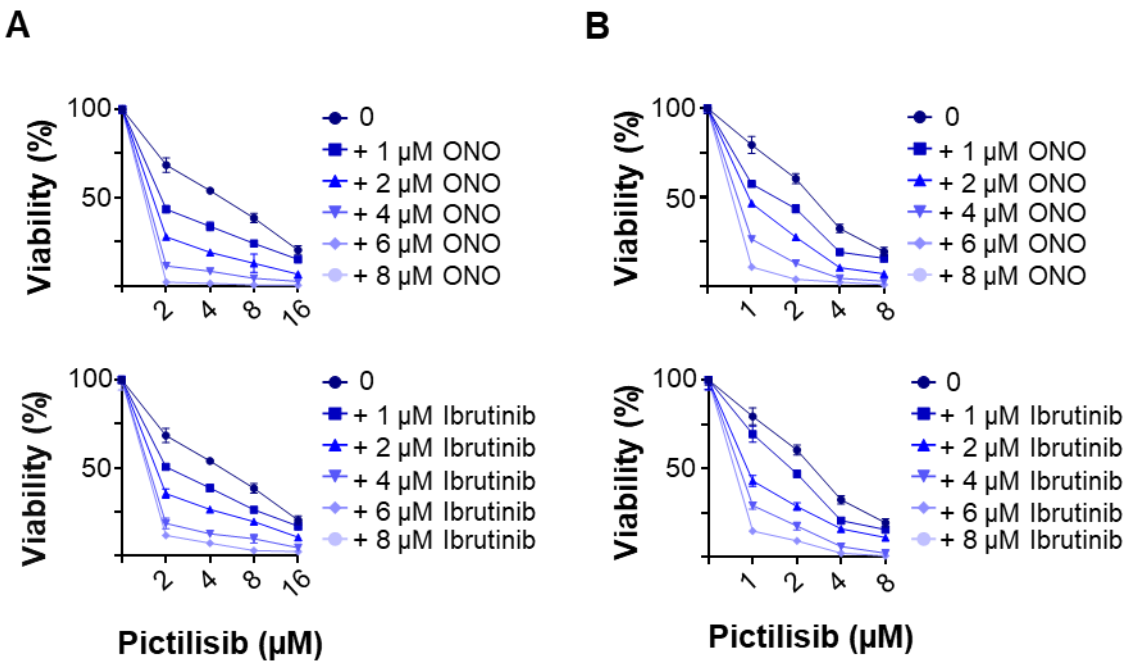


Figure 6.11. The addition of ITK inhibitors to pictilisib can reduce the viability of Jurkat and MOLT-4 cells. A) Viability assay shows additional of ONO7790500 and Ibrutinib to pictilisib decreased the viability IC50 of pictilisib in Jurkat cell line. B) Similar result is seen with MOLT-4 cell line. Cell lines were treated in 96-well plates for 48 h using CTG assay, and the data represent mean ± SEM, n = 3. ONO: ONO7790500.

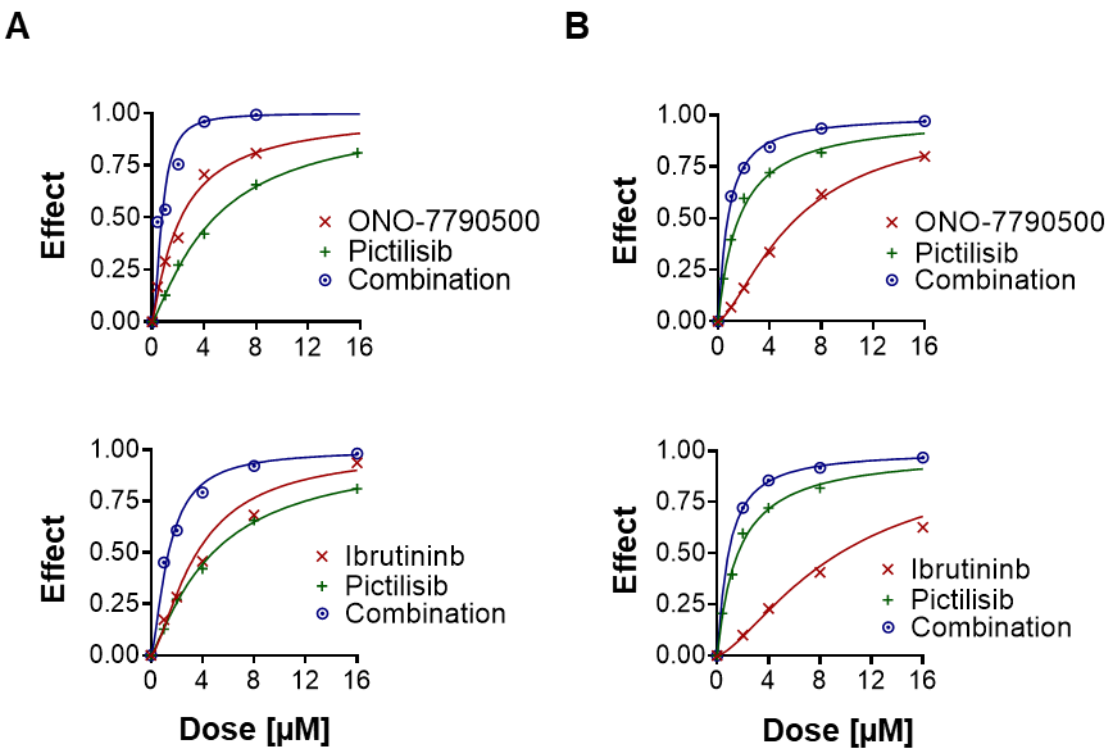


Figure 6.12. ITK inhibitors synergise with idelalisib on Jurkat and MOLT-4 T cells. After treatment of A) Jurkat and B) MOLT-4 cell lines for 48 h, dose response curves obtained from ONO7790500 or Ibrutinib and their combination with idelalisib using a fixed concentration. Data were obtained from three independent experiments.

In this experiment, Jurkat and MOLT-4 T cell lines were chosen to evaluate the effects of ITK inhibitors and PI3K inhibitors in combination. Cell viability assessments show that the IC50 for Idelalisib and Pictilisib inhibitors is 18 µM and 5.2 µM respectively, in Jurkat cells (Figure 6.9A and 6.13A) but the sensitivity of MOLT-4 cells was different with Idelalisib having an IC50 of 25 µM whereas the IC50 for Pictilisib was 2.5 µM (Figure 6.9B). Both PI3K inhibitors showed synergy in combination with ITK inhibitors in both cell lines. The addition of ITK inhibitors (1 µM) to the PI3K inhibitor Idelalisib in Jurkat cell line shifted the IC50 from 18 µM to 5 µM, and from 25 µM to 10 µM in MOLT-4 cells (Figure 6.10). When ITK inhibitors were added in combination at the minimum concentration ($C_{min,}$ 1 µM), IC50 of Pictilisib shifted from 5 µM to 1.8 µM in Jurkat cell line. In MOLT-4, the addition of ITK inhibitors (1 µM) produced only a minor change to IC50 of pictilisib from 2.8 to 1.5 µM (Figure 6.11). These data observed synergism effect on cell viability with the combination of ITK inhibitors and PI3K inhibitors.

The effect of a single agent and in combination is indicated by the Fa curve to show a growth inhibition curve (Figure 6.10 and 6.12). In this experiment, the drug interactions were tested with a fixed ratio due to both ITK and PI3K inhibitors have relatively the same type of growth inhibition curves. The combination index (CI) was ~ 0.4 in Jurkat and ~ 0.6 in MOLT-4 when the effective dose of both drugs inhibited the cell proliferation to 50% (Figure 6.13 and 6.14). The dose of ITK inhibitors (supplemental table C, D, E and F) substantially enhanced the activity of PI3K inhibitors with a CI value ranging from ~ 0.2 to 0.6 (CI < 1), indicating the synergistic effects of their combination on the suppression of malignant T cell proliferation.

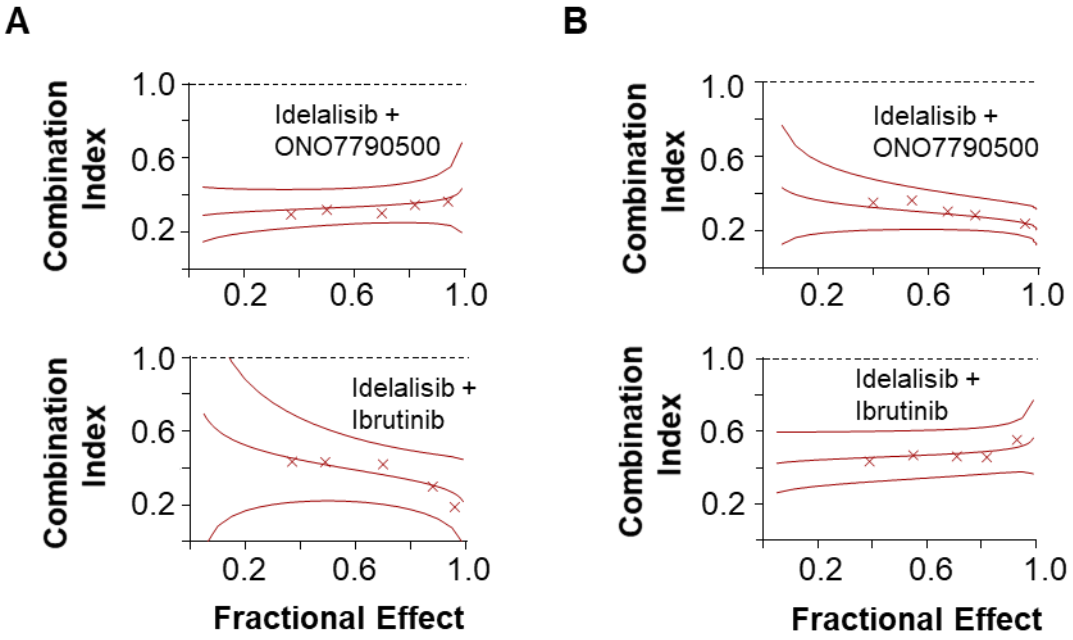


Figure 6.13. Generation of index-Fractional affected (Fa) plots from combination of ITK inhibitors and idelalisib demonstrate synergy. ITK inhibitors and idelalisib were diluted 1:2 around their IC50 values. CI was analysed by CalcuSyn software. Column A and B represent CI data on Jurkat and MOLT-4 cells respectively.

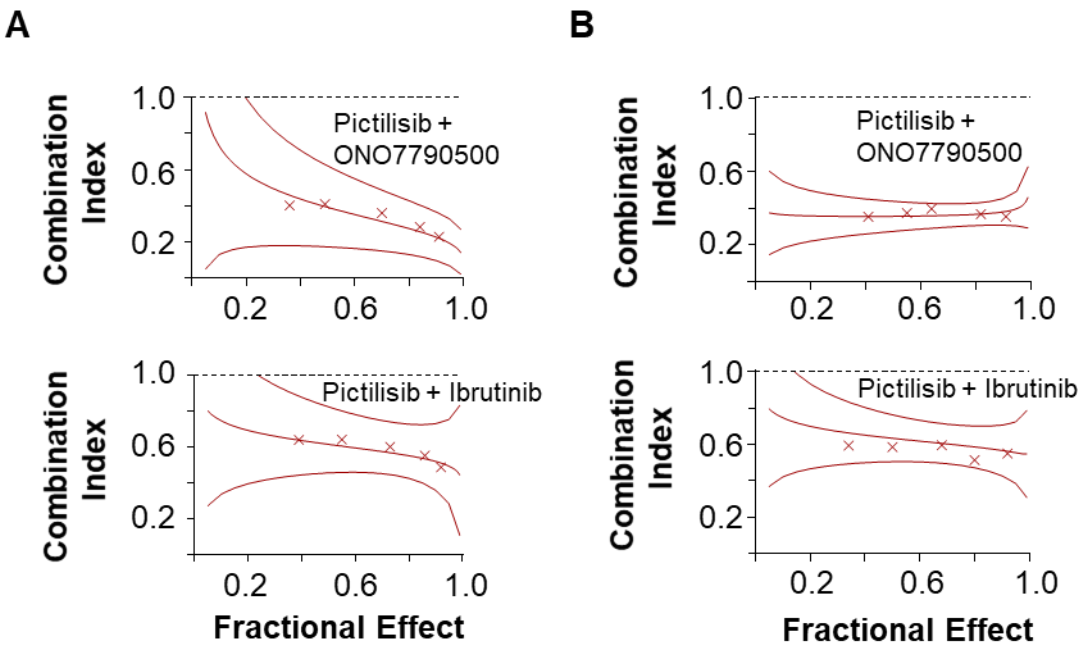


Figure 6.14. Interaction between ITK inhibitors and pictilisib is synergistic. A) Jurkat, and B) MOLT-4 T cells were treated for 2 days with indicated doses at fixed ratio, as seen in figure 6.13 and Supplemental Table E and F. Combination index (CI)/Fractional-effect (Fa) were generated by CalcuSyn software method. CIs for Jurkat and Molt-4 treated with combinations of ONO7790500 or Ibrutinib with pictilisib concentrations show synergistic effects, CI < 1.

6.2.4 ITK inhibitors and CXCR4 inhibitor are synergistic and result in loss of Jurkat T cell migratory ability.

Tissue invasion is a feature of aggressive tumours and a modifier of lymphocyte egress is a potential therapeutic in autoimmunity (Schwab and Cyster, 2007). ITK inhibitors reduce migration of T-cell lines in response to CXCL12 in a transwell assay (Chapter 3, page 3.2.7). CXCR4 is the T-cell surface molecule that is the receptor for CXCL12. To investigate the synergistic effects of Plerixafor, a CXCR4 antagonist, and ITK inhibitors, migration assays were carried out using the Jurkat T cell line. As previously shown (chapter 3, section 3.2.7), treatment of Jurkat cells and CD4 T cells with ITK inhibitors (2 µM) significantly reduced the percentage of migrated cells (transwell migration assay) but lower doses did not significantly decrease cell migration. Therefore, we sought to investigate the combinatorial effects of ITK inhibitors and Plerixafor on CXCL12-induced migration activity of Jurkat T cells. Plerixafor is approved to use by the U. S. Food and Drug Administration (FDA) for stem cell mobilisation in combination with granulocyte-colony stimulating factor (G-CSF) in patients with non-Hodgkin lymphoma and multiple myeloma.

Firstly, the cytotoxic activity of Plerixafor, the small-molecule inhibitor of CXCR4, was analysed using CTG viability assay. Our analysis shows that Plerixafor had no effects on the proliferation of Jurkat cells (Figure 6.15).

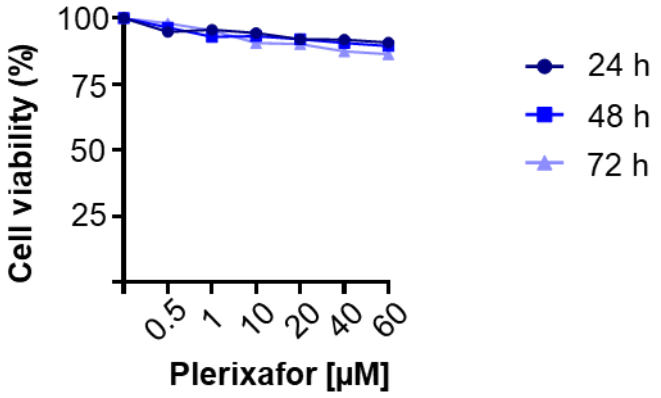


Figure 6.15. Effect of plerixafor (CXCR4 inhibitor) on viability of Jurkat T cells. Jurkat cells were treated with plerixafor as indicated concentrations at different time points (24, 48 and 72 h). Cell viability was analysed by CTG viability assay

Secondly, the effect of Plerixafor alone was investigated up on CXCL12 stimulation and figure 6.16A shows that 50 nM of Plerixafor was only reduced 28% of migrated Jukrat cells. We next investigated the synergy between ITK inhibitors and Plerixafor *in vitro*. ITK inhibitors (ONO-7790500 and Ibrutinib) substantially inhibited cell migration in response to CXCL-12. The migration was reduced by the addition of Plerixafor (50 nM) in a dose-dependent manner (Figure 6.16B and C). The data suggest that combining CXCR4 antagonist and ITK inhibitors have more effects on tumour cell migration compared to Plerixafor alone and may be an effective strategy to reduce cell migration in T cell lymphoma with possible benefits to patient survival.

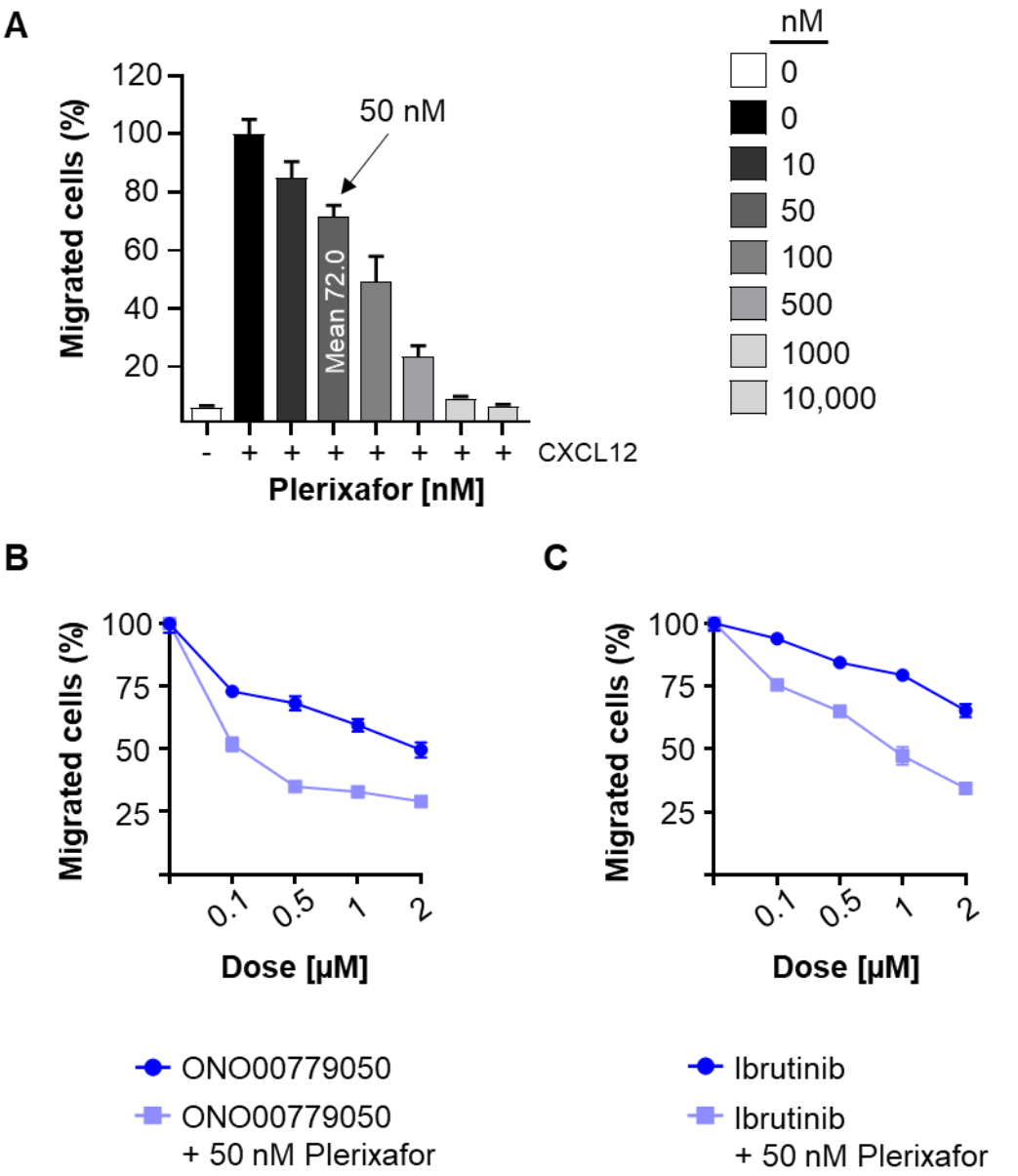


Figure 6.16. Plerixafor (CXCR4 inhibitor) enhances the effect of ITK inhibitors in Jurkat cell line. (A) Jurkat cells were treated with variuous concetrations of plerixafor up on 100 nM CXCL12 stimulation for 3 h using transwell migration experiment. (B and C) Jurkat cells were treated with ITK inhibitors alone and their combination at the concentration of 0.1, 0.5, 1 and 2 μM with 50 nM plerixafor for 3 h. Transwell migration assays demonstrate that the addition of 50 nM plerixafor to ONO00779050 and Ibrutinib remarkably decreased the ability of Jurkat cell migration.

6.3 Discussion

In many cancers including PTCL there is a need for more effective therapies, which are also well tolerated by patients. In addition, resistance to chemotherapeutic agents is a major cause of treatment failure. The use of combination chemotherapy is a route to promoting efficacy while maintaining toxicities at tolerable levels and minimising opportunities for drug resistance. However, while there have been attempts to produce rational combinations with small molecule inhibitors (Griner *et al*., 2014) there is at present no standard route to achieving this result.

Taking a candidate compound approach, chemotherapy agents or small molecule inhibitors were tested for their effects in enhancing loss of viability induced by ITK inhibitors. Also, the combination of Plerixafor and ITK inhibitors was tested for potential for inhibition of chemokine induced migration.

The definition of synergism is that the drug combination is more effective than the additive effects of individual agents, while antagonism describes the situation in which the effects of drugs in combination is less than each agent separately. One of the widely used pharmacological approaches to evaluate the effect of combination is the median-drug effect analysis method by calculation a combination index (CI) from growth inhibition curves or drug cytotoxicity (Bijnsdorp *et al*., 2011).

The frontline treatment for PTCL consists of doxorubicin with cyclophosphamide, prednisone and vincristine (CHOP). Therapeutic responses are not adequate or durable, with rapid relapse for many of the subtypes (Schmitz *et al*., 2010; Abouyabis *et al*., 2011). Our study demonstrated synergy for two combinations with ITK inhibitors:

1) Doxorubicin, and

2) PI3Kδ inhibitors, Idelalisib and Pictilisib.

We observed no significant enhancement of ITK inhibitors activity in combination with agents (Romidepsin, Pralatrexate and Gemcitabine) that are included in current therapeutic regimens for relapsed or refractory T cell lymphomas. ITK inhibitors did not influence the toxicity of these chemotherapy agents for T cell lymphoma cell lines (as measured by annexin V binding) but we do not completely understand the mechanisms that underlie the observed antagonism. It is likely that the large differences in IC50

contribute to these effects. The IC50 of these agents (Romidepsin, Pralatrexate and Gemcitabine) was much less (~ 500-1000-fold) than that of the ITK inhibitors for the cell line employed in this study and their effects, therefore, overwhelm the assay results.

Another agent, cyclosporin A, is employed clinically as an immune suppressant after transplantation of solid organs or haemopoietic stem cells and has a similar IC50 to the ITK inhibitors. However, the combination with ITK inhibitors again did not enhance loss of viability. In this case there is a possible explanation: Cyclosporin A acts downstream of ITK to inhibit the calcium signalling pathway, and this implies that many of the effects of ITK inhibitors on cell viability are largely mediated by calcium signalling. Taken together, these data do not provide evidence for the inclusion of ITK inhibitors in combination with these drugs in future regimens.

CXCR4 expression is associated with the progression of many haematologic and non-haematologic malignancies (O'Callaghan *et al*., 2012) and several studies identified it on the surface of various human tumours. CXCR4 is a poor prognostic factor in many cancers (Yasuoka *et al*., 2008; Scala *et al*., 2005; Kim *et al*., 2005; Spoo *et al*., 2007; Konoplev *et al*., 2007) and is a target for therapy in various malignancies because of its key role in mediating metastatic spread (Burger and Kipps, 2006). ITK is activated by CXCR4 in a PI3K dependent manner (Fischer *et al*., 2004). ITK inhibitors might be useful agents *in vivo* to perturb migration (section 3.2.7 in chapter III), and could enhance the effects of cytotoxic chemotherapy. Here we found that Plerixafor, a CXCR4 antagonist, enhanced the effects of ITK inhibitors in repressing migration of Jurkat T cells while not having an effect on survival. An intriguing possibility is that adding Plerixafor and an ITK inhibitor to current regimens might enhance effectiveness by reducing migration.

In Summary, the combination of ITK inhibitors and some chemotherapeutic agents led to impressive therapeutic effects *in vitro*. We suggest that ITK inhibitors might be useful when added to chemotherapy regimens for the treatment of some types of PTCL. Based on our preclinical results, validation of drug combination synergy between ITK inhibitors and conventional chemotherapies employed in this study needs to be assessed in animal models of lymphoma.

Chapter VII. Discussion and future work

7.1 Cell lines and primary cells.

There is very little work involving the study of primary human PTCL cells. This is primarily due to the rarity of these conditions, and secondly because it is very difficult to obtain pure populations of cells as the lymphoma cells are present in an environment of non-malignant B-cells. Therefore, a large amount of work on T-cell functions including TCR signalling is carried out on T-cell lines of which Jurkat is one of the most frequently used (Abraham and Weiss, 2004). Jurkat cells expresses ITK and proved useful for the work described in this thesis. As one approach to control for the non-specific effects of the small molecule inhibitors, a panel of human T-cell lines containing varying levels of ITK, was obtained: K299, MOLT-4 and CCRF CEM.

siRNA knockdown of ITK in Jurkat cells caused reduced growth rates with less impressive effects on apoptosis. Although siRNA knockdown can be associated with off-target effects, the fact that the results obtained were similar (for both proliferation and apoptosis) to those seen with the small molecule inhibitors does suggest that the inhibitors do have the expected on-target effects. As knockdown of ITK caused reduced calcium flux and IL-2 production in the Jurkat T cell line, it is possible that ITK promotes cellular proliferation through the promotion of calcium mobilisation and cytokine production. In agreement with this argument, previously published data demonstrate that Itk-deficient mice have reduced IL-2 production (Schaeffer *et al*., 1999; von Bonin *et al*., 2011).

TEC kinases are required for normal CD4+ T cell differentiation (Schwartzberg *et al*., 2005; Schaeffer *et al*., 2001) and there may be a complex interplay between ITK and other members of the family such as RLK. Following a report that Itk-deficient mice showed abnormal differentiation of CD4+ T-cells with a skew from activating to suppressive subsets (Gomez-Rodriguez *et al*., 2014), we wished to find out if the small molecule ITK inhibitors had similar effects.

Because cell lines are unsuitable for these experiments human tonsillar CD4+ T-cells were used to investigate effects on normal T-cell populations. We were also fortunate to be able to use two unique patients with CD4+ T-cell lymphoma: one patient had a malignant pleural effusion from which it was possible to obtain purified lymphoma cells and the second patient had circulating lymphoma cells, which could be purified.

7.2 Small molecule ITK inhibitors

Few ITK inhibitors are commercially available, and some of them have been examined in other animal models such as inflammation, asthma and skin dermatitis (Lin *et al*., 2004). Although none of those ITK inhibitors has been entered clinical trials. In general, small molecule inhibitors for ITK irreversibly bind to cysteine residues within the ATP binding site in ITK. Cys-442 of ITK is located at the end of the C-lobe α-helix, which is shared with other TEC family members including RLK and TEC in T cells, so achieving high selectivity/specificity has been shown to be difficult (Charrier *et al*., 2011; Das *et al*., 2006; Snow *et al*., 2007; Velankar *et al*., 2010).

The tyrosine kinase inhibitor, ibrutinib, was primarily developed as a BTK inhibitor for use in B-cell non-Hodgkin's lymphomas. It is very effective in these conditions (Byrd *et al*., 2013), but it is also apparent that it is active against ITK (Dubovsky *et al*., 2013; Savig-Barfi et a., 2015). Given the initial success of Ibrutinib in *in vivo* models in T-cells, ITK could be an appealing target for T-cell disorders. In this research, in additional to Ibrutinib, PF-06465469 and BMS-509744, a novel ITK inhibitor, ONO7790500, has been studied which has more specificity toward ITK in T-cell malignancies and selectivity targets ITK, while having little effect on other cancer cell lines.

7.3 Effects of ITK inhibitors

Although the small molecule inhibitors are very potent and they are likely to have off-target effects particularly on other kinases. Indeed, ibrutinib is known to target two similar tyrosine kinases: one expressed in B-cells, BTK, and the other in T-cells, ITK. Two of the compounds used here (ONO7790500 and BMS-509744) are believed to be more specific for ITK than PF-06465469 and Ibrutinib. In order to gain some understanding of on- and off-target effects we compared some effects of small molecules with ITK knockdown. Our findings demonstrate that ITK inhibitors appear to have more profound effects on cell apoptosis than ITK knockdown does. However, we found that treatment of T cell lines with the more specific inhibitors; ONO-7790500 and BMS-509744 was not enough to cause apoptosis. Similarly, there was more reduction in IL-2 production with non-specific ITK inhibitors (PF-06465469 and Ibrutinib) compared to either ITK knockdown or the more specific inhibitors. As a result, it appears that induction of

apoptosis and reduced IL-2 secretion could partly reflect off-target effects of the non-specific inhibitors.

ITK is critically essential for cell migration. According to Takesono *et al*. (2004), Jurkat cells expressing ITKKD-GFP (Kinase-dead Itk-GFP; cell transiently transfected with green fluorescent protein (GFP)-tagged Itk) results in impaired RAC and CDC42 activation and decreased migration in response to CXCL12. This finding is in agreement with data for Jurkat cell and primary $CD4^+$ T cells presented in this project. ITK inhibitors partially blocked migration and suppressed signalling to the CXCL12-activated GTPases, RAC and CDC42, but have slightly less effect on RHOA activity.

In addition, Jurkat cells also demonstrated reduced IL-21 expression following administration of ITK inhibitors. IL-21 has essential roles in $CD4^+$ T-cell differentiation and suggests a mechanism whereby ITK inhibitors could modify $CD4^+$ T-cell plasticity.

Previous work often employing mice deficient for Itk, has shown roles for this kinase in Treg and Th17 differentiation (Gomez-Rodriguez *et al*., 2014) and in Th2 differentiation (Fowell *et al*., 1999). However, animal studies have not reported the effects of ITK deficiency on Tfh differentiation. Using tonsillar $CD4^+$ T-cells as a source of normal T-cells, it was possible to show that ITK inhibitors reproduced the effects observed in ITK deficient animals on reciprocal differentiation of Th17 and Treg subsets. Under appropriate culture conditions tonsillar T-cells differentiate towards Th17 but the administration of ITK inhibitor promotes Treg differentiation at the expense of Th17. Similarly, using culture conditions to promote Th2 differentiation, it was possible to show that ITK inhibitors prevented this. ITK is not believed to have a role in Th1 differentiation and the small molecule inhibitors did not inhibit differentiation to this subset *in vitro*. Therefore, the experiments described in this thesis suggest, for the first time, that small molecule ITK inhibitors have the potential to alter CD4+ T-cell plasticity. Overall the effects of inhibiting ITK might be expected to suppress the activating subsets while promoting repressive Tregs.

It is surprising that the mouse studies have not included an analysis of Tfh cell differentiation. However, ITK inhibitors strongly prevented differentiation to Tfh cells by tonsillar T-cells (Figure 7.1). This is a potentially important observation because numerous studies have shown that a proportion of PTCL-NOS, in particular, the follicular variant, express Tfh markers including PD1, BCL6, CXCL113, ICOS and SAP (Ma and

Deenick, 2014). Similarly, cutaneous T-cell lymphomas mainly, mycosis fungoides, Sezary syndrome (Roncador *et al*., 2007; Meyerson *et al*., 2012) and primary cutaneous CD4-positive small and medium cell T-Cell Lymphoma (Rodríguez *et al*., 2009), have been reported to express Tfh markers. ITK is highly expressed in the newly recognised entity of "nodal PTCL with Tfh phenotype" (Swerdlow *et al*., 2016).

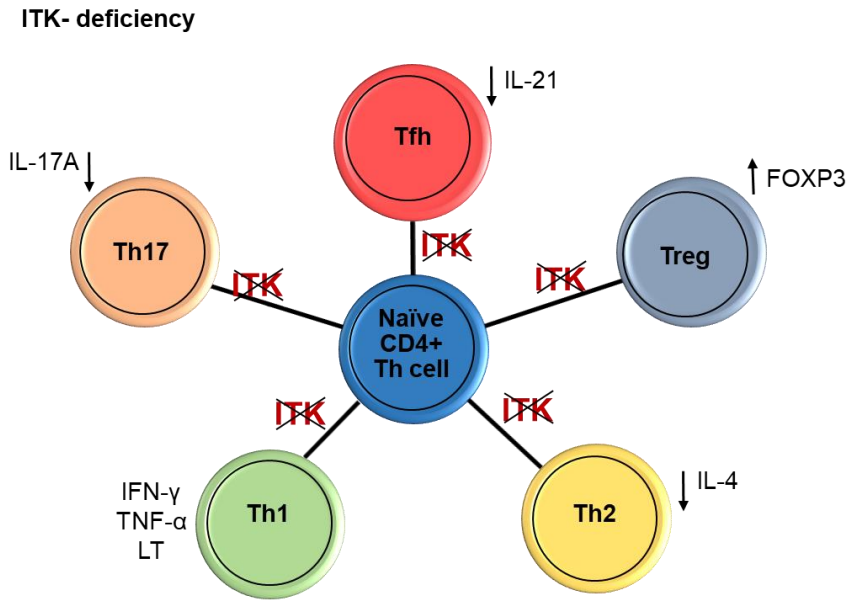


Figure 7.1. The effect of ITK inhibitors on CD4+ T cell differentiation. Unlike Th1 cells, differentiation of Th2, Th17 and Tfh cells are impaired by ITK inhibitors employed in this study, whereas the proportion of Treg cells are increased. Th1 differentiation was not impaired due to ITK inhibitors, this is most likely because RLK, another Tec protein-tyrosine kinase family in T cells, provides a compensatory platform for Th1 activation.

There is very little, if any, experimental work using primary human PTCL cells. In this thesis the effects of differentiating culture conditions were tested on purified $CD4^+$ T-cells from two patients. The results showed firstly, that lymphoma cells can be differentiated *in vitro*. There was strong differentiation to Tfh cells and lesser degrees to Th17 and Treg. Secondly, ITK inhibitors were able to prevent differentiation to activating T-cell subsets, especially Tfh cells, while promoting Treg differentiation.

The research reported here reveals several potential mechanisms of action for ITK inhibitors in PTCL. These are:

1) reduction in proliferation and promotion of apoptosis

2) reduction in chemokine induced migration, and

3) inhibition of Tfh differentiation and promotion of suppressive Tregs.

To test these ideas further, mouse models of T-cell lymphoma are required. There are no standard animal models for these diseases but a transgenic mouse bearing an ITK:SYK fusion gene has been produced, and it has been shown that this fusion protein mimics a TCR signal and is sufficient to drive development of T-cell lymphomas (Dierks *et al*., 2010; Pechloff *et al*., 2010). TET2 deficient mice have also been produced (Muto *et al*., 2014), which produce T-cell lymphomas with a long latency of 14 months.

Finally, it appears that certain activating T-cell mutations may be sufficient to produce T-cell lymphomas in mice. Animals that have a nitrogen mustard induced mutation in the E3-ubiquitin ligase, Roquin, show increased surface expression of ICOS and have increased numbers of Tfh cells (Vinuesa *et al*., 2005). Mice homozygous for the mutation display severe autoimmunity but heterozygotes do not. However, heterozygous animals ($Roquin^{san/+}$) develop T-cell lymphomas with similarities to human AITL in about 50% by six months of age (Ellyard *et al*., 2012). Interesting studies could administer ITK inhibitors to these animal models and investigate the frequency of tumour development or, in already established tumours, to investigate T-cell differentiation or migration in the presence and absence of ITK inhibitors.

Work described here raises the exciting possibility that ITK inhibitors could be additional agents to employ in the treatment of PTCL and one mode of action may be to alter the tumour environment to promote suppressive Tregs.

7.4 Synergy between ITK inhibitors and other agents

The analysis of chromosomal aberrations has been important to the understanding of B-cell lymphomas and the major translocations e.g. t(8;14) involving c-MYC in Burkitt lymphoma, demonstrate the driver oncogenes in these diseases. A few translocations have been described in PTCL, which include the t(5;9), fusing ITK to SYK, translocation (Streubel *et al*., 2006) and, even more rarely, an internal rearrangement of chromosome 2 causing a fusion between CTLA4 and CD28 genes (Yoo *et al*., 2016). It could, therefore, be argued that ITK is a driver in a minority of PTCL and ITK inhibitors might be highly effective single agents in these diseases.

However, ITK is highly expressed in a larger proportion of PTCL and evidence is provided in this thesis and elsewhere that it might play a role in T-cell proliferation by

modulating TCR signalling (Readinger *et al*., 2009). Therefore, it is reasonable to suppose that ITK inhibitors may be a useful addition to other treatments either conventional chemotherapy or novel agents.

The role of ITK in $CD4^+$ T-cell differentiation has been exploited in pre-clinical work in B-cell lymphomas in which it has been shown to synergise with anti-PD-L1 antibodies (Sagiv-Barfi *et al*., 2015). In this study, Ibrutinib and PF-06465469 were tested in combination Cyclosporin A, pralatrexate, gemcitabine, and romidepsin agents *in vitro*. The reason for choosing pralatrexate and romidepsin is that these agents have a licence for use in the treatment of refractory or relapsed PTCL in the USA (Coiffier *et al*., 2012; O'Connor *et al*., 2011). Gemcitabine is a pyrimidine derivative that has activity in PTCL (Arkenau *et al*., 2007) and is a component of the regimen (GEM-P, gemcitabine, cisplatin and methylprednisolone) that has recently been trialled in comparison with CHOP (cyclophosphamide, adriamycin, vincristine and prednisolone).

No convincing synergistic activity with ITK inhibitors was obtained with any of these agents. Cyclosporin A is an immune modulator that inhibits T-cell activity by blocking calcium signalling and, as described in chapter 3, acts downstream of ITK and would not be expected to enhance the effects of ITK inhibitors. Romidepsin, pralatrexate and gemcitabine had IC50 values that were ~500-fold lower than that of the ITK inhibitors making it impossible to demonstrate synergy *in vitro*.

Doxorubicin is a chemotherapeutic agent used to treat a wide range of cancers, which is chemically related to adriamycin, and remains a first-line antineoplastic drug (Outomuro *et al*., 2007; Moretti *et al*., 2009). Although it is an effective chemotherapeutic, doxorubicin is linked to cardiotoxicity (Hanušová *et al*., 2011) and therefore, attempts to reduce the dose of this drug while maintaining efficacy of the regimen will be worthwhile for patients. Significant synergy was obtained between ITK inhibitors and doxorubicin, suggesting that these small molecule inhibitors might be useful adjuncts to current treatment. Although in this study, ITK inhibitors have been shown to synergise in combination with doxorubicin, the mechanism of effect of combination therapy is not known and has not been investigated.

Idelalisib and pictilisib are small molecule inhibitors active against PI3K; Idelalisib is specifically active against the PI3Kδ isoform whereas Pictilisib is also active against PI3Kα. PI3K and specifically PI3Kδ are required for Tfh development and function in

normal germinal centres (Rolf *et al*., 2010) and it is, therefore, a good candidate for therapy in PTCL. Here we reported that ITK inhibitors synergistically enhanced the anti-tumour efficacy of both Idelalisib and Pictilisib in T-cell lines. The molecular mechanisms by which ITK and PI3K inhibitors exert their combined antitumor effects were not investigated but we speculate that inhibition of ITK might enhance the suppression signalling downstream of PI3K including the AKT and mTOR pathway which is critical for cell proliferation and survival.

Finally, ITK has important roles in mediating chemokine signalling as well as TCR signalling. Trafficking of lymphocytes might be an important phenotype to target in the treatment of PTCL although there is no information on this. Ibrutinib repressed migration to the chemokine CXCL12 and plerixafor, an antagonist of CXCR4 (the receptor for CXCL12) enhanced this effect suggesting that CXCR4 signalling for migration is not completely through ITK. The findings suggest that combining an ITK inhibitor and CXCR4 antagonist may be useful in PTCL.

7.5 Could ITK be a target for therapy in PTCL?

Peripheral T cell lymphomas (PTCLs) are a diverse group of neoplasms with different clinical and biological behaviour. Their biology is poorly understood; as a result, they are not easy to classify, and no effective and standard therapies are available for PTCLs, and patients with PTCLs are generally treated with multi-agent chemotherapy regimens (Vose *et al*., 2008). Due to a poor clinical outcome for patients with most of the subtypes of these lymphomas and lack of potentially curative approaches, new modalities and novel agents are required to improve survival in these patients. Analysis of molecules modulating T-cell functions, and drugs that can interrupt this pathway may lead to the discovery of better therapeutic targets for malignant T cell lymphomas.

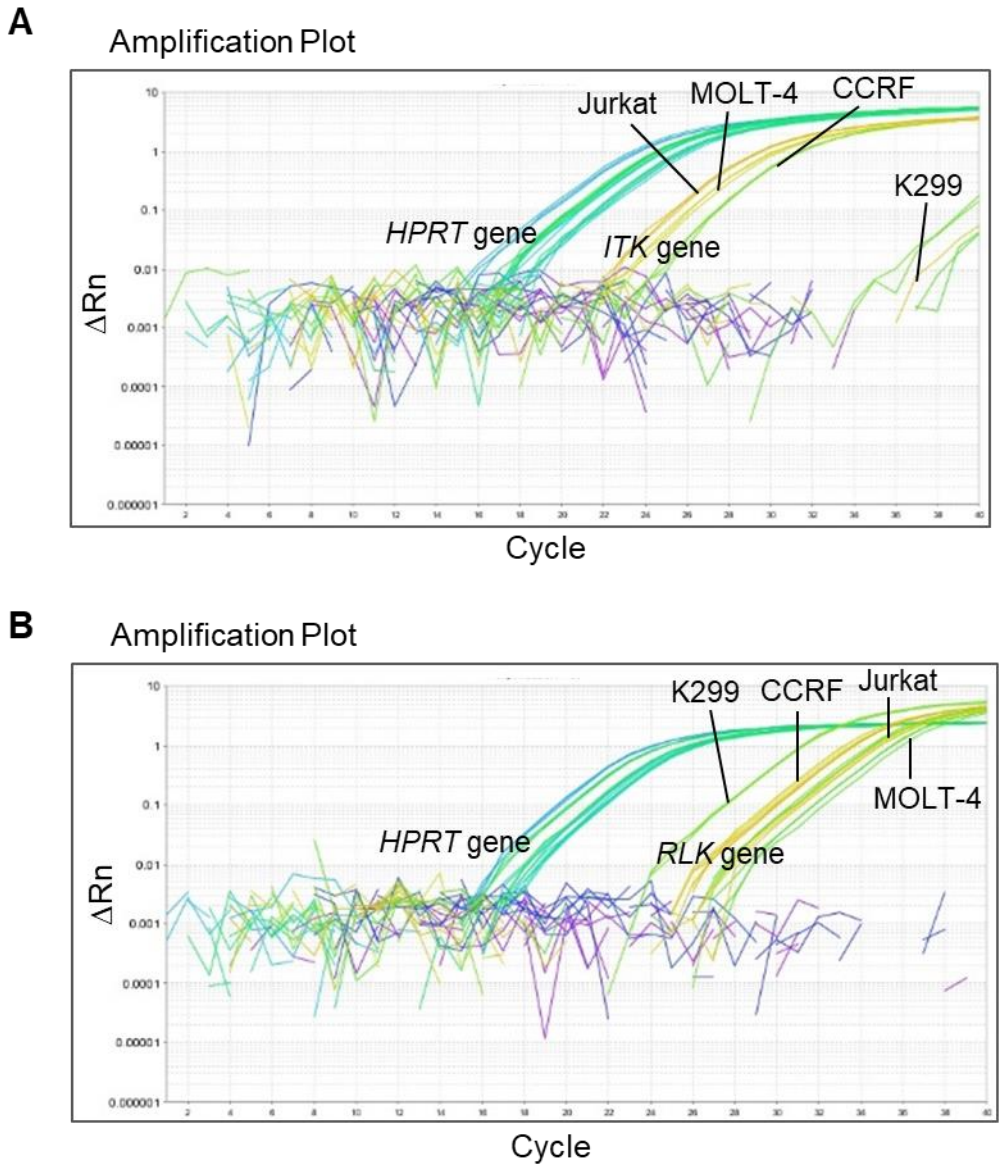
As well as roles in TCR signalling and migration, ITK is also essential for normal $CD4^+$ T-cell differentiation (Readinger *et al*., 2009; Bluestone *et al*., 2009). By employing mice bearing homozygous disruptions of Itk it has been demonstrated that this gene regulates the balance between Th17 and Treg subsets (Gomez-Rodriguez *et al*., 2014) as well as being important in Th2 (Fowell *et al*., 1999) and Th9 (Gomez-Rodriguez *et al*., 2016) differentiation. This is functionally important and $Itk^{-/-}$ mice have an altered balance between activating and suppressive T-cell functions (Gomez-Rodriguez *et al*., 2016).

Therefore, interruption of ITK activity by an orally bioavailable small molecule inhibitor with an acceptable side effect profile might be expected to repress TCR mediated functions such as proliferation or migration but could also modify $CD4^+$ T-cell differentiation to alter the environment such that repressive functions predominate. Modification of $CD4^+$ T-cell differentiation to treat PTCL is a novel suggested mechanism of action. Interestingly the inhibitory effects of ibrutinib on ITK function are already beginning to be exploited. Based on work in mouse models of graft versus host disease (GVHD) (Dubovsky *et al*., 2014) and clinical studies the FDA has now approved ibrutinib for the treatment of chronic GVHD.

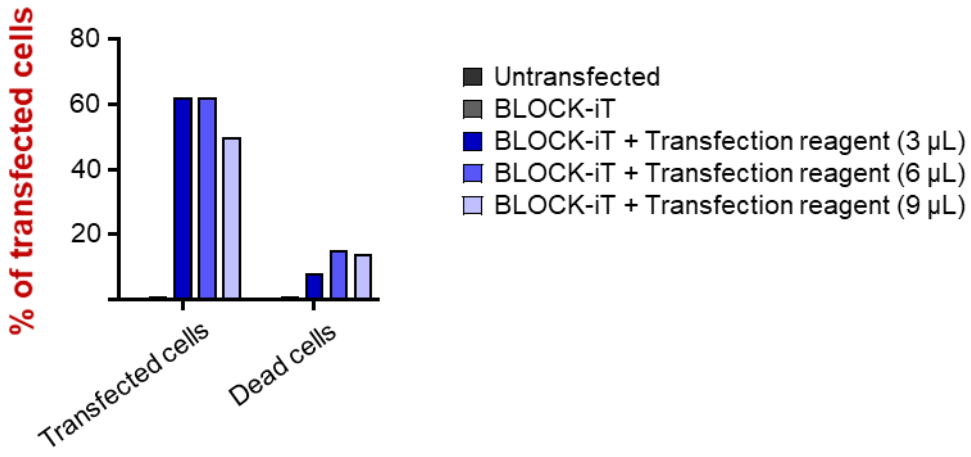
7.6 Conclusions

In summary, this project has focused on the role of ITK in T-cell signalling, migration and differentiation. Collectively the evidence indicates a potential therapeutic effect of ITK inhibitors mediated by inhibition of TCR signalling, repression of migration and alteration of differentiation to a more repressive environment. Also, the synergy of ITK inhibitors with some chemotherapeutic or novel agents might lead to impressive therapeutic effects and would be a better option for patients with some types of PTCL than current standard methods of chemotherapy treatment. From this body of research, two key areas for future work are particularly important. First of all, it is essential to investigate the role of ITK inhibitor *in vivo*; therefore, a mouse/animal model of T cell lymphomas would be required in order to validate the result obtained with small molecule inhibitors *in vitro*. Secondly, it will be interesting to explore the combined effects of ITK inhibitor with PI3K inhibitors or Plerixafor in mice prone to develop peripheral T cell lymphomas. Since the mechanism of action for these combinations remains unknown, the investigation of the mechanism behind the synergy will be required. This will help to further understand the pathogenesis of PTCL and also provide more effective treatment approach for these diseases.

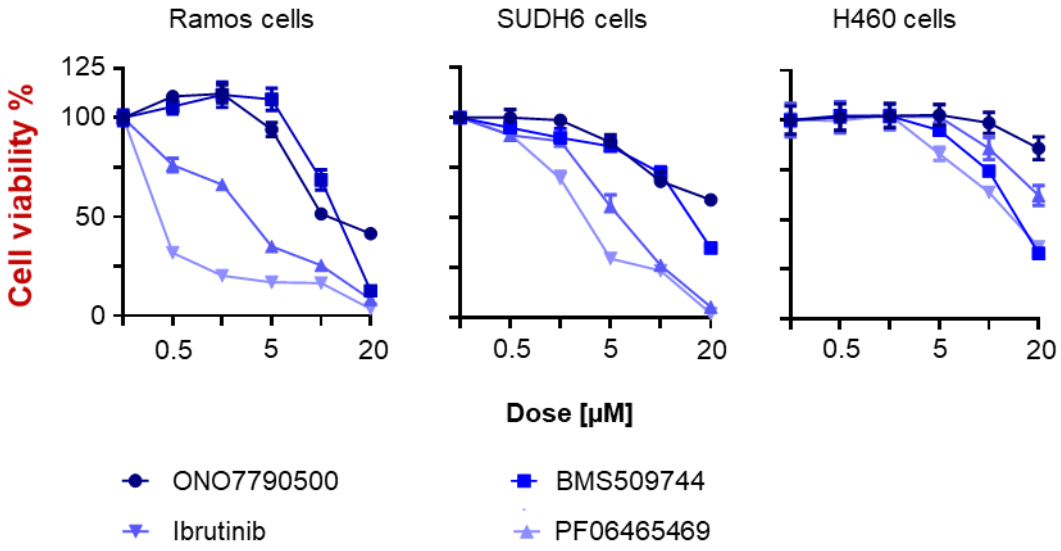
Appendices



Supplemental figure A. Example of Ct values of the data that are obtained from a RT-PCR experiment. Amplification plots show Ct curves of *ITK*, *RLK* and *HPRT* (housekeeping genes) in four T-cell lymphoma/leukaemia cell lines and Ramos and HEK293 cell lines (Figure 3.1A). The Ct values of *ITK* target (C) and *RLK* (B) are determined by Taqman RT-PCR. Y-axis (ΔRn = normalized fluorescence intensity) and X-axis (number of cycles).



Supplemental figure B. siRNA transfection in Jurkat cells was optimized using Block-iT Fluorescence Oligo with DharmaFECT 1 transfection reagent. Briefly, Jurkat cells were transfected at the density 2, 4 and 8 x 10^5 cells/mL with 100 nM Block-iT using various amount of DharmaFECT transfection reagent (3, 6 and 9 µL) overnight. The transfected rate was around 60 % by conducting 3 µL DharmaFECT in 2-4 x 10^5 cell/mL per well in 24 well plates. The results presented are representative of two experiments.



Supplemental figure C. Effects of small molecule ITK inhibitors on non-T cell lymphoma cell lines (Ramos, SUDH6 and H469 cell lines).
Cells were treated with ONO7790500, BMS509744, Ibrutinib and PF-06465469 for 48 h at the indicated concentrations of the drugs. The graph presents that ONO7790500 and BMS509744 were less sensitive to Ramos and SUDH6 cell lines, whereas H460 cells seem to be more resistant to ONO-7790500 and Ibrutinib. Cell viability was evaluated by using the CellTiter-Glo (CTG) luminescent cell viability assay. The experiments were repeated twice.

Jurkat cells, Drug µM			Fractional Inhibition, fa	Combination ratio
ONO00779050	Ibrutinib	Doxorubicin		
1			0.195000	Combination with a non-fixed ratio
2			0.276000	
4			0.584000	
6			0.799000	
8			0.890000	
	1		0.208000	
	2		0.361000	
	4		0.444000	
	6		0.663000	
	8		0.816000	
		0.02	0.11500	
		0.04	0.310000	
		0.08	0.440000	
		0.16	0.667000	
		0.32	0.840000	
ONO00779050 + Doxorubicin				
1	0.02		0.32600	non-fixed ratio
2	0.04		0.558000	
4	0.08		0.874000	
6	0.16		0.983000	
8	0.32		0.992000	
Ibrutinib + Doxorubicin				
1	0.02		0.374000	non-fixed ratio
2	0.04		0.676000	
4	0.08		0.942000	
6	0.16		0.992000	
8	0.32		0.994000	

Supplemental table A. This table show experimental design and dose relationships of ONO-00779050, ibrutinib, doxorubicin and their two-drug combinations on growth inhibition of Jurkat T cells after 48 h exposure.

MOLT-4 cell, Drug µM			Fractional Inhibition, fa	Combination ratio
ONO00779050	**Ibrutinib**	**Doxorubicin**		
2			0.141000	Combination with a non-fixed ratio
4			0.255000	
6			0.458000	
8			0.590000	
16			0.840000	
	2		0.152000	
	4		0.223000	
	6		0.322000	
	8		0.464000	
	16		0.702000	
		0.01	0.222000	
		0.02	0.398000	
		0.04	0.456000	
		0.08	0.596000	
		0.16	0.766000	
ONO00779050 + Doxorubicin				
2	0.01		0.589000	non-fixed ratio
4	0.02		0.685000	
6	0.04		0.843000	
8	0.08		0.927000	
16	0.16		0.974000	
Ibrutinib + Doxorubicin				
2	0.01		0.590000	non-fixed ratio
4	0.02		0.703000	
6	0.04		0.838000	
8	0.08		0.907000	
16	0.16		0.977000	

Supplemental table B. This table show experimental design and dose relationships of ONO-00779050, ibrutinib, doxorubicin and their two-drug combinations on growth inhibition of MOLT-4 cells after 48 h exposure.

Jurkat cell, Drug µM			Fractional Inhibition, fa	Combination ratio
ONO00779050	Ibrutinib	Idelalisib		
0.5			0.117000	Combination with a fixed constant ratio
1			0.230000	
2			0.372000	
4			0.728000	
8			0.948000	
16			0.984000	
	0.5		0.164000	
	1		0.225000	
	2		0.296000	
	4		0.471000	
	8		0.661000	
	16		0.965000	
		1.25	0.108000	
		2.5	0.210000	
		5	0.251000	
		10	0.445000	
		20	0.660000	
		40	0.864000	
ONO00779050 + Idelalisib				
0.5		1.25	0.307000	fixed constant ratio
1		2.5	0.423000	
2		5	0.664000	
4		10	0.897000	
8		20	0.988000	
16		40	0.989000	
Ibrutinib + Idelalisib				
0.5		1.25	0.310000	fixed constant ratio
1		2.5	0.347000	
2		5	0.453000	
4		10	0.702000	
8		20	0.937000	
16		40	0.990000	

Supplemental table C. This table demonstrates design the experiment for drug combination and shows dose relationships (synergism, additive or antagonism) of ONO-00779050, ibrutinib, idelalisib and their two-drug combinations on growth inhibition of Jurkat T cells after 48 h exposure.

MOLT-4 cell, Drug µM			Fractional Inhibition, fa	Combination ratio
ONO00779050	Ibrutinib	Idelalisib		
2			0.211000	Combination with a fixed constant ratio
4			0.581000	
8			0.766000	
16			0.901000	
32			0.920000	
	2		0.169000	
	4		0.412000	
	8		0.625000	
	16		0.869000	
	32		0.984000	
		4	0.092000	
		8	0.241000	
		16	0.389000	
		32	0.610000	
		64	0.907000	
ONO00779050 + Idelalisib				
2	4		0.742000	fixed constant ratio
4	8		0.875000	
8	16		0.977000	
16	32		0.993000	
32	64		0.997000	
Ibrutinib + Idelalisib				
2	4		0.655000	fixed constant ratio
4	8		0.816000	
8	16		0.943000	
16	32		0.984000	
32	64		0.992000	

Supplemental table D. This table demonstrates design and dose relationships of each drug alone (ONO-00779050, ibrutinib and idelalisib) and their combinations on growth inhibition of Jurkat T cells after 48 h exposure.

Jurkat cells, Drug µM			Fractional Inhibition, fa	Combination ratio
ONO00779050	**Ibrutinib**	**Pictilisib**		
0.5			0.167000	Combination with a fixed constant ratio
1			0.290000	
2			0.403000	
4			0.706000	
8			0.808000	
	1		0.174000	
	2		0.284000	
	4		0.458000	
	8		0.683000	
	16		0.937000	
		1	0.127000	
		2	0.272000	
		4	0.421000	
		8	0.657000	
		16	0.810000	
ONO00779050 + Pictilisib				
0.5	1		0.479000	fixed constant ratio
1	2		0.538000	
2	4		0.755000	
4	8		0.960000	
8	16		0.993000	
Ibrutinib + Pictilisib				
1	1		0.452000	fixed constant ratio
2	2		0.608000	
4	4		0.793000	
8	8		0.922000	
16	16		0.982000	

Supplemental table E. This table shows design and dose relationships of each individual drug ONO-00779050, ibrutinib and pictilisib, and their combinations on growth inhibition of Jurkat T cells after 48 h exposure.

MOLT-4 cells, Drug µM			Fractional Inhibition, fa	Combination ratio
ONO00779050	**Ibrutinib**	**Pictilisib**		
1			0.069000	Combination with a fixed constant ratio
2			0.161000	
4			0.335000	
8			0.617000	
16			0.800000	
	2		0.100000	
	4		0.230000	
	8		0.407000	
	16		0.627000	
	32		0.880000	
		0.5	0.206000	
		1	0.396000	
		2	0.597000	
		4	0.721000	
		8	0.819000	
ONO00779050 + **Pictilisib**				
1	0.5		0.607000	fixed constant ratio
2	1		0.745000	
4	2		0.846000	
8	4		0.936000	
16	8		0.972000	
Ibrutinib + **Pictilisib**				
2	0.5		0.722000	fixed constant ratio
4	1		0.856000	
8	2		0.918000	
16	4		0.968000	
32	8		0.984000	

Supplemental table F. This table shows experimental design and dose relationships of each individual drug ONO-00779050, ibrutinib and pictilisib, and their combinations on growth inhibition of MOLT-4 cells after 48 h exposure.

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