Disarming tissue factor: generating a model tumour antigen

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Abstract

Coagulative abnormalities are a well-recognised feature of malignancy. The risk of developing thrombotic disorders is increased four-fold in cancer patients and more than six-fold higher in those receiving chemotherapy. Consequently, coagulative disorders are the second leading cause of death in cancer patients. One of the principal mechanisms through which cancer cells promote coagulation is through the upregulation of tissue factor (TF), a transmembrane receptor expressed on cells surrounding the blood vessels and a potent initiator of coagulation. Upon binding to circulating coagulation factor VIIa (FVIIa), TF and FVIIa form a catalytic complex, initiating the extrinsic coagulation pathway and resulting in the rapid formation of blood clots.

Chimeric Antigen Receptor (CAR) T cell therapy is an emerging form of immunotherapy which combines the specificity of antibodies with the cytolytic activity of T cells, enabling potent killing of tumours in an antigen-antibody defined interaction. TF has been explored as a therapeutic target for a range of cancer treatments, however its potential as a target antigen for CAR T cell therapy is yet to be realised. The anti-TF monoclonal antibody, TF8-5G9, has been shown to effectively bind to and inhibit the procoagulant function of human TF making it an ideal candidate for a novel CAR structure. The efficacy of the construct would first need to be demonstrated in a preclinical setting. However, human TF is highly efficient at complexing with murine FVIIia. Thus, expression of the antigen in a live model could result in major coagulopathies.

This research tackles this issue by generating a truncated form of human TF, using inverse PCR, thereby deleting the FVIIia binding sites while disabling any TF-driven coagulation. Flow cytometric and western blot analyses showed that the expression of the truncated TF was mainly intracellular. However, no coagulation was triggered by the low-level surface expression of the truncated TF, as shown by our in vitro coagulation assay. Failure to efficiently export TF to the surface may be attributed to protein misfolding and thus demands different strategies to truncate the TF gene. Together, these results provide the model framework for TF as a novel target in immunotherapy.
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<th>Description</th>
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<tbody>
<tr>
<td>Δ1-106huTF</td>
<td>Truncated human tissue factor gene</td>
</tr>
<tr>
<td>ACT</td>
<td>Adoptive cell therapy</td>
</tr>
<tr>
<td>AF488</td>
<td>Alexa Fluor 488 conjugated to goat IgG</td>
</tr>
<tr>
<td>AF680</td>
<td>Alexa Fluor 680 conjugated to donkey IgG</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric antigen receptor</td>
</tr>
<tr>
<td>CD19</td>
<td>Cluster of differentiation 19</td>
</tr>
<tr>
<td>CD28</td>
<td>Cluster of differentiation 28</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>CTL-associated protein 4</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FGA</td>
<td>Fibrin generation assay</td>
</tr>
<tr>
<td>FVα</td>
<td>Activated coagulation factor V</td>
</tr>
<tr>
<td>FVII</td>
<td>Coagulation factor VII</td>
</tr>
<tr>
<td>FVIIa</td>
<td>Activated FVII</td>
</tr>
<tr>
<td>FVII-PPP</td>
<td>FVII-depleted platelet poor plasma</td>
</tr>
<tr>
<td>FIX</td>
<td>Coagulation factor IX</td>
</tr>
<tr>
<td>FX</td>
<td>Coagulation factor X</td>
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<td>Coagulation factor XI</td>
</tr>
<tr>
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<td>Coagulation factor XII</td>
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<td>GOI2</td>
<td>Gene of interest 2</td>
</tr>
<tr>
<td>huTF</td>
<td>Full-length human tissue factor gene</td>
</tr>
<tr>
<td>IDT</td>
<td>Integrated DNA Technologies</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IPCR</td>
<td>Inverse polymerase chain reaction</td>
</tr>
<tr>
<td>ITR</td>
<td>Inverted tandem repeat</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>luc</td>
<td>Luciferase gene</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>P2A</td>
<td>2A peptide derived from Porcine teschovirus-1</td>
</tr>
<tr>
<td>pac</td>
<td>Puromycin N-acetyltransferase gene from <em>Streptomyces alboniger</em></td>
</tr>
<tr>
<td>PAR2</td>
<td>Protease-activated receptor 2</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death protein 1</td>
</tr>
<tr>
<td>PD-L1/PD-L2</td>
<td>Programmed death-ligand 1/Programmed death-ligand 2/</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet poor plasma</td>
</tr>
<tr>
<td>R5</td>
<td>RPMI medium supplemented with 5% FCS</td>
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<tr>
<td>R10</td>
<td>RPMI medium supplemented with 10% FCS</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein (dTomato)</td>
</tr>
<tr>
<td>rSAP</td>
<td>Recombinant shrimp alkaline phosphatase</td>
</tr>
<tr>
<td>SB</td>
<td><em>Sleeping beauty</em></td>
</tr>
<tr>
<td>scFv</td>
<td>Short chain variable fragment of antibody</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA buffer</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TF8-5G9</td>
<td>Anti-TF antibody</td>
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Chapter One:

Introduction
1.0 Introduction

1.1 Cancer

Cancer refers to a group of diseases characterised by the uncontrolled proliferation of abnormal cells with the potential to invade tissues [1]. Cells become transformed through the acquisition of genetic mutations or epigenetic changes in genes which regulate the cell cycle and promote malignancy [2]. Hereditary and environmental elements are both contributors to the causes of cancer, with the majority of cases stemming from the latter [3]. These include lifestyle factors such as tobacco use, diet and exercise, sun exposure, chronic inflammation and infection by oncogenic viruses [3]. Globally, cancer ranks among the top leading cause of deaths, estimated to develop in one in six people [4]. Standard treatment options include chemotherapy, radiation and/or surgery; however, these are most often not curative and pose a high risk of adverse side effects [5]. Researchers around the world are endeavouring to create new therapies which meet the demand for safer and more effective treatments.

1.2 The immune response and tumours

The human immune system consists of two branches: the innate arm and the adaptive arm [6]. In general, the innate response is rapid, non-specific and does not confer lasting immunity [6]. In contrast, the adaptive arm is slower to respond but capable of generating specific immunological memory [6]. In response to infection, the innate arm clears foreign material by phagocytosis or apoptosis [7]. Innate leukocytes such as macrophages may engulf or release enzymes which destroy extracellular pathogens, while natural killer (NK) cells induce apoptosis in intracellularly infected or transformed malignant cells. Dendritic cells (DCs) and other members of the antigen-presenting cell (APC) family act as a bridge between the innate and adaptive arms [6, 7]. APCs sample native antigen and present processed peptides on major histocompatibility complex (MHC) molecules to cells of the adaptive immune system [6].

The two primary mediators of adaptive immunity are T and B lymphocytes [6]. The adaptive immune response is initiated when antigen-specific T cells encounter peptide bound to MHC, stimulating signalling through the T cell receptor (TCR; ref. [6, 8]). Additional costimulatory signalling through receptors such as cluster of differentiation
28 (CD28) is required to enable full activation of T cells [6]. T cells mediate diverse functions in the immune system and are broadly classed into helper, cytotoxic or regulatory subsets based on their differential expression of receptors, cytokines and transcriptional profiles [6]. B lymphocytes provide humoural immunity through the production of soluble immunoglobulin (Ig), also known as antibody [9]. Antibodies can directly neutralise pathogens or tag them for phagocytosis by innate leukocytes thus, have a crucial role in enhancing pathogen clearance [8, 10]. On naïve B cells, immunoglobulins exist as membrane bound receptors which recognise native antigens [8]. Antigen binding and cytokine activation signals provided by T helper cells stimulate B cell differentiation into antibody secreting plasma subsets, or memory subsets which persist long-term and rapidly expand upon re-exposure to specific antigens [6, 11].

With regard to cancer, the anti-tumour immune response is primarily mediated by NK cells and cytotoxic T lymphocytes (CTLs; ref. [12]). Growing tumours may release inflammatory cytokines which recruit CTLs and NK cells to the tumour site [13]. Infiltrating CTLs engage MHC-tumour peptide complexes through their antigen-specific TCRs and in response, release molecules which kill and eliminate tumours. Tumour cells which evade immune attack enter an immune-mediated equilibrium phase in which genetic and epigenetic changes accumulate [13, 14]. During this phase, poorly immunogenic variants are selected for, sculpting tumours into genetically diverse, heterogeneous and immune-resistant clonal populations [13, 15]. Tumour cells exhibit a myriad of mechanisms which enable escape from host immune responses [5, 12]. These include downregulation of MHC, upregulation of immune checkpoint ligands and upregulation of suppressive cell subsets that limit CTL and NK cell activation [12, 13]. Cancer cells exploit these safety mechanisms, meant to protect against autoimmune reactivity, and suppress the immune response to favour survival [15, 16]. The complex, immunosuppressive network established by cancer poses a barrier to the natural host anti-tumour immune response as well as potential immune-based treatments [12, 16, 17].

### 1.3 Cancer immunotherapies and chimeric antigen receptor (CAR) T cells

Immunological approaches to therapy aim to augment the natural host response against cancer cells [18]. Various immune-based strategies have gained traction over the years including cancer vaccines, immune checkpoint inhibitors and most recently, adoptive cell therapies (ACT; ref. [19]). As these treatments have gradually been introduced into the
In the clinic, interest has been sparked from both the public and cancer researchers alike as to whether these approaches can be more broadly generalised to treat all cancers.

Despite considerable efforts, therapeutic cancer vaccines for the treatment of patients who have already developed cancer have found limited success over the years [20]. With the exception of Sipuleucel-T, an APC-based vaccine which targets prostate antigen, most vaccines have proven to be ineffective due to issues such as immune suppression by the tumour microenvironment and lack of unique tumour-associated antigens [20, 21]. Interestingly, phase I clinical trial data have rarely revealed immune-related toxicities, validating the relative safety of this approach. Hope remains that the efficacy of cancer vaccines can be improved with the use of adjuvants or by combination therapy with other anti-cancer treatments [20, 22].

Perhaps the most well-known of the aforementioned immunotherapies are inhibitors of the immune checkpoints programmed cell death protein 1 (PD-1) and CTLA-associated protein 4 (CTLA-4). Immune checkpoints function as negative regulators of T cell activation and are upregulated by activated T cells to limit autoimmunity [23, 24]. The inhibitory pathways stimulated by PD-1 activation are triggered by receptor binding to its ligands PD-L1 or PD-L2 [25]. These ligands are broadly expressed by immune cells and healthy tissues to limit excessive inflammatory responses that lead to autoimmune destruction of tissues and chronic inflammation [26]. However, tumour cells frequently exploit PD-1 activation on T cells as an escape mechanism, upregulating the inhibitory ligand PD-L1 to suppress the immune response against cancer [25]. The mechanism of the CTLA-4 checkpoint is distinct from this, preventing immune cell activation by attenuating the costimulatory signalling required for effective T cell responses [27]. CTLA-4 shares structural homology with the T cell costimulatory receptor CD28 and competes for binding to members of the B7 protein family on APCs. CTLA-4 displays a higher affinity for these ligands, thereby outcompeting CD28 for binding and resulting in reduced costimulatory signalling and T cell activation [28]. Since their inception, monoclonal antibodies which block these immune checkpoints to reengage the anti-tumour response have proven strikingly effective, recently earning the two researchers behind concept the Nobel Prize in Physiology or Medicine for 2018 [29]. The FDA approved anti-PD-1 antibodies nivolumab and pembrolizumab are currently in clinical use for the treatment of advanced malignancies such as melanoma, non-small cell lung cancer and Hodgkin’s lymphoma to name a few [27, 30]. Clinical trial data of PD-1
blockade show long-lasting and clinically meaningful responses, translating into improved overall and progression-free survival in a subset of patients with advanced stage disease [31-34]. Similarly, the CTLA-4 antagonist ipilimumab, approved for the treatment of metastatic melanoma, has demonstrated reduced tumour progression and survival benefits both in patients with previously treated or untreated melanoma [35, 36]. Unfortunately, objective responses are infrequently observed therefore, the prolonged effects of treatment extend to only a small number of patients [32, 33, 37, 38]. Moreover, given that immune checkpoint inhibitors act to promote unrestrained T cell proliferation, use of these monoclonal antibodies has been associated with a high risk of immune-related adverse events [32, 33, 35, 39]. Approximately 70-80% of patients experience side effects from treatment; however, most are low-grade and clinically manageable [39, 40]. Immune checkpoint inhibitors are a clinical success story and mark a turning point in the way that cancer can be treated. Despite the remarkable ability of immune checkpoint inhibitors to extend survival and deliver durable and potentially curative effects, poor efficacy in the majority of patients as well as issues regarding safety warrant further investigation into alternative approaches in order to meet the needs of a broader range of patients.

Another developing field of immunotherapy research is ACT which involves the administration of live, anti-tumour immune cells into a host [41, 42]. The biological approach to ACT involves treating patients with autologous tumour infiltrating lymphocytes; cells derived from resections of their own solid tumours and clonally expanded ex vivo [41, 42]. Preliminary findings from melanoma patients have been positive, with 40% of patients from all stages of disease exhibiting complete and durable regressions [43, 44]. Limiting the expansion of this therapy to other tumour types is accessibility to tumour infiltrating lymphocyte populations. Unlike melanoma, many epithelial-derived tumours cannot easily be excised therefore, sources from which tumour-specific cells can be derived are restricted. To overcome these limitations, researchers have extended the scope of ACT by applying genetic approaches that break the limitations of biology [45]. Chimeric antigen receptors (CAR), constructs designed to express on T cells and specifically target tumour-associated antigens, provide an engineered approach to ACT by offering an alternative means of generating tumour-specific populations of lymphocytes [45].
First proposed by Zelig Eshhar and colleagues in 1989, CARs are one of most recent additions to the toolbox of immunotherapy [46]. The basic CAR structure comprises three regions: the extracellular antigen recognition domain derived from single chain variable fragments (scFv) of antibodies, the transmembrane domain which anchors the protein to the cell surface and the intracellular domain consisting of T cell signalling components which enable activation of cytotoxic functions (Figure 1; ref. [47-49]). Direct recognition of native antigen can occur through the antibody-derived receptor, bypassing the need for MHC-restricted antigen peptide presentation which is usually downregulated in tumour environments [45, 50]. By this design, scFv can be selected to target any tumour-associated antigen desired, redirecting CARs T cells against a wide array of tumour antigens which may previously have been resistant to immune attack, while overcoming the complexities of MHC polymorphisms between patients.

One of the biggest challenges in CAR T cell development is selection of an appropriate target antigen [22, 45, 50]. As most proteins expressed by tumours are also present on healthy cells, anti-tumour CARs can inadvertently attack self-tissue, resulting in severe off-tumour but on-target toxicities [43, 47]. Even minute expression levels by healthy cells can trigger full T cell activation and therefore, the ideal candidate protein is a surface antigen whose expression is fully restricted to tumour cells or non-vital cells [22, 51]. An antigen which closely meets these requirements is the B cell marker cluster of differentiation 19 (CD19), which has been a leading target of choice in early CAR T cell trials [45]. CD19 expression is a unique to B cells and is upregulated in most haematological malignancies including acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia and B cell lymphomas [52]. Targeting of tumours with second generation anti-CD19 CAR T cells has delivered remarkable response rates [53, 54]. One trial conducted by Maude and colleagues demonstrated complete remission in 90% of children and adults with relapsed or refractory ALL [53]. Of these patients, 70% had sustained remissions of up to two years which were associated with persistence of the reinfused cell subset. Results from this study demonstrated that engineered T cells populations could expand in vivo, have potent activity against advanced disease and persist long-term in hosts. While long-term effector functions are advantageous for eradicating larger tumour masses, the continued targeting of healthy CD19-expressing cells resulted in B cell aplasia in all patients who had a response. For most people, the effects of B cell aplasia are not life-threatening and can be clinically managed with intravenous Ig replacement therapy [45, 47]. Moreover, cytokine release syndrome
arising from infusional toxicities can be effectively controlled with interleukin 6-targeted therapies. Favourable outcomes seen from CD19 CAR trials highlight the therapeutic potential of immune cell engineering. Despite their success in haematological settings, application of CAR T cells to the solid tumour context has proven more challenging [47, 51, 55-57]. Epithelial solid cancers account for 90% of all cancer-related deaths yet studies evaluating solid tumour antigens form the minority of CAR clinical trials [41, 58]. As mentioned previously, expression of tumour antigen on healthy tissues creates specificity issues for solid tumour CARs [45, 50]. Immune attack of vital tissues has been the cause of multiple fatalities and as such, created valid concerns for patient safety in CAR trials going forward [59, 60]. Because few truly tumour-specific antigens exist, researchers are looking for other targets and features which discriminate normal from malignant cells [45, 50].
Figure 1: Prototypical structure of a chimeric antigen receptor (CAR).

The basic CAR construct consists of an antigen recognition domain, derived from antibody fragments specific to tumour-associated antigen, spliced onto T cell signalling domains. When expressed on patient T cells, these engineered constructs redirect T cell cytotoxic functions with antibody-mediated specificity towards antigen-expressing tumour cells, thus enabling potent cell killing.
1.4 Overview of coagulation

Haemostasis is the physiological response which maintains blood circulation and prevents bleeding (by clotting) upon injury to the vascular system [61]. This complex process is highly regulated by cellular and molecular components, which interact to promote procoagulant or anticoagulant functions at varying stages of the haemostatic process [62]. Under normal physiological conditions, coagulation factors circulate in the blood in their precursor states [61]. Damage to vessel walls exposes collagen, present within the endothelium, to the luminal contents resulting in adhesion of circulating platelets to endothelial collagen [63]. Contact between platelets and collagen fibres results in platelet activation, stimulating the release of dense granules which contain active factors that further promote the aggregation of platelets. A temporary platelet plug is formed at the site of injury to prevent bleeding and becomes moderately stabilised through the binding of fibrinogen in the blood to activated platelets [61, 64]. The plug becomes fully reinforced by the crosslinking of fibrin monomers, following conversion of fibrinogen to fibrin through the intrinsic and/or extrinsic coagulation cascade.

The extrinsic pathway is triggered by the binding of two coagulation factors; active/inactive factor VII (FVIIa/FVII respectively) and tissue factor (TF; also known as FIII; ref. [65]). TF is a transmembrane receptor which provides haemostatic protection. Expression of TF is predominantly on the subendothelial cells surrounding blood vessels and on vital organs such as the brain, lungs and heart [66]. External trauma to vessel walls exposes TF to FVIIa circulating in the blood, forming a catalytic TF-FVIIa complex which activates other components of the cascade including FIX and FX [61, 65]. The intrinsic pathway, also known as the TF-independent pathway, is engaged upon exposure of coagulation FXII to anionic/negatively charged surfaces such as endothelial collagen, exposed by vascular damage, or polyphosphates, released by activated platelets [61, 64]. In cascading fashion, activated FXII catalyses the activation of factor XI, which in turn activates FIX, which then finally activates FX [61, 64]. At FXa, the intrinsic and extrinsic pathways converge to form the common prothrombinase pathway [67]. Activated FX complexes with thrombin and FVa, forming the prothrombinase complex that cleaves prothrombin to thrombin [68, 69]. Thrombin catalyses the conversion from fibrinogen to fibrin; the final step in the coagulation cascade [69].
While it is known that TF is a potent initiator of coagulation, the majority of TF expressed in the body exists in a non-coagulant or cryptic form [70-72]. Mechanisms of TF decryption have yet to be elucidated; however, some theories suggest structural dissimilarities between non-coagulant / cryptic TF and procoagulant / decrypted TF [71]. There is variable evidence on whether cryptic TF exists in a dimerised or oligomerised state [70]. Conformational changes may expose a hidden binding cleft within the protein complex to enable binding to FX and conversion to the active decrypted state [71]. The mechanisms which support the conversion between encrypted and decrypted states may underlie the cause of some coagulation-related pathologies.

1.5 Tissue factor: a model antigen for anti-cancer immunotherapy

Although coagulation is a vital physiological process, coagulative abnormalities feature prominently in malignancy [73-75]. Excessive coagulation can lead to thrombosis; the formation of clots within blood vessels that occlude blood flow [76]. The association between cancer and thrombosis, first characterised by Armand Trousseau in 1865, has now been made more evident with studies showing the risk of thrombotic events is increased four-fold in cancer patients and exacerbated to more than six-fold in those receiving chemotherapy [77-81]. Presently, thrombotic disorders are recognised as the second leading cause of death in cancer patients [82].

At the centre of the relationship between cancer and thrombosis is TF-mediated coagulation. TF expression is often dysregulated in tumour cells resulting in aberrant upregulation, particularly in aggressive cancer types such as breast, melanoma and lung cancer [83-85]. Strikingly, the level of overexpression can be up to $10^5$-fold that of non-malignant tissues and is correlated with an increased risk of thrombosis [67]. Independent of TF-mediated hypercoagulation, signalling through the cytoplasmic domain of TF activates protease-activated receptor 2 (PAR-2) pathways which function to promote tumour growth, metastasis, angiogenesis and inflammation [86, 87]. Given the clear role of TF in pathogenesis and mortality, targeting of TF represents a useful therapeutic option for anti-cancer therapy.

Current anti-cancer therapies targeting TF include FVII-immunoconjugates, which bind to TF and induce antibody-mediated cytotoxicity, and photodynamic therapy which trafficks photosensitising drugs to TF-expressing tumours, allowing localised reactive
oxygen species to induce cell death upon light activation [86, 88, 89]. Direct injection of immunoconjugates was effective at reducing tumour volumes in a preclinical setting; however, these chimeric molecules exhibited procoagulant effects through TF-FVII binding, posing a high risk for thrombosis [88]. Studies of photodynamic therapy with TF-targeting nanoparticles showed successful localisation of photosensitising drugs to TF-overexpressing tumours [89]. Though photodynamic therapy is an attractive and minimally invasive approach, the activating light cannot penetrate deeply through skin, therefore application of this approach is limited to relatively superficial tumours. An alternate method of targeting TF is through the use of monoclonal antibodies. Screening of numerous TF-neutralising antibodies has revealed that the clone TF8-5G9 is the most effective inhibitor of TF procoagulant function [90, 91]. TF8-5G9 does not directly inhibit the binding of TF to FVIIa but instead neutralises the catalytic function of the TF-FVIIa complex by binding and exerting steric hindrance, preventing complex interaction with subsequent coagulation factors in the cascade; FIX and FX [92]. In application with CAR technology, a TF8-5G9-derived CAR T cell could have substantial therapeutic potential. Exploiting the overexpression of TF as a target, an anti-TF TF8-5G9 CAR employed against tumours could mitigate increased thrombotic and metastatic risk whilst destroying TF-expressing cancer cells.

The development of such a construct would firstly require refinement in a preclinical model. A study by Petersen et al. demonstrated that in vivo, human TF and murine TF have similar binding affinities to murine FVIIa, therefore both act as potent initiators of coagulation in mice [93]. Moreover, a tumour overexpressing the human TF in mice could result in excessive coagulative events. This is particularly problematic for experimental models which are administered directly into the bloodstream, such as the pulmonary metastatic tumour model [94]. In order to create a viable model, it would therefore be necessary to negate the procoagulant activity of TF. We propose that this limitation can be overcome by expressing a truncated form of the TF protein.

Full-length TF is comprised of three domains: extracellular, transmembrane and cytoplasmic [95]. The extracellular domain consists of 219 amino acid residues which assemble into two protein modules: the membrane-associated carboxyl (C)-terminal module and the amino (N)-terminal module [95, 96]. These are separated by a disordered polypeptide linker at position 102-107 [97]. As shown in Figure 2, the binding epitope of FVIIa, resides predominantly in the N-terminal module. The most energetically
significant residues involved in this interaction have been identified as lysine-20, isoleucine-22 and phenylalanine-140 [98]. By contrast, the epitope for TF8-5G9 resides completely in the C-terminal module (Figure 2). In this research, we truncated TF at position 106 in the linker strand, removing the N-terminal module of the protein. Since FVIIa and TF8-5G9 binding regions are relatively distinct, truncation at the interdomain strand removes most of the sites involved in triggering coagulation while simultaneously retaining the epitope required for TF8-5G9 CAR T cell activation. For simplification, removal of the N-terminal module of TF will be referred to throughout this thesis as removal of FVII/FVIIa binding sites. In actuality, some residues involved in the binding interaction with FVII/FVIIa will invariably remain in the truncated mutant protein.
Figure 2: Crystal structure of the extracellular domain of tissue factor (TF).

Stick representation of the extracellular domain of human tissue factor (red). Protein orientation is with the C-terminus at the bottom of the image. (A) Residues involved in the interaction between TF to activated factor VII (FVIIa; yellow), reside predominantly in the N-terminal module. (B) Residues involved in the interaction between the anti-TF antibody TF8-5G9 to TF (blue) reside in the membrane-associated C-terminal module. Figure generated by PyMOL using the sequences from Huang et al., 1997 and Xie et al, 2018 [99, 100].
1.6 Aims and hypothesis

Application of CAR T cell therapy to the solid tumour context is currently limited by a lack of appropriate targets. Since tumour antigens are generally derived from self-proteins, off-target, off-tumour toxicities have been a common feature in clinical trials [47, 51, 55-57]. In this regard, the overexpression of TF on cancer cells presents a novel means of distinguishing malignant from non-malignant cells. TF is a relevant molecule in cancer and coagulation and warrants exploration as a potential therapeutic target. In this research, we aim to:

1. Create a truncated form of the TF antigen
2. Demonstrate surface expression of TF on a murine TF-negative cell line, detectable by TF8-5G9 antibody
3. Compare the coagulative function of the truncated TF to full-length TF
4. Develop a luciferase-based cytotoxicity assay for future testing with TF8-5G9 CAR T cells

We hypothesise that truncated TF will be capable of surface expressing on murine cancer cells, disarmed of its coagulative function and sufficiently antigenic to induce CAR T cell activation.
Chapter Two:

Materials and Methods
2.0 Methods and Materials

2.1 Media and solutions

2.1.1 RPMI-1640 Medium
For 1 L: 1 × RPMI-1640 Medium sachet (Gibco #31800-022)
2 g of sodium bicarbonate
10 mL Penicillin / Streptomycin (100 U/mL / 100 µg/mL; Gibco #15140)
55 µM β-mercaptoethanol (Gibco #21985)
Total volume of 1 L made up using milliQ water
pH 7.3
Filter sterilised (0.22 µm pore filters; Cole-Parmer® #EW-02915-52)

2.1.2 RPMI-1640 + Foetal Calf Serum (FCS)
R5: 95% RPMI-1640 medium
5% FCS (Pan Biotech #P30-3312)
R10: 90% RPMI-1640 medium
10% FCS

2.1.3 Dulbecco’s Phosphate Buffered Saline (PBS)
For 1 L: 1 × PBS sachet (Gibco #21600-010)
Total volume of 1 L was made up using milliQ water
pH 7.3
Filter sterilised (0.22 µm pore filters)

2.1.4 Bovine serum albumin (10% BSA; in PBS)
For 100 mL: 10 g of BSA (Gibco # 30063-572)
Total volume made up to 100 mL using PBS
Filter sterilised (0.22 µm pore filters)

2.1.5 Luria-Bertani Medium (LB)
For 1 L: 10 g of Bacto™ Tryptone, Enzymatic digest of casein (BD #211705)
5 g of Bacto™ Yeast extract (BD #212750)
5 g of NaCl (Scharlau #SO0227)
Total volume of 1 L was made up using milliQ water

2.1.6 50× Tris-acetate-EDTA Buffer (TAE)
For 1 L:
272 g of Tris
57 mL of glacial acetic acid
100 mL of 0.5 M EDTA pH 8.0
Total volume of 1 L was made up using milliQ water
pH 8.0 with NaOH

2.1.7 2× Ammonium sulphate [(NH₄)₂SO₄] buffer
150 mM Tris-HCl pH 8.8
40 mM (NH₄)₂SO₄
0.02% Tween20

2.1.8 2× Polymerase chain reaction (PCR) master mix
2× (NH₄)₂SO₄ buffer
4 mM MgCl₂
0.05 U/µL Taq polymerase (Thermo Scientific #EP0402)
0.4 mM dNTP (NEB #N0477L)
Total volume was made up using milliQ water

2.1.9 Resazurin
0.12 g/L resazurin (Sigma-Aldrich # R7017-1G)
0.01 g/L methylene blue
0.1 M phosphate buffer
0.4 mM potassium ferricyanide
0.4 mM potassium ferrocyanide
pH 7.4

2.1.10 Cell lysis buffer
0.02% azide
150 mM NaCl
0.25% CHAPS detergent
0.5% Triton-X100
100 mM Tris
pH 8.0
cOmplete™ protease inhibitor (1 tablet / 10 mL; Roche
#11-697-498-001)

2.1.11 Western blot reagents
4× Bolt LDS Sample Buffer (Invitrogen #B0007)
10× Bolt Sample Reducing Agent (Invitrogen #B0009)
Bolt 4-12% Bis-Tris Plus gels (Invitrogen #NW04120BOX)
Bolt MOPS SDS Running Buffer (Invitrogen #B000102)
Bolt Antioxidant (Invitrogen #BT0005)
SeeBlue Plus2 Pre-stained Protein Standard (Invitrogen #LC5925)
Nitrocellulose membrane (GE Healthcare #GE10600018)
Bolt Transfer Buffer (Invitrogen #BT0006)

2.1.12 Flow cytometry fixation buffer
1% paraformaldehyde (PFA)
1% BSA/PBS
0.02% azide
Total volume was made up using PBS

2.1.13 Permeabilisation buffer
0.2% Tween20
1% BSA
0.02% azide
5% FCS
Total volume was made up using PBS
2.1.14 Coagulation-specific reagents

Human thrombin (Sigma #T-6884)

Human citrated platelet-poor plasma (PPP; Siemens #ORKL17)

Coagulation FVII-deficient PPP (Siemens #OTXV13)

10 mM calcium chloride (CaCl$_2$)
2.2 **Restriction enzymes**

**Table 2.1 List of restriction enzymes used in experiments**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction buffer</th>
<th>Incubation</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AccI (New England Biolabs; NEB #R0161S)</td>
<td>Cutsmart® (NEB #B7204S)</td>
<td>37°C, 15 minutes</td>
<td>80°C, 20 minutes</td>
</tr>
<tr>
<td>BsmBI (NEB #R0580S)</td>
<td>NEBuffer 3.1 (NEB #B7203S)</td>
<td>55°C, 15 minutes</td>
<td>80°C, 20 minutes</td>
</tr>
<tr>
<td>DpnI (NEB #R0176S)</td>
<td>Cutsmart®</td>
<td>37°C, overnight</td>
<td>80°C, 20 minutes</td>
</tr>
<tr>
<td>SfiI (NEB #R0123S)</td>
<td>Cutsmart®</td>
<td>15°C, 15 minutes</td>
<td>-</td>
</tr>
</tbody>
</table>

2.3 **Primer sequences**

**Table 2.2 List of primer sequences used in experiments**

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>5’ – 3’ sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sleeping beauty (SB)</td>
<td>GCCTCAGACAGTGTTCAAAG</td>
</tr>
<tr>
<td></td>
<td>AGGCACAGTGCGGCTGTA</td>
</tr>
<tr>
<td>IPCR</td>
<td>ACAAAACCTCGGACAGCACAATTCAGAGTTTTGAAC</td>
</tr>
<tr>
<td></td>
<td>ACCAGTGGAACCTGGAACCCAGACAGCAGC</td>
</tr>
<tr>
<td>BsmBI overhang</td>
<td>CCTCTGACGTCTCCGGCCACCATGGACAGACACA</td>
</tr>
<tr>
<td></td>
<td>GGCCCTGACGTCTCCGGATCCAGGCCTTATGAAACATTCA</td>
</tr>
<tr>
<td>Gene of interest 2 (GOI2)</td>
<td>GCCCTTCTCTCTTTCCCTGTAG</td>
</tr>
<tr>
<td></td>
<td>GTACACCACCGCTGCTTCAG</td>
</tr>
</tbody>
</table>
2.4 Antibodies

Table 2.3 List of antibodies used in experiments

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Conjugation</th>
<th>Clone</th>
<th>Isotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human TF</td>
<td>-</td>
<td>TF8-5G9</td>
<td>Mouse IgG1κ</td>
<td>Purified in-house</td>
</tr>
<tr>
<td>Anti-mouse IgG</td>
<td>Alexa Fluor 488 (AF488)</td>
<td>-</td>
<td>Goat IgG</td>
<td>Invitrogen #A-11029</td>
</tr>
<tr>
<td>Anti-mouse β-actin</td>
<td>-</td>
<td>AC-15</td>
<td>Mouse IgG1</td>
<td>Sigma #A1978</td>
</tr>
<tr>
<td>Anti-mouse IgG</td>
<td>Alexa Fluor 680 (AF680)</td>
<td>-</td>
<td>Donkey IgG</td>
<td>Invitrogen #SA5-10170</td>
</tr>
<tr>
<td>Anti-human IL-12</td>
<td>-</td>
<td>C8.6</td>
<td>Mouse IgG1</td>
<td>BD Pharmedingen #554659</td>
</tr>
</tbody>
</table>

2.5 Plasmids

Table 2.4 List of plasmids made for experiments

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Backbone</th>
<th>Gene</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSBbi-RP:TF</td>
<td>pSBbi-RP [101]</td>
<td>Full-length human TF (huTF; Integrated DNA Technologies; IDT)</td>
<td>SfiI-SfiI insertion of gBlock (encoding amino acids 1-263; NM_001993.4)</td>
</tr>
<tr>
<td>pSBbi-RP:ATF</td>
<td>pSBbi-RP</td>
<td>Δ1-106huTF (IDT)</td>
<td>SfiI-SfiI insertion of gBlock (encoding amino acids 107-263; NM_001993.4)</td>
</tr>
<tr>
<td>pSBbi-RP:ATF-I</td>
<td>pSBbi-RP:TF</td>
<td>Δ1-106huTF</td>
<td>Deletion of amino acids 1-106 by IPCR</td>
</tr>
<tr>
<td>pSBbi-BsmBI:TF</td>
<td>pSBbi-BsmBI</td>
<td>huTF (IDT)</td>
<td>BsmBI-BsmBI insertion of gene (encoding amino acids 1-263; NM_001993.4)</td>
</tr>
<tr>
<td>pSBbi-BsmBI:TF+luc</td>
<td>pSBbi-BsmBI:TF</td>
<td>Luciferase (IDT)</td>
<td>SfiI-SfiI insertion of gBlock encoding amino acids 1-550; AHC94771.1)</td>
</tr>
</tbody>
</table>
2.6 *Tumour cell lines*

**Table 2.5 List of tumour cell lines used in experiments**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Disease</th>
<th>Tissue</th>
<th>Organism</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL4</td>
<td>Lymphoma</td>
<td>T cells</td>
<td>Murine (C57BL/6)</td>
<td>ATCC #TIB-39</td>
</tr>
<tr>
<td>EL4-TF</td>
<td>Lymphoma</td>
<td>T cells</td>
<td>Murine (C57BL/6)</td>
<td>EL4 transfected with pSBbi-RP:TF</td>
</tr>
<tr>
<td>EL4-ΔTF</td>
<td>Lymphoma</td>
<td>T cells</td>
<td>Murine (C57BL/6)</td>
<td>EL4 transfected with pSBbi-RP:ΔTF</td>
</tr>
<tr>
<td>EL4-ΔTF-I</td>
<td>Lymphoma</td>
<td>T cells</td>
<td>Murine (C57BL/6)</td>
<td>EL4 transfected with pSBbi-RP:ΔTF-I</td>
</tr>
</tbody>
</table>
2.8 **Restriction enzyme digest and dephosphorylation**

gBlocks (IDT) or plasmid pSBbi-RP were first reconstituted at 10 ng/µL with sterile milliQ or 100 ng/µL in TE buffer (pH 8.0) respectively. All restriction enzyme digests were performed as per the manufacturer’s instructions. In brief, 200 ng of gBlock DNA or 2 µg of vector DNA (30 µL or 50 µL reaction volume respectively) was digested with 10 U of SfiI enzyme or 10 U of BsmBI enzyme (see Table 2.1 for conditions). Following digestion, vector DNA was dephosphorylated using 4 U of shrimp alkaline phosphatase (rSAP; NEB #M03715) and incubated at 30°C for 30 minutes then at 65°C for 5 minutes to heat inactivate the enzyme.

2.9 **DNA purification**

Digested DNA fragments were purified using the Monarch PCR & DNA Cleanup Kit (NEB #T10305) as per the manufacturer’s instructions. In brief, the samples were diluted in a 5:1 ratio of “DNA Cleanup Binding Buffer”, loaded onto a filter column and centrifuged at 16,000 ×g for one minute. The DNA was washed twice by adding 200 µL of “DNA Wash Buffer” to the column prior to centrifugation at 16,000 ×g for one minute. The sample was eluted by adding 15 µL of warm “DNA Elution Buffer” to the centre of the filter, incubating for one minute at room temperature, then centrifuged at 16,000 ×g for one minute. The DNA concentration of the sample was quantified using the Nanodrop ND-1000 instrument at 260 nm.

2.10 **Gel electrophoresis**

DNA samples (15 µL) were stained with 3 µL of DNA Loading Dye (Thermo Fisher Scientific #R0611) and 15-20 µL of sample was loaded into a 1% agarose gel consisting of 1% SeaKem LE agarose (Lonza #50004), TAE buffer and 1× SYBR Safe DNA Gel Stain (Invitrogen #S33102). The size of DNA fragments was referenced against the Quick-Load Purple 2-log DNA ladder (NEB #N05505). Gel electrophoreses were run at 100 V, 300 mA for 60 min then imaged using the LiCor Biosciences Odyssey Fc instrument at 600 nm.
2.11 Extraction of DNA from agarose gel

DNA was extracted and purified from agarose gels using the Monarch DNA Gel Extraction Kit (NEB #T10205) as per manufacturer’s guidelines. In brief, DNA bands were excised from agarose gels using a scalpel, weighed, then dissolved in 4× volumes of “Gel Dissolving Buffer”. Gel slices were incubated at 50°C and vortexed occasionally. Once completely dissolved, the sample was loaded onto a filter column and centrifuged at 16,000 ×g for one minute. The DNA was washed twice by adding 200 µL of “DNA Wash Buffer” to the column and centrifuged at 16,000 ×g for one minute. The sample was eluted by adding 15 µL of warm “DNA Elution Buffer” to the centre of the filter, incubating for one minute at room temperature, then centrifuged at 16,000 ×g for one minute. The DNA concentration of the sample was quantified using the Nanodrop ND-1000 instrument at 260 nm.

2.12 DNA ligation

Restriction enzyme-digested plasmids and gBlock inserts were ligated using T4 DNA Ligase (NEB #M0202S) according to the manufacturer’s instructions. In brief, a molar ratio of 3:1 of insert to vector DNA (90 ng:30 ng respectively) was supplemented with 400 U (1 µL) of T4 DNA Ligase, 2 µL of T4 DNA Ligase Reaction Buffer (NEB #M0202S) and milliQ water to make up a total volume of 20 µL. The reaction mixture was incubated at 16°C overnight prior to heat inactivation of the ligase at 65°C for 10 min. The ligated DNA was then used to transform bacteria (section 2.14).

2.13 Calcium chloride (CaCl2) competent Escherichia coli

LB media (2 mL) was inoculated with a colony of *E. coli* TOP10 and incubated overnight at 37°C with shaking at 200 rpm. The starter culture was added to 200 mL of LB media and incubated at 37°C with shaking at 200 rpm until an approximate OD600nm of 0.35-0.45 was reached. The large culture was incubated in an ice water bath for 20 min then centrifuged at 2,000 ×g and 4°C for 10 min. The pelleted bacteria were resuspended in 20 mL of pre-cooled 75 mM CaCl2/10 mM 1,4-Piperazinediethanesulfonic acid (PIPES)/15% glycerol, incubated in an ice bath for 20 min then centrifuged as previously mentioned. The bacterial pellet was resuspended in 2 mL of pre-cooled
CaCl$_2$/PIPES/glycerol solution then aliquoted into microfuge tubes (pre-cooled to -80°C) and stored at -80°C.

2.14 Transformation of CaCl$_2$ competent E. coli

All 20 µL of ligated DNA was added to 50 µL of thawed CaCl$_2$ competent E. coli (sections 2.12 and 2.13), gently mixed by pipetting and incubated on ice for 30 min. The bacteria were then heat shocked at 42°C for 90 s and immediately plunged into ice for five minutes. To regenerate the transformed bacteria, the sample was added to 1 mL of pre-warmed LB media (37°C) and incubated at 37°C for one hour. A 100 µL and 900 µL aliquot of regenerated bacteria were plated on LB agar plates containing 100 µg/mL of ampicillin (AppliChem #A0839,0025) and incubated overnight at 37°C, 1% CO$_2$.

2.15 Colony PCR

Transformed E. coli TOP10 bacterial colonies were touched with a sterile pipette tip, streaked onto a LB agar plate containing ampicillin (100 µg/mL) then incubated overnight at 37°C, 1% CO$_2$. The remaining bacteria on the pipette tip were resuspended in 15 µL of a PCR solution containing 250 nM of each respective forward primer and reverse primer (Table 2.2) and sterile milliQ water as a diluent. The parameters used for colony PCR were: 95°C for two minutes; 35 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for one minute per kb; 72°C for five minutes. PCR products were gel electrophoresed on a 1% agarose gel containing 1× SYBR Safe DNA Gel Stain and visualised using the LiCor Biosciences Odyssey Fc instrument at 600 nm.

2.16 Miniprep plasmid purification

Transformed E. coli TOP10 bacterial colonies were touched with a sterile loop, inoculated in 10 mL of LB broth with ampicillin (100 µg/mL) and grown overnight in a shaking incubator at 37°C, 200 rpm. Plasmids (Table 2.4) were purified from bacteria using the Monarch Plasmid Miniprep Kit (NEB # T1010L) in accordance with the manufacturer’s instructions. In brief, overnight bacterial cultures were first pelleted at 6000 × g for 15 min at 4°C then resuspended in 200 µL of “Plasmid Resuspension Buffer”. Cells were then lysed using 200 µL of “Plasmid Lysis Buffer”, gently inverting the
suspension until a uniform colour was achieved prior to incubation at room temperature for one minute. The lysate was then neutralised using 400 µL of “Plasmid Neutralisation Buffer”, gently inverting the suspension until a precipitate was formed and a uniform colour was achieved prior to incubation at room temperature for two minutes. The lysate was then centrifuged at 16,000 ×g for at least five minutes until the pellet was compact. The supernatant was then transferred to a filter column and spun for one minute. DNA was then washed by using 200 µL of “Plasmid Wash Buffer 1” followed by 400 µL of “Plasmid Wash Buffer 2”, with a one-minute 16,000 ×g centrifugation between each wash. Plasmid DNA was then eluted by adding 40 µL of pre-warmed “DNA Elution Buffer” to the centre of the filter, incubated for one minute at room temperature, then centrifuged at 16,000 ×g for one minute. The DNA concentration of the sample was quantified using the Nanodrop ND-1000 instrument at 260 nm.

### 2.17 Midiprep plasmid purification

Transformed *E. coli* TOP10 bacterial colonies were touched with a sterile loop, inoculated in 150 mL of LB broth with ampicillin (100 µg/mL) and grown for 12-16 h in a shaking incubator at 37°C, 200 rpm. Plasmids (Table 2.4) were purified from bacteria using the NucleoBond Xtra Midi Plus kit (Macherey-Nagel #740412.50) as per the manufacturer’s instructions. In brief, bacterial starter cultures were pelleted at 6000 ×g for 15 min at 4°C then resuspended in 8 mL of “Resuspension Buffer RES + RNase A”. Cells were then lysed by adding the equivalent volume (8 mL) of “Lysis Buffer LYS” and gently inverting the suspension five times prior to incubation at room temperature for five minutes. The lysate was neutralised by adding an equivalent volume (8 mL) of “Neutralisation Buffer NEU” and gently inverting the tube until the suspension turned colourless. This suspension was loaded into a column filter, pre-equilibrated with 12 mL of “Equilibration Buffer EQU” and filtered by gravity flow to clarify the lysate and remove precipitate. The filter was then washed with 5 mL of “Equilibration Buffer EQU” and discarded, leaving the DNA in the remaining column. Plasmid DNA was washed by adding 8 mL of “Wash Buffer WASH” and eluted using 5 mL of pre-warmed “Elution Buffer ELU”. The eluted plasmid was precipitated by adding 3.5 mL of isopropanol, vortexed then incubated for two minutes at room temperature. The precipitation solution was loaded into a “NucleoBond Finalizer” and washed with 2 mL of 70% ethanol. The Finalizer containing the DNA pellet was dried, to remove ethanol, and the plasmid was
finally eluted with 600 µL of “Redissolving Buffer TRIS”. Eluted DNA concentration was quantified using the Nanodrop ND-1000 instrument at 260 nm.

2.18 **Cell culture**

EL4 (Table 2.5) cells stored in liquid nitrogen were thawed in R10 media (pre-warmed to 37°C) then centrifuged at 453 × g for five minutes. Pellet was resuspended in 10 mL of R10 media, transferred to a 75cm² cell culture flask and incubated at 37°C, 5% CO₂. Cells were maintained at an approximate concentration of 1 × 10⁶ cells/mL.

2.19 **Electroporation using the Neon transfection system**

EL4 cells were transfected with plasmid DNA using the Neon Transfection System (Invitrogen #MPK10025) according to the manufacturer’s instructions. In brief, cells were washed once in PBS then resuspended at 2 × 10⁷ cells/mL in “Resuspension Buffer R”. Resuspended cells (110 µL), 1 µg of transposase DNA (SB100X) and 9 µg of plasmid DNA were combined in a microfuge tube and 100 µL of the mix was added to the Neon Tube (pre-filled with 3 mL of Electrolytic Buffer) for electroporation. EL4 cells were then electroporated with one pulse at 1080 V for 50 ms and immediately transferred to a 6-well plate containing 4 mL of pre-warmed R10.

2.20 **Puromycin selection**

To determine the optimal concentration at which puromycin inhibits the growth of untransfected EL4 cells, a resazurin cell viability assay was performed. Puromycin (InvivoGen #ant-pr-5) was added to a 96-well flat-bottom plate (Thermo Fisher) in a 2-fold serial dilution with R5 from 16,000 ng/mL to 15.625 ng/mL (50 µL/well). EL4 cells were resuspended in R5 at 2 × 10⁵ cells/mL and 50 µL of cells were added to each well resulting in a total maximal concentration of 8,000 ng/mL of puromycin and 1 × 10⁵ cells/mL. The plate was incubated for 68 hours at 37°C, 5% CO₂ before 10% resazurin (11 µL) was added to each well. The plate was incubated for a further four hours then fluorescence was measured using a microplate reader (Varioskan LUX; Thermo Fisher Scientific) with excitation 540 nm/emission 585 nm. All samples were run in duplicate.
2.21 Fluorescence microscopy

Cells (in 200 uL of R5) were first mounted on glass slides and incubated at 37°C with 5% CO$_2$ for 30 min in a humid box. A cover slip was then placed on the slides and visualised using an Olympus BX-51 upright microscope. Bright-field images were captured with an approximate shutter speed of 5 ms, while the red fluorescent protein (RFP) dTomato was visualised with a mercury/xenon lamp with an approximate shutter speed of 400 ms at 200× magnification.

2.22 Site-directed mutagenesis by inverse polymerase chain reaction (IPCR)

The pSBbi-RP:TF plasmid (Table 2.4) was mutated by IPCR to generate a plasmid containing a truncated form of the human TF gene (pSBbi-RP:ΔTF-I). pSBbi-RP:TF template DNA (30 ng) was added to 0.5 µM of IPCR forward and reverse primers respectively (Table 2.2), 200 µM of dNTPs (NEB #N0447S), 0.02 U/µL of Q5 High-fidelity DNA polymerase (NEB #M0491S), 5 µL of 5× Q5 High GC enhancer (NEB #B9028S), 5 µL of 5× Q5 Reaction Buffer (NEB #B9027S) and nuclease free water was supplemented to a total volume of 25 µL. The parameters used for IPCR were: 98°C for 30 s; 30 cycles of 98°C for 10 s, 65°C for one minute, 72°C for six minutes; 72°C for two minutes. IPCR products (5 µL) were gel electrophoresed on a 1% agarose gel containing 1× SYBR Safe DNA Gel Stain and visualised using the LiCor Biosciences Odyssey Fc instrument at 600 nm for the presence of a 6.95 kb band (corresponding to pSBbi-RP:ΔTF-I). Due to the small size difference between amplified pSBbi-RP:ΔTF-I DNA and unamplified pSBbi-RP:TF template (6.95 kb and 7.26 kb respectively), IPCR products (300 ng) were discriminated by AccI digestion (Table 2.1). pSBbi-RP:ΔTF-I has one AccI restriction site whereas pSBbi-RP: TF has two AccI restriction sites (Appendix 3Appendix 4). Following heat inactivation, the samples were gel electrophoresed and visualised as mentioned above. Bands corresponding to the size of AccI-digested pSBbi-RP:ΔTF-I (6.95 kb) were excised and purified from the gel (see section 2.11). Purified DNA was digested overnight with 10 U of DpnI enzyme to degrade any remaining template. Prior to ligation, the DNA was purified (see section 2.9) and the 5’ end was phosphorylated for blunt-end ligation with T4 Polynucleotide Kinase (PNK; Thermo Scientific #EK0031) as per manufacturer’s instructions. In brief, a 20 µL reaction containing all 15 µL of sample, 10 U of PNK, 2 µL of 10× T4 PNK Reaction
Buffer A and 2 µL of 10 mM ATP (NEB #B0202S) was incubated at 37°C for 20 min. The kinase was then inactivated at 75°C for 10 min and DNA was ligated overnight (see section 2.12). Ligase was heat inactivated at 65°C for 10 min before the DNA was used to transform bacteria.

2.23 Immunostaining for flow cytometry

All centrifugations were executed at 453 xg for five minutes at 4°C. For surface staining, cells were first washed by centrifugation then resuspended in 0.1% BSA/PBS and kept on ice throughout the protocol. Samples were prepared by aliquoting 2 x 10^5 cells into each tube then centrifuged. Pelleted cells were resuspended in 100 µL of 2 µg/mL of TF8-5G9 antibody, 2 µg/mL of anti-human IL-12 (Table 2.3) or 0.1% BSA/PBS and incubated on ice for 15 min. For cold inhibition experiments, TF8-5G9 was incubated with 10 µg/mL of TF-Fc (Callaghan Innovation) prior to primary staining. In addition, anti-mouse Ig compensation beads (two drops; BD #55284) were stained with 2 µg/mL of TF8-5G9 antibody. Cells and beads were washed by centrifugation, resuspended in 100 µL of 2 µg/mL of anti-mouse-AF488 (Table 2.3) or 0.1% BSA/PBS and incubated on ice, in the dark, for 15 min. Cells and beads were finally washed by centrifugation, resuspended in 500 µL of 0.1% BSA/PBS and stored on ice, in the dark until flow cytometric analysis. For intracellular staining, cells were fixed in 200 µL of flow cytometry fixation buffer prior to staining. Antibody dilutions were prepared in permeabilisation buffer and cells were stained as per the surface group.

2.24 Flow cytometry

Sample acquisition of stained, unstained, single stained, compensation and isotype stained cells was performed using a BD LSRFortessa flow cytometer with the BD FACSDiva software. Voltages were set using unstained and single stained cells. For all samples, 10,000 events were acquired. Using FlowJo software version 10.5, data were subjected to forward scatter (FSc) and side scatter (SSc) doublet discrimination prior to gating of cell populations and fluorophore analysis.
2.25 Preparation of lysate from cell culture

Protein lysates were prepared by adding $1 \times 10^7$ cells (EL4 or EL4 transfectants; Table 2.5) to 120 µL of cell lysis buffer followed by 30 minutes incubation on ice. The samples were centrifuged at 16,000 $\times$ g for one minute, pellets were discarded and concentration of the supernatant (containing lysate) was measured (see section 2.26 below). Lysates were then stored at -80°C.

2.26 Bicinchoninic acid assay (BCA) for protein lysate quantification

Protein concentrations were measured following the microplate procedure of the Pierce BCA Protein Assay Kit (Thermo Scientific #23227). In brief, a working reagent of 50 parts of “BCA Reagent A” with 1 part of “BCA Reagent B” was prepared. A BSA protein standard was prepared in doubling dilutions from 2 mg/mL to 0.0625 mg/mL. Protein sample or BSA standard (10 µL) was added to a 96-well flat-bottom clear plate in duplicate then 200 µL of working reagent was added to each well. The plate was incubated at 37°C in the dark for 30 min. Absorbance was measured on a microplate reader at 562 nm and data were analysed using GraphPad Prism 7 software. Protein concentrations were determined by comparing each unknown sample to the standard curve.

2.27 Western blot analysis

EL4 cell lysates (10 µg) or soluble TF-Fc (1 µg) samples were resuspended in 15 µL of PBS and added to 5 µL of 4× Bolt LDS Sample Buffer. For samples to be boiled/reduced, 10 µg of samples were resuspended in 13 µL of PBS and supplemented with 5 µL of 4× Bolt LDS Sample Buffer and 2 µL of 10× Bolt Sample Reducing Agent. Reduced samples were then boiled for five minutes and plunged on ice. A 4-12% Bis-Tris gel was inserted into an electrophoresis tank (XCell SureLock Mini-Cell; Invitrogen #EI0001) and the tank was filled with Bolt MOPS SDS Running Buffer and 0.5 mL of Bolt antioxidant. Wells were washed thoroughly before lysate samples (20 µL) and molecular weight marker (7 µL) were loaded. The gel was run for 40 minutes at 200 V, 300 mA on ice. Proteins were transferred from the gel to a nitrocellulose membrane using the XCell II Blot Module (Invitrogen #EI9051). The blot module was placed in an electrophoresis tank, filled with Bolt Transfer Buffer and the transfer was run for one hour at 30 V, 400
mA on ice. Membranes were submerged in PBS overnight at 4°C to increase the binding of the proteins to the membrane. Following overnight incubation, membranes were blocked with 1% BSA/PBS for one hour on a rocker then incubated in 1 µg/mL of TF8-5G9 antibody or anti-β-actin (Table 2.3) antibody diluted in 1% BSA/PBS overnight at 4°C on a rocker. Membranes were washed in 0.1% Tween20/PBS three times for 15 min on a shaker then incubated in a 1/10,000 dilution of anti-mouse-AF680 (Table 2.3) for one hour on a rocker and in the dark. Wash steps were performed as described above and fluorescently labelled protein was visualised using the LiCor Biosciences Odyssey Fc instrument at 600, 700 and 800 nm.

2.28 Fibrin generation assay (FGA)

EL4 or EL4 transfectants (Table 2.5) were resuspended in PBS at 2500 cells/µL and 20 µL of each sample was added to 100 µL of PPP in a 96-well flat-bottom clear plate. Coagulation was initiated by adding 14 µL of 10 mM of CaCl₂ and absorbance was immediately measured on a microplate reader at 562 nm. Measurements were taken every 10 s for 30 min at room temperature. Area under the curve was calculated from the initiation point until the timepoint at which the absorbance of the PBS control began to rise, indicating triggering of the TF-independent intrinsic coagulation pathway (5% of maximum fibrin; ref. [102]). For the blocking experiments, cells were incubated with 20 µg/mL or 40 µg/mL of either TF8-5G9 antibody or anti-human IgG isotype control (Table 2.3) on ice for 30 min prior to addition to the welled-plate. All samples were performed in triplicate.

2.29 Overhang PCR

BsmBI overhang primers (Table 2.2) were used to add BsmBI restriction sites to the full-length huTF gene. Template DNA (1 ng of pSBbi-RP:TF), 1 µL of 0.5 µM of each forward and reverse primer (see Table 2.2), 25 µL of 2× PCR master mix were mixed together and milliQ water was supplemented to reach a total volume of 50 µL. The parameters used for overhang PCR were: 95°C for two minutes; 35 cycles of 95°C for 30 s, 65°C for 30 s, 72°C for one minute per kb; 72°C for five minutes. DNA products (5 µL) were gel electrophoresed on a 1% agarose gel containing 1× SYBR Safe DNA Gel Stain and visualised using the LiCor Biosciences Odyssey Fc instrument at 600 nm.
2.30 DNA sequencing

DNA (200 ng) was amplified using 3.2 pmol/5 µL of forward and reverse primers respectively and sent to the Genetic Analysis Service by the Department of Anatomy at the University of Otago. Sanger sequencing was performed using an ABI 3730xl DNA Analyser.

2.31 Descriptive statistics, data analysis and presentation

DNA sequences were analysed using Geneious 11.1.4 software. A$_{562}$nm levels measured by BCA assay, A$_{405}$nm levels measured by FGA and A$_{585}$nm levels measured by resazurin assay were analysed in GraphPad Prism 7 (GraphPad, San Diego, CA). Flow cytometric data were analysed using FlowJo 10.5 software.
Chapter Three: 

Results
3.0 Results

3.1 Cloning of the full-length human TF gene (huTF)

For stable and efficient integration of human genes into the genome of murine EL4 cells, genes were cloned into Sleeping beauty (SB) transposon vectors for SB-mediated transposition. The SB transposon system consists of two plasmids: one encoding the SB transposase gene SB100X and the other containing the genes of interest. The SB transposase excises the gene of interest flanked by inverted tandem repeats (ITR) and inserts gene cassettes at TA-nucleotide sites randomly across the genome of target cells [103]. Synthesised gBlock DNA encoding the full-length huTF gene and the bidirectional SB vector pSBbi-RP were digested with SfiI. To ensure the correct orientation of cloning, the two SfiI sites were asymmetric (Appendix 1). Following digestion, SB vector DNA was dephosphorylated to prevent self-ligation. Prior to ligation, digested SB vector and huTF insert were subjected to gel electrophoresis and correct DNA band sizes were excised and purified (data not shown). E. coli TOP10 were then transformed with ligated DNA (pSBbi-RP:TF) and five colonies were subjected to a colony PCR using SB primers flanking the SfiI insertion sites (Table 2.2 and Figure 3A). Three colonies displayed the correct band size corresponding to the huTF gene insert (996 bp), while two colonies displayed band sizes corresponding to the previously inserted gene (1.7 kb), suggesting poor SfiI digestion and self-ligation of the vector. Two huTF-positive colonies were subjected to Miniprep plasmid amplification and purification for verification by DNA sequencing. Sequencing results showed consensus in the huTF gene of both plasmids (Figure 3B). Plasmid purified from colony 2 (Figure 3A) was chosen for further work.
Figure 3: Colony polymerase chain reaction (PCR) and sequencing results for the pSBbi-RP:TF plasmid.

Full-length tissue factor (huTF) gene was cloned into pSBbi-RP (Sleeping beauty; SB) plasmid using restriction cloning with SfiI resulting in pSBbi-RP:TF plasmid. (A) Using SB primers, colony PCR was performed to screen transformed *E. coli* colonies (1-5) for the presence of the full-length huTF within SfiI sites. Lane MW: 2-log DNA ladder, lanes 2, 4 and 5: PCR-positive colonies for full-length huTF (996 bp), lane 6: milliQ water negative control, lane 7: vector backbone (pSBbi-RP) positive control, lane 8: pSBbi-RP:nil insert ligation. (B) Sequencing results to verify the positive transformants. Plasmids from transformants 2 and 5 were purified by Miniprep plasmid purification and sequenced using SB primers. Black bars represent consensus between the huTF gene, murine Igκ signal sequence (orange) and the sequenced plasmids.
3.2 Puromycin selection of untransfected EL4

Murine EL4 cells are susceptible to the antibiotic puromycin which causes cell death by inhibiting protein synthesis [104]. All plasmids used in this research contain the puromycin N-acetyltransferase (pac) gene from *Streptomyces alboniger*, conferring resistance to puromycin selection in all transfected cells (Appendix 3 Appendix 6). To determine the optimal concentration at which puromycin induces untransfected EL4 cell death, EL4 cells were incubated in a titration of the antibiotic from 7.8125 ng/mL to 8,000 ng/mL or R5 media only (negative control). Resazurin, a weakly fluorescent blue dye which is reduced to a highly fluorescent pink chemical by metabolically active cells, was used as an indicator of cell viability [105]. Viable cells, which reduce resazurin, strongly fluoresce at A_{585nm} whereas dead cells with no metabolic activity produce no fluorescence. Resazurin was added during the final four hours of the 72-h incubation after which cell viability was quantified by fluorometric analysis. Maximal death of EL4 cells was sufficiently induced by a puromycin concentration of 2,000 ng/mL, as indicated by the lowest level of resazurin fluorescence (Figure 4). Increasing concentrations of puromycin beyond this point up to 8,000 ng/mL did not further decrease fluorescence levels. Thus 2,000 ng/mL was the optimal cytotoxic concentration of puromycin necessary to select out all untransfected EL4 cells and was therefore utilised for transfected EL4 selection.
Figure 4: Determination of the optimal puromycin concentration to induce cell death within untransfected EL4 cells.

EL4 cells were treated with puromycin with a titrating concentration from 7.8125 ng/mL to 8000 ng/mL for 72 h (final concentration $1 \times 10^5$ cells/mL). Resazurin (10%) was added at the final four hours and fluorescence was measured using a microplate reader (excitation 540 nm / emission 585 nm). Data are expressed as the means of duplicates from one optimisation pilot experiment. Error bars represent mean ± SD.
3.3 Transfection of EL4 cells with the full-length huTF gene.

All plasmids used in this research to transfect EL4 contain an RFP reporter gene as an indicator for successful transfection (Appendix 3 Appendix 4). Transfection of EL4 cells with the pSBbi-RP:TF plasmid (Table 2.4 and Figure 3) was carried out using the Neon transfection system. To ensure healthy growth, transfected EL4 cells were cultured at an increasing concentration of puromycin, from 1 µg/mL to 2 µg/mL at days one and two respectively. Images of cells were captured by fluorescence microscopy to observe the expression of RFP, using untransfected EL4 to normalise background fluorescence levels (Figure 5). Cells emitting red fluorescence represent puromycin-resistant cells successfully transfected with pSBbi-RP:TF and expressing the RFP gene within the plasmid.
Figure 5: Microscopy images showing red fluorescent protein (RFP) expression in pSBbi-RP:TF transfected EL4 cells.

Full-length huTF gene was transfected into EL4 from the RFP-expressing plasmid pSBbi-RP:TF using the SB transposon and Neon transfection systems. Upright fluorescence microscopy with 200× magnification was used to visualise the cells using (A, C) bright-field view or (B, D) fluorescence of the reporter gene RFP. EL4: untransfected; EL4-TF: transfected. Scale bar represents 200 µm. Images are representative of two experiments.
3.4 Truncation of the full-length huTF gene to Δ1-106huTF

To delete the FVII/VIIa binding site from the huTF protein, site-directed mutagenesis of the pSBbi-RP:TF plasmid was performed by IPCR. IPCR primers (Table 2.2) were designed to bind to pSBbi-RP:TF, flanking the locus of the huTF gene encoding amino acids 1-106 (FVII/FVIIa binding sites). Primers annealed in back-to-back orientation, amplifying the pSBbi-RP backbone and amino acids 107-219 of the huTF insert. The resulting IPCR product consisted of a pSBbi-RP backbone with Δ1-106huTF insert which was re-circularised by blunt-end ligation to form the pSBbi-RP:ΔTF-I plasmid. A schematic diagram of the gene truncation strategy is summarised in Figure 6.

Initially, PCR was performed according to the manufacturer’s instructions for the Q5 High-fidelity DNA polymerase. However, this approach yielded little to no DNA amplification when observed by gel electrophoresis (data not shown). Several studies using the IPCR technique on large plasmids, albeit with lower fidelity DNA polymerases, have suggested that longer PCR extension times provide more efficient elongation of DNA fragments [106, 107]. This approach was tested in the current study by prolonging the extension time from 3.5 minutes per cycle to 6 minutes per cycle. Results from the optimised method successfully generated DNA which approximately corresponded to the size of linearised pSBbi-RP:ΔTF-I (6.97 kb; Figure 7A). Due to the small difference of the DNA fragment removed by IPCR (315 bp), it was difficult to distinguish between gel bands from the IPCR-generated and template plasmids (pSBbi-RP:ΔTF-I and pSBbi-RP:TF respectively; Figure 7A). Since one of the two AccI sites is located in the deletion site and is lost during amplification, we therefore screened for IPCR-positive DNA by digesting amplified DNA with AccI (Figure 6, Figure 7B). pSBbi-RP:ΔTF-I plasmids with a single AccI restriction site should yield a 6.97 kb linear fragment, while pSBbi-RP:TF templates with two AccI restriction sites, produce fragments 5.46 kb and 1.82 kb in length (Figure 6, Appendix 3, Appendix 4). Following AccI digestion of pSBbi-RP:ΔTF-I, a mixture of amplified and unamplified DNA products corresponding to all three possible band sizes was observed. The 6.97 kb band from the AccI digested pSBbi-RP:ΔTF-I was excised for ligation (Figure 7B).
Figure 6: Site-directed mutagenesis of the TF gene by inverse PCR (IPCR).

IPCR primers (Table 2.2) were used to delete the gene locus of TF that codes for the factor VII/VIIa (FVII/FVIIa) binding site. The IPCR primers anneal to the plasmid in reverse orientation, flanking the region to be deleted (Appendix 1). DNA amplification occurs outwards, excluding this region from the resulting PCR product. The DNA product is then re-ligated forming a plasmid with the desired gene deletion. Red sections represent AccI restriction sites.
Figure 7: IPCR truncation of the pSBbi-RP:TF plasmid to produce Δ1-106huTF.

The full-length huTF gene was truncated to Δ1-106huTF by performing IPCR on pSBbi-RP:TF which served as the template for amplification. (A) Using IPCR primers, pSBbi-RP:TF plasmid was amplified resulting in pSBbi-RP:ΔTF-I plasmid. Lanes MW: 2-log DNA ladder, lanes 1-2: DNA products generated using IPCR primers (6.96 kb), lanes 3-4: DNA products generated using SB primers positive control (996 bp), lane 5: milliQ water negative control, lane 6: vector backbone positive control (pSBbi-RP:TF; 7.28kb). (B) Screening to verify IPCR-generated products by AccI digestion. Successfully amplified DNA (pSBbi-RP:ΔTF-I) produces a single band at 6.96 kb. Unamplified DNA (pSBbi-RP:TF) produces two bands at 5.46 kb and 1.82 kb. Lanes MW: 2-log DNA ladder, lanes 1-2: digestion of IPCR-generated DNA, lanes 3-4: digestion of vector backbone (pSBbi-RP:TF) for size comparison.
3.5 Transformation of E. coli Top10 with commercially or IPCR-derived Δ1-106huTF gene

Due to initial challenges with the IPCR strategy, a synthetic gBlock of the Δ1-106huTF gene was commercially obtained. This gBlock was designed to encode the same nucleotide sequence as the Δ1-106huTF insert of the pSBbi-RP:ΔTF-I plasmid generated by IPCR. As IPCR was optimised prior to the arrival of the gBlock, both forms of the mutant Δ1-106huTF gene (gBlock-derived Δ1-106huTF and IPCR-truncated Δ1-106huTF) were processed in parallel.

The synthetic gBlock DNA encoding the Δ1-106huTF gene was designed with asymmetrical SfiI flanking sites (Appendix 2), identical to the full-length huTF gBlock (section 3.1). The Δ1-106huTF gBlock was cloned into the SB vector pSBbi-RP following the identical methods used for the full-length huTF (see section 3.1; Cloning of the full-length huTF gene). Both plasmids containing the Δ1-106huTF insert (pSBbi-RP:ΔTF and pSBbi-RP:ΔTF-I) were used to transform E. coli TOP10. Colony PCR was performed using SB primers (Figure 8). Among six pSBbi-RP:ΔTF transformant colonies, three displayed bands approximate to the expected 681 bp Δ1-106huTF gene insert (Figure 8A). Despite the slim size difference observed, DNA sequencing confirmed the truncated sequence Δ1-106huTF gene insert for two colonies (Figure 8C). As for pSBbi-RP:ΔTF-I transformant colonies, all ten colonies displayed the correct band sizes corresponding to the Δ1-106huTF gene insert (Figure 8B; 681 bp). One colony was selected for sequencing and results confirmed the Δ1-106huTF gene insert sequence within the pSBbi-RP:ΔTF-I plasmid (Figure 8C).

Sequencing results revealed one ambiguity in the forward read of the Δ1-106huTF gene of colony 1 pSBbi-RP:ΔTF plasmids (Figure 8C), identified to be a G/T nucleotide. The reverse read of the gene showed full consensus with Δ1-106huTF and identified the ambiguity was a G nucleotide, indicating that a mutation was not introduced. Colonies 3 (pSBbi-RP:ΔTF-I) and 5 (pSBbi-RP:ΔTF) showed full consensus with the Δ1-106huTF gene and therefore were chosen for further work.
Truncated huTF (Δ1-106huTF) gene was cloned into pSBbi-RP plasmid using SfiI restriction cloning or generated by IPCR, resulting in pSBbi-RP:ΔTF or pSBbi-RP:ΔTF-I plasmids respectively. Using SB primers, colony PCR was performed to screen *E. coli* colonies transformed with either plasmid. Truncated huTF band (681 bp) was detected in selected colonies (A) 1, 3, and 5, transformed with pSBbi-RP:ΔTF and (B) 1-10, transformed with pSBbi-RP:ΔTF-I plasmids. Lanes MW: 2-log DNA ladder; pSBbi-RP:nil insert ligation negative control: lanes 7, 8 (for A) and 12 (for B); vector backbone (pSBbi-RP) positive control: lanes 9 and 11 (for A and B respectively); milliQ water negative control: lanes 10 and 12 (for A and B respectively). (C) Sequencing results to verify selected positive transformants. Plasmids from transformants 1 and 5 in figure A and transformant 3 in figure B were purified by Miniprep plasmid purification and sequenced using SB primers. Black bars represent consensus between the Δ1-106huTF gene, murine Igκ signal sequence (orange) and the sequenced plasmids.
3.6 Transfection of EL4 cells with the ΔI-106huTF gene.

Transfection of EL4 cells with the pSBbi-RP:ΔTF and pSBbi-RP:ΔTF-I plasmids (Table 2.4) was carried out using the Neon transfection system. The transfection and selection were carried out following the identical methods used for the full-length huTF (see section 3.3; Transfection of EL4 cells with the full-length huTF gene). Fluorescence microscopy images of transfected cells showed that the majority of EL4-TF and EL4-ΔTF transfectants appeared to express RFP while only some EL4-ΔTF-I transfectants expressed RFP (Figure 9). This observation was later quantified by flow cytometric analysis (Figure 10B).
Figure 9: Microscopy images showing RFP expression in pSBbi-RP:TF, pSBbi-RP:ΔTF, or pSBbi-RP:ΔTF-I transfected EL4 cells.

Full-length or truncated huTF gene was transfected into EL4 from the RFP-expressing plasmids pSBbi-RP:ΔTF and pSBbi-RP:ΔTF-I using the SB transposon and Neon transfection systems. Upright fluorescence microscopy with 200× magnification was used to visualise and compare the cells using (A, C, E, G) bright-field view or (B, D, F, H) fluorescence of the reporter gene RFP. EL4: untransfected; EL4-TF: transfected with full-length TF; EL4-ΔTF: transfected with truncated huTF; EL4-ΔTF-I: transfected with truncated huTF. Scale bar represents 200 µm. Images are representative of two experiments.
3.7 Expression of TF in EL4 transfected with pSBbi-RP:TF, pSBbi-RP:ΔTF or pSBbi-RP:ΔTF-I

To confirm the ability of transfected cells to express full-length or truncated TF, we analysed the surface and intracellular expression of TF by flow cytometry (Figure 10A). Cells were stained for TF using the anti-TF antibody TF8-5G9. EL4-TF cells exhibited high levels of surface expressed TF. The corresponding RFP expression profile in EL4-TF showed equivalently high expression of fluorescence genes (Figure 10B), consistent with the images of the cells captured by fluorescence microscopy (Figure 9D).

EL4-ΔTF cells exhibited no detectable surface expressed TF. This was in contrast to the high RFP expression of EL4-ΔTF cells (Figure 9F and Figure 10B). Surprisingly, EL4-ΔTF-I cells exhibited low levels of surface expressed TF (Figure 10A), despite deficiencies in RFP gene expression that were observed by both fluorescence microscopy and flow cytometric analysis (Figure 9H and Figure 10B).

To determine whether TF expression was being limited to the intracellular environment, cells were fixed and permeabilised to facilitate the access of detection antibodies to the intracellular level of the cells. TF expression in permeabilised cells was highest in EL4-TF cells and lowest in EL4-ΔTF-I with EL4-ΔTF cells as intermediary (Figure 10A). Mirroring observations made from images in Figure 9, RFP expression of EL4-ΔTF-I cells was downregulated compared to EL4-TF and EL4-ΔTF cells. TF and RFP expression was directly proportional in these cells; TF-positive cells were RFP-positive, TF-negative cells were RFP-negative (Appendix 7).

To further confirm that truncated TF was present in EL4-ΔTF and EL4-ΔTF-I cells, we performed western blot analyses on whole cell lysates (Figure 11A). Lysates were stained for TF using the anti-TF antibody TF8-5G9. Since the extracellular domain of TF has three potential glycosylation sites, the molecular weight of TF can vary from around 35 kDa to anywhere upwards of 47 kDa [108]. The results showed low but detectable TF expression in EL4-TF cells, within the range of glycosylated TF, but not in EL4-ΔTF nor EL4-ΔTF-I cells. Cellular TF was only detectable by TF8-5G9 in the native state (non-boiled/non-reduced) whereas soluble TF-Fc was detectable in both native and denatured states. Differences in the molecular weight of cellular TF and soluble TF are due to
conjugation with Fc (26 kDa). Since EL4-TF cells were previously shown to express high levels of TF (Figure 10A), the presence of only a weak band for TF demonstrated a reduced sensitivity of the western blot assay for antigen detection using the TF8-5G9 antibody in comparison to flow cytometric analysis.
Figure 10: Analysis of TF expression level between EL4 transfectants using flow cytometry.

Flow cytometry was used for the detection of TF in untransfected EL4, EL4-TF, EL4-ΔTF and EL4-ΔTF-I using surface and intracellular staining. (A) Cells were incubated with a mouse anti-human TF antibody (TF8-5G9; αTF) and detected using the secondary antibody anti-mouse IgG-Alexa Fluor 488 (AF488). For negative controls, cells were incubated with secondary antibody only, pre-incubated αTF with TF-Fc (cold inhibition), or with TF-Fc alone. For intracellular detection, cells were treated with fix buffer prior to staining and antibodies were prepared in permeabilisation buffer. (B) RFP expression corresponding to the surface stained cells. Data are representative of three experiments.
Figure 11: Detection of full-length or truncated huTF in EL4 cell lysates by western blot.

(A) Boiled/reduced (+) or non-boiled/non-reduced (-) whole cell lysates (20 µg) were subjected to polyacrylamide gel electrophoresis (PAGE) and western blotted using mouse anti-human TF antibody (clone TF8-5G9) or anti-mouse β-actin IgG, both detected with anti-mouse IgG-AF680 (Lanes 1-8). Detectable TF band (Lane 3) is indicated by the red arrow. (B) Soluble TF-Fc (1 µg) was used as a positive control (Lanes 9 and 10). Results are representative of three experiments.
3.8 The procoagulant activity of transfected EL4 cells

We next determined the procoagulant potential of all four EL4 cell types (EL4, EL4-TF, EL4-ΔTF, and EL4-ΔTF-I) using a fibrin generation assay (FGA; Figure 12A). FGA measures plasma clot turbidity, determined by the rate of fibrin deposition, as an indicator of functional coagulant activity. EL4-TF cells were significantly more procoagulant than their untransfected cell of origin (EL4) whereas EL4-ΔTF and EL4-ΔTF-I failed to induce any detectable fibrin (Figure 12A).

To confirm that the procoagulant activity of EL4-TF cells was driven by TF, we tested cells with a titrating dose of the anti-TF antibody TF8-5G9 (Figure 12B). Fibrin deposition of cells was significantly suppressed when blocked with either concentration of the antibody (20 µg/mL or 40 µg/mL). Equivalent concentrations of a monoclonal mouse IgG control failed to inhibit the FGA.

The importance of the TF-FVII interaction for initiating the extrinsic pathway was tested by comparing the activity of procoagulant EL4-TF cells in FVII-depleted (FVII-) plasma versus standard plasma (Figure 12C). Since FVII is critical for initiating the TF-driven extrinsic pathway, fibrin generation was significantly suppressed by removal of this clotting factor from plasma.
Figure 12: Fibrin generation assays (FGA) comparing transfected EL4 cells.

Cells (50,000) were added to platelet-poor plasma (PPP) and calcium chloride was supplemented to initiate coagulation. Fibrin generation was monitored at 405 nm until the negative control (PBS) started to generate detectable fibrin. (A) FGA of EL4, EL4-TF, EL4-Δ, EL4-ΔI cells. (B) FGA of EL4-TF with inclusion of 20 μg/mL or 40 μg/mL of αTF antibody (TF8-5G9) or IgG isotype control. (C) FGA of EL4-TF using factor VII-depleted plasma (FVII-PPP) and standard PPP (Ctrl). One-way ANOVA with Bonferroni multiple comparisons test performed on the area under the curves (AUC): ns = not significant, ***P < 0.0001 ****P < 0.0001. Error bars represent mean ± SD. Samples were loaded in triplicates. Results are representative of three experiments (for A and B); experiment performed once (for C).
3.9 Development of the antigen expression system for TF8-5G9 CAR cytotoxicity assays

Since the purpose of the truncated antigen model is to be compatible with TF8-5G9 CARs, it is critical to ensure that cells expressing the antigen are capable of inducing anti-TF T cell responses. A luciferase-based cytotoxicity assay was proposed involving co-expression of the luciferase reporter gene in target tumour cells. In active, ATP-producing cells, luciferase oxidises its substrate luciferin and emits a luminescent signal [109]. Upon cell death when intracellular ATP is no longer available, the luminescent signal is lost [110]. In this manner, tumour cell lines which co-express TF antigen and luciferase can be challenged with TF8-5G9 CAR-expressing peripheral blood mononuclear cells, allowing cytotoxic activity to be detected as a decrease in bioluminescence.

For optimisation of the cytotoxicity assay, the platform was initially developed using the unaltered full-length TF antigen. This could later be used as a control to compare the cytotoxic activity of TF8-5G9 CAR T cells against truncated TF versus full-length TF. To facilitate the co-transfection of the huTF gene with the luciferase reporter gene (luc) using a single vector, huTF was cloned into pSBbi-BsmBI by Golden Gate assembly. Firstly, BsmBI overhang primers were designed to bind to huTF and create overhangs which contain BsmBI restriction sites. BsmBI is a type IIS restriction enzyme which cleaves DNA at a site separate to the recognition sequence, leaving overhangs which directly assemble to the plasmid [111]. In this way, BsmBI digestion cleaves recognition sites from the final DNA product, allowing scarless cloning of the huTF gene into pSBbi-BsmBI (pSBbi-BsmBI:TF). A schematic diagram of the Golden Gate cloning strategy is summarised in Figure 13.

gBlock DNA encoding the huTF gene was cloned into the BsmBI restriction sites of the plasmid, upstream of a P2A coding sequence derived from Porcine teschovirus-1. The P2A “self-cleaving” peptide facilitates the translation of two separate polypeptides from one mRNA transcript through a conserved sequence at the C-terminus which creates steric hindrance, causing ribosome skipping or fall-off [112, 113]. P2A was thus used to mediate the simultaneous expression and cleavage of the upstream huTF and downstream pac resistance genes. E. coli TOP10 were transformed with ligated DNA (pSBbi-BsmBI:TF) and seven colonies were subjected to a colony PCR using GOI2 primers.
(Table 2.2) flanking BsmBI insertion sites (Figure 14A). All seven colonies displayed correct band sizes corresponding to the amplified region containing the huTF gene insert (943 bp). Three huTF-positive colonies were subjected to amplification and purification for verification by DNA sequencing.

Sequencing results revealed an ambiguity in the huTF gene of colony 1 pSBbi-BsmBI:TF plasmids (Figure 14B), identified as a pyrimidine (C/T) by both the forward and reverse reads of the sequence. When aligned to the reference sequence, the original base was shown into be a T nucleotide. In addition to this, non-consensus in the murine Igκ signal sequence was detected, likely as result of errors incorporated when nearing the maximum read length. As we could not verify that the huTF sequence of the plasmid DNA matched the reference huTF sequence, plasmids from this colony were not chosen for further work. Purified plasmids from colony 3 showed full consensus with the huTF gene, indicating no mutations were introduced. In colony 5-purified plasmids, a mutation was identified in the huTF sequence, indicating a transition from the purine nucleotide A to G. Due to redundancy in the genetic code, this mutation did not cause a change in the amino acid sequence of the protein produced (GCA to GCG). However, since plasmids from colony 3 were identified to be mutation-free, we continued the cloning strategy using these plasmids as the backbone for insertion of a second gene, luc, and excluded colony 5-purified plasmids from further work.

To obtain the luc insert, plasmid DNA encoding the luc gene was digested with SfiI and subjected to gel electrophoresis. Bands corresponding to the size of the luc insert (1.8 kb) were excised and purified from the gel. pSBbi-BsmBI:TF plasmids purified from colony 3 (Figure 14) were SfiI digested and subjected to gel electrophoresis. Bands corresponding to the size of backbone DNA (6.6 kb) were excised for ligation. The luc insert was cloned into the asymmetric SfiI sites of the pSBbi-BsmBI:TF resulting in the pSBbi-BsmBI:TF+luc plasmid which encodes two transgenes: the huTF antigen and luciferase reporter. E. coli TOP10 were transformed with ligated DNA (pSBbi-BsmBI:TF+luc) and a colony PCR was performed using SB primers to amplify the luc insert (Figure 15A). All four selected colonies displayed band sizes corresponding to the luc gene insert (1.8 bp). Concurrently, DNA from the selected colonies was amplified with GOI2 primers to verify the presence of huTF within the same plasmids. All four colonies displayed band sizes corresponding to the huTF gene insert, confirming that luc and huTF genes were co-expressed within the same plasmid. All luc-positive colonies...
were plasmid purified for verification by DNA sequencing. Sequencing results revealed no mutations in the luc gene of any plasmid (Figure 15B).

Due to time restrictions, transfection of the pSBbi-BsmBI:TF+luc construct in to tumour cell lines did not proceed and cytotoxicity assays were not able to be completed.
Figure 13: Overhang PCR with BsmBI restriction sites for Golden Gate assembly of huTF into a SB transposon vector.

Primers to amplify the huTF gene were designed with overhang regions containing BsmBI restriction sites (BsmBI overhang primers; Table 2.2). BsmBI cleaves at a separate site to the recognition sequence and removes the restriction site from digested DNA products. The overhangs produced by BsmBI digestion are designed to be complementary between the insert and plasmid, allowing seamless assembly of the huTF gene fragment into the SB vector.
Full-length huTF gene was cloned into pSBbi-BsmBI plasmid using restriction cloning with BsmBI resulting in pSBbi-BsmBI:TF plasmid. (A) Using gene of interest 2 (GOI2) primers, colony PCR was performed to screen transformed E. coli colonies (1-7) for the presence of the full-length huTF within BsmBI sites. Lanes MW: 2-log DNA ladder, lanes 1-7: PCR-positive colonies for huTF (943 bp), lane 8: pSBbi-BsmBI: nil insert ligation negative control, lane 9: vector backbone (pSBbi-BsmBI) negative control, lane 10: milliQ water negative control. (B) Sequencing results to verify the positive transformants. Plasmids from transformants 1, 3 and 5 were purified by Miniprep plasmid purification and sequenced using GOI2 primers. Black bars represent consensus between the huTF gene, murine Igκ signal sequence (orange) and the sequenced plasmids.

Figure 14: Colony PCR and sequencing results for the pSBbi-BsmBI:TF plasmid.
Luciferase (luc) gene was cloned into pSBbi-BsmBI:TF using restriction cloning with SfiI resulting in pSBbi-BsmBI:TF+luc plasmid. (A) Using SB and GOI2 primers, colony PCR was performed to screen transformed *E. coli* colonies (1-4) for the presence of *luc* gene within SfiI sites and huTF gene within BsmBI sites, respectively. Lane MW: 2-log DNA ladder; lanes 1-4: *luc*-positive colonies (1.8 kb); lanes 1-4: huTF-positive colonies (943 bp); lane 5: pSBbi-BsmBI:TF:nil insert ligation negative control; lane 6: vector backbone (pSBbi-BsmBI) negative control; lane 7: milliQ water negative control. (B) Sequencing results to verify the positive transformants. Plasmids from colonies 1-4 were purified by Miniprep plasmid purification and sequenced using SB primers. Black bars represent consensus between the *luc* gene and the sequenced plasmids.
Chapter Four:

Discussion
4.0 Discussion

4.1 Discussion

Rapid innovations in the field of immunotherapy have brought forth a myriad of novel treatments to the clinic [17, 58, 114]. Thus far, two of the most successful strategies, immune checkpoint inhibitors and CAR T cell therapy, have both focused on engaging the cytotoxic actions of T lymphocytes [24]. In haematological cancers, CARs have demonstrated durable responses in a broad group of patients; however, widespread application of CAR T cell therapy continues to be marred by their limited success against solid epithelial cancers, as well as concerns for patient safety [41, 49, 53, 59]. Appropriate choice of a specific, targetable antigen is one of the defining factors for limiting on-tumour, off-target effects and delivering successful/safe CAR therapy [55]. However, in epithelial cancers, most tumour antigens expressed are derived from self-proteins, usually expressed at other sites around the body on healthy tissues [50]. Clinical trials for CARs against solid tumours have been met with severe cases of adverse side effects alongside instances of patient fatality [59, 115]. In this regard, it would be useful to explore other potential targets or features which may more easily distinguish cancerous cells from healthy tissue.

In this research, we presented TF as a novel target antigen for solid cancer immunotherapy and developed the foundations for preclinical testing. We aimed to address the hypercoagulant nature of TF in vivo by truncating the antigen to remove the catalytic FVII/FVIIa binding sites which induce coagulative pathways. This was carried out by deleting the gene locus of FVII/FVIIa binding sites encoded within the huTF gene using IPCR.

Site-directed mutagenesis by IPCR was initially favoured for its relatively rapid execution and taken as an opportunity to explore an alternative mutagenesis technique for SB plasmids (previously untested in the McLellan laboratory; ref. [106]). However, the failure of this method to produce reliable amplification suggested an incompatibility with the plasmid (Figure 6). Previous work in the McLellan laboratory has shown that SB plasmids were poorly compatible with another PCR mutagenesis technique, quick-change PCR. The SB plasmids used in this research contained AT-rich regions, particularly at the ITR sites which approached nearly 70% AT-content. AT-rich
sequences are known to be difficult to amplify by PCR owing to the thermodynamic instability of this base pairing [116, 117]. Traditionally, this can be compensated for by lowering primer annealing and extension temperatures; however, this comes at the cost of compromising the quality of amplified DNA [117-119]. We trialled multiple conditions to optimise the protocol and found that prolonged extension time was crucial for successful amplification (data not shown). Consequently, this resulted in an increased chance of error-prone replication which could alter the desired PCR product.

Throughout this research, we observed defective expression of the RFP reporter in EL4-ΔTF-I mutants, both by fluorescence microscopy and flow cytometric analysis (Figure 9 and Figure 10). Since the transfectants had been subjected to puromycin selection, all imaged cells were known to be expressing the pac gene and hence, were successfully transfected with pSBbi-RP:ΔTF-I expression plasmids containing the Δ1-106huTF gene. Flow cytometric analysis revealed a wide range of RFP expression within EL4-ΔTF-I cells, correlating with the range of TF expressed by intracellularly stained populations (Appendix 7). This suggested proportional expression of the transgenes encoded in the plasmid; i.e RFP-high populations were TF-positive, RFP-low populations were TF-negative. These results indicated that mutations were likely to have been introduced into the RFP gene of the plasmid during amplification by IPCR, potentially causing a frameshift. Another explanation for poor RFP expression may have been low transfection efficiency which results in downregulated transgene expression. Sequencing of the RFP gene could have been performed to confirm the presence of mutations; however, error-prone replication would most likely have affected regions of the plasmid other than the reporter gene. Therefore, a more favourable approach would have been to clone the sequenced Δ1-106huTF gene into a new SB backbone (pSBbi-RP) using SfiI restriction sites.

Alternatively, the truncated huTF gene could have been generated by a traditional PCR approach. Primers could be designed to amplify an insert region encoding amino acids 107-219 of the huTF gene in pSBbi-RP:TF. Amplification of a shorter DNA fragment would reduce the likelihood of mutations being introduced; however, this would require the addition of restriction sites flanking the insert to facilitate cloning back in the plasmid. Scarless cloning would be desirable to prevent the introduction of excess base pairs between the signal sequence and the protein coding region of the gene which could affect
the ability of the protein to surface express. For this, a Golden Gate cloning approach similar to the one described in Figure 13 could be taken.

To facilitate the transduction of gene cassettes into the murine genome, the SB-transposase system was utilised. Traditional viral delivery methods, such as retroviral and lentiviral systems, promote high transduction efficiency and sustained gene expression; however, these have several major drawbacks [120]. Viral vectors show high preference for insertion into gene encoding regions and thus, can disrupt recipient host cell functions and elicit unwanted adverse effects [121]. Additionally, viral vectors are restricted by the size of DNA they may carry, have associated immunogenicity and carcinogenicity and are costly to purify [122]. By comparison, SB-mediated non-viral delivery methods are cheaper, relatively safe and have the capacity to transfer larger genes [123]. Initially, non-viral modes such as SB plasmid-based delivery had reduced transduction efficiency compared to viral delivery systems [120]. This has since been overcome through the engineering of SB transposases into more hyperactive variants such as the SB100X which was used in this research [120]. Risk of insertion into transcriptionally active sites remains possible; however, SB transposase systems have been shown to integrate more randomly than all vectors currently in use for gene therapy and therefore convey better safety from adverse events [120, 124, 125]. Following integration into recipient genomes, the potential for remobilisation of transposons is low [120]. Additionally, reintegration remains relatively random and is no more likely to disrupt active genes than the original insertion [120]. SB was chosen for this study to facilitate the expression of multiple genes; ampicillin resistance, required for selection of positive-transformants, pac resistance, required for selection of positive-transfectants and crucially, the RFP reporter gene, which in this research was the crucial feature illustrating the problems encountered throughout execution.

In consultation with Dr Yoshio Nakatani (Structural Biologist; Department of Microbiology and Immunology, University of Otago), threonine-106 in the interdomain strand was proposed to be the optimal site for truncation. The disordered linker strand separates the protein modules comprising the extracellular domain of TF and by nature, is not involved in tertiary structures of either module. The rationale for this truncation site was four-fold:

1. To conserve the conformation of the C-terminal module such that translocation of truncated protein to the cell surface would not be disrupted
2. To remove the majority of interactions involved in FVII-FVIIa binding, including two out of three of the most energetically important residues; lysine-20 and isoleucine-22
3. To retain the binding epitope for activation of a TF8-5G9 CAR
4. To mitigate the chance for spontaneous tumour rejection in mice by removing the maximal amount of immunogenic material

Flow cytometric analysis detected surface expression of truncated-TF by EL4-ΔTF-I cells at comparatively low-levels to the full-length expressing cell line EL4-TF, whereas TF expression was completely undetectable in the gBlock mutant EL4-ΔTF cells (Figure 10A). It is possible that low/nil-detection resulted from lack of surface expressed protein. When cells were intracellularly stained, higher levels of the antigen could be detected, suggesting that truncated protein was being produced in the endoplasmic reticulum (ER) but protein folding had been compromised by truncation. Alternatively, low/nil-detection may have been observed if truncated antigen was surface expressed, but the conformation of the antigen was no longer compatible with the TF-detection antibody, TF8-5G9. Equally in both cases, lack of targetable antigen does not fulfil the purposes of this antigen model.

Although there was a minor shift in TF fluorescence once cells were fixed and permeabilised, previous work in the McLellan laboratory has never shown EL4 cells to be procoagulant or express TF. This shift was therefore most likely due to non-specific binding of the TF8-5G9 antibody to the intracellular contents of EL4 cells. An IgG1 isotype control was included in these experiments to determine the influence of non-specific binding; however, this showed more reactivity with intracellular components than the TF8-5G9 detection antibody once cells were fixed and permeabilised (data not shown). We concluded that the isotype control used did not accurately reflect the background non-specific staining levels of TF8-5G9 and therefore, was not an appropriate control. Single staining with anti-mouse-AF488 and cold inhibition of TF8-5G9 were determined to be more relevant negative controls for these experiments.

Reasons for the differences in the level of surface expressed antigen between EL4-ΔTF and EL4-ΔTF-I are not entirely clear, despite both cell lines expressing identical Δ1-106huTF sequences. This finding was particularly surprising as the IPCR-derived
mutants EL4-ΔTF-I cells simultaneously demonstrated superior surface expression of antigen while expressing defects in reporter gene function which may reflect abnormalities in the plasmid (Figure 9Figure 10). We theorise that lack of surface expression of truncated TF may have been due to a bottleneck effect, caused by the overexpression of loose or slow folding protein. Unaffected pSBbi-RP:ΔTF plasmids may have allowed for overproduction of the truncated TF. Due to folding difficulties, the efficiency of processing the truncated protein may have been compromised, overloading the ER with misfolded protein [126]. In this manner, enzymes and components recruited in processing and transport of proteins from the ER to the surface membrane may have been fully saturated, preventing the translocation of any mature forms of truncated protein that may have been present. Paradoxically, mutations to pSBbi-RP:ΔTF-I plasmids affecting transgene expression levels, could have allowed for a lower production of truncated protein. Without the oversaturation of proteins at the ER, complete folding and processing into mature protein may have been allowed to occur, thereby permitting surface expression of truncated antigen.

In conjunction with flow cytometric analysis, western blotting was carried out using the TF8-5G9 antibody to detect TF production in cell lines transfected with the truncated Δ1-106huTF gene (Figure 11). Despite strong expression of TF observed by flow cytometry, only a weak band was detected in the strongly TF-positive cell line EL4-TF. Given this discrepancy, we determined that the poorly detectable TF band represented a reduced sensitivity of the western blot assay for detection of TF. Though TF-Fc was strongly detectable with TF8-5G9, variations in protein processing and post-translational modifications (e.g. glycosylation of the extracellular domain) in the cell-associated TF may have affected the affinity of the antibody for TF antigen. Other studies which have used TF8-5G9 to detect TF by western blot have coupled the antibody to beads or substrates which increase the affinity of binding [127, 128]. Additionally, cellular TF was detectable only in the native state. This showed that antibody binding was dependent on protein conformation, which was likely to be altered in truncated TF. Absence of bands in EL4-ΔTF and EL4-ΔTF-I lysates were therefore attributed to the presence of TF at levels below the detectable limit, as opposed to a lack of TF production by EL4-ΔTF and EL4-ΔTF-I cells. The TF8-5G9 antibody had not previously been tested in the McLellan laboratory for western blot assays and from these results, we concluded that direct use of this antibody for western blot analysis was not compatible. Flow cytometry
was determined to be a more accurate indicator of TF expression and results from this assay were deemed inconclusive.

The procoagulant activity of each cell type was assessed by a functional FGA (Figure 12A). Neither cell line transfected with truncated TF expressed coagulative ability compared to their cell of origin EL4. Combined with observations from flow cytometric analysis, this was perhaps the most significant finding from this research. Since EL4-\(\Delta\)TF cells had not been shown to surface express TF, these cells were not expected to have coagulant activity. Strikingly, since EL4-\(\Delta\)TF-I cells had been identified to express low levels of TF and did not induce coagulation, these results showed that the coagulative function of any surface expressed antigen had been effectively abolished. Despite low surface expression levels, previous studies have demonstrated that low molar concentrations of TF (3 - 10 molecules per \(\mu\text{m}^2\)) are sufficient to induce fibrin deposition [129, 130]. Previous work in our laboratory has shown that apoptotic vesicles expressing TF at levels undetectable by flow cytometry, demonstrate potent coagulative activity when measured using the same functional assay [102].

Blocking experiments were performed with only the cell type which exhibited procoagulant function, EL4-TF (Figure 12B). Pre-incubation with TF8-5G9 significantly delayed the onset of fibrin generation but did not completely inhibit the FGA. Given the potency of TF, the concentrations of antibody chosen and incubation times may not have been sufficient to saturate the binding sites which inhibit the interaction with subsequent coagulation factors. Additionally cells were blocked with another anti-TF antibody; however, TF8-5G9 proved to be a superior inhibitor of procoagulant activity (data not shown). This observation was consistent with previous works and validated the choice of antibody to be used as structural basis of a novel CAR design. Similarly, removal of the FVII ligand from the FGA neutralised the procoagulant function of TF by restricting formation of the catalytic complex (Figure 12C). Whilst fibrin generation was not completely abrogated, the supplier does not guarantee 100% removal from plasma and therefore, up to 1% of residual FVII may have remained present to activate extrinsic coagulation pathways.
4.2 Future work

Preliminary work for the development of a truncated TF antigen was carried out on murine TF-negative EL4. This was necessary to optimise the expression of the truncated antigen and characterise the coagulant activity of the cells. However, EL4 cells are derived from murine T cell lymphoma and therefore, do not constitute a classical solid tumour model. Once the truncated TF antigen has been optimised, future work should continue in a solid cancer cell line such as B16 melanoma. B16 expresses murine TF and is a well-established, readily transplantable tumour model [131]. The cumulative effects of expressing truncated human TF and the murine TF should be assessed for safety.

The point of truncation chosen appears to address our initial aim to remove the coagulative function of the antigen. However, results from this research did not demonstrate that truncated TF could be reliably surface expressed, warranting further optimisation to stably express the mutant protein. In this regard, generating a protein crystal structure may be an effective way of analysing the folding or misfolding of the truncated protein and characterise binding capacity with FVII/FVIIa and TF8-5G9. It may be desirable to explore other truncation sites which impart less effect of protein conformation. Alternative approaches to protein truncation which may neutralise the procoagulant effects of human TF in murine models would be to generate knock-in mouse models or use immunodeficient mice. Replacing the expression of murine TF with human TF is advantageous in providing a way to examine on-target, off-tumour effects. Immunosuppressed mice such as NSG model would allow transplantation of human tumour cells, displaying physiological levels of antigen which more accurately reflect the disease state.

Due to time constraints we were not able to optimise the truncated antigen nor complete the necessary framework for the development of cytotoxicity assays. The BsmBI cloning strategy performed in this research provides a scaffold for future in vitro cytotoxicity assays that will enable optimisation of TF8-5G9 CARs. The finishing steps in developing this assay include transfection of the pSBbi-BsmBI:TF+luc plasmid into EL4 and optimisation of the assay, firstly with the full-length antigen. The cloning procedures could then be repeated with optimised expression plasmids encoding Δ1-106huTF genes.
Concluding remarks

In this research, a truncated form of TF was generated for future in vivo testing of a TF8-5G9 anti-TF CAR. Two forms of the truncated huTF gene were compared; an IPCR-derived mutant and a commercially manufactured gBlock mutant. Cells transfected with IPCR-derived TF (EL4-ΔTF-I) had defective RFP expression but strikingly, displayed low levels of surface expressed TF that was functionally inactive. By comparison, cells transfected with gBlock-derived TF (EL4-ΔTF) expressed no surface detectable antigen despite having high RFP expression, suggestive of high transfection efficiency. These preliminary findings warrant further work to optimise surface expression of the truncated antigen. The efficiency of TF8-5G9 CARs can then be tested against the full-length or truncated antigen, co-expressed with luciferase using BsmBI Golden Gate assembly.
References

40. Rizvi, N.A., et al., Activity and safety of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for patients with advanced, refractory squamous non-small-


Appendix

Appendix 1: huTF sequence of pSBbi-RP:TF
Appendix 2: Δ1-106huTF sequence of pSBbi-RP:ΔTF and pSBbi-RP:ΔTF-I
Appendix 3: Plasmid map of pSBbi-RP:TF

Appendix 4: Plasmid map of pSBbi-RP:ΔTF and pSBbi-RP:ΔTF-I
Appendix 5: Plasmid map of pSBbi-BsmBI:TF

Appendix 6: Plasmid map of pSBbi-BsmBI:TF+luc
Appendix 7: Dot plot of EL4-ΔTF-I cells transfected with pSBbi-RP:ΔTF-I

Cells show direct correlation between RFP and TF expression (see Figure 10).