

# **Conjugate Vaccines Targeting Tumour Vasculature: CLEC14A and ROBO4 as Novel Immunotherapeutic Antigens**

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## **Abstract**

Tumour angiogenesis plays a fundamental role in the progression of solid tumours and their metastasis through the leaky vasculature, supplying nutrients and oxygen via these vessels. Targeting tumour endothelial markers (TEMs) such as CLEC14A and ROBO4 presents a promising immunotherapeutic strategy to disrupt angiogenesis and inhibit tumour growth. TEMs are distinct proteins predominantly or exclusively expressed on the endothelial cells that form tumour blood vessels, often showing limited or no expression in healthy vasculature, making them attractive targets. This thesis investigates the development of active vaccination approaches aimed at stimulating immune responses against CLEC14A and ROBO4, aiming to impair tumour vasculature and improve cancer treatment outcomes.

To achieve this, protein-based conjugate vaccine platforms were designed and optimised. This approach was chosen to address the inherent challenge of targeting self-antigens like CLEC14A and ROBO4, which typically have low immunogenicity and struggle to elicit robust immune responses due to insufficient T-cell support. Conjugate vaccines overcome this limitation by covalently linking these weak antigens to highly immunogenic carrier proteins, thereby recruiting essential T cell help and leading to a more robust and long-lasting immune response. Tetanus toxin fragment C (TTc) was selected as the carrier protein due to its well-established ability to elicit robust CD4+ T-cell responses and its safety profile in humans. Furthermore, widespread pre-existing T cell memory to tetanus toxoid (TT) in the general population ensures efficient and rapid recruitment of T cell help in vaccinated patients. Recombinant proteins were successfully expressed, purified, and characterised before being used in vivo immunisation studies.

The results demonstrate that vaccination against CLEC14A and ROBO4 induces a strong humoral immune response. Specifically, ROBO4-TTc-immunised mice exhibited significantly increased ROBO4-specific IgG1 antibody titres. Additionally, the ROBO4-TTc vaccine induces cellular immune responses.

A mouse model of triple-negative breast cancer (TNBC) was employed to evaluate the efficacy of the ROBO4-TTc vaccine in eliciting tumour-specific immune responses. TNBC is an aggressive and heterogeneous subtype of breast cancer that poses a significant clinical challenge due to the absence of the oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), and its limited immune response to immune checkpoint inhibitor treatment (ICT). This model closely mimics human TNBC, making it valuable for studying tumour biology and evaluating novel treatments. The efficacy of the ROBO4-TTc vaccine was evaluated by analysing tumour burden. This was complemented by a comprehensive assessment of immune activation, including antibody production, T and B cell responses, and immune cell infiltration into the tumour, using flow cytometry, enzyme-linked immunosorbent assay (ELISA), and histological techniques. Additionally, the mechanistic basis of ROBO4-targeted vaccination was investigated to determine its effects on tumour vasculature, endothelial cell function, and overall tumour microenvironment.

The ROBO4-TTc vaccine significantly inhibited tumour growth in the TNBC model (4T1). ROBO4-TTc-immunised mice exhibited reduced tumour vascularisation and increased immune cell infiltration, contributing to delayed tumour progression. This is likely due to vascular normalization, where the irregular, leaky tumour blood vessels become more structured and functional, improving immune cell access to the tumour

microenvironment and reducing hypoxia-driven immunosuppression. These findings highlight the potential of active vaccination strategies targeting tumour endothelium as a well-characterised approach to cancer therapy. By leveraging immune-mediated destruction of tumour vasculature, this study provides a foundation for further exploration of endothelial-targeted immunotherapies in treating solid malignancies.

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## List of Abbreviations

|                         |                                          |
|-------------------------|------------------------------------------|
| °C                      | Degree Celsius                           |
| 4T1                     | Murine mammary carcinoma cell line       |
| Alum                    | Aluminium potassium sulphate             |
| ANOVA                   | Analysis of variance                     |
| APC                     | Antigen-presenting cell                  |
| ATTC                    | American Type Culture Collection         |
| BCA                     | Bicinchoninic acid                       |
| BCR                     | B Cell Receptor                          |
| bp                      | Base pairs                               |
| Breg                    | Regulatory B cells                       |
| BSA                     | Bovine Serum Albumin                     |
| CAR-T                   | Chimeric antigen receptor T-cell therapy |
| CD                      | Cluster of Differentiation               |
| cDNA                    | Complementary DNA                        |
| CGG                     | Chicken Gamma Globulin                   |
| CLEC14a                 | C-Type Lectin Domain Family 14 Member A  |
| CMV                     | Cytomegalovirus                          |
| CpG                     | Cytosine triphosphate guanine            |
| Ct                      | Cycle threshold                          |
| CXCR                    | C-X-C Motif Chemokine Receptor           |
| DAPI                    | 4',6-diamidino-2-phenylindole            |
| DCs                     | Dendritic cells                          |
| Delta Ct ( $\Delta$ Ct) | Cycle threshold delta                    |
| DMEM                    | Dulbecco's Modified Eagle Medium         |
| DNA                     | Deoxyribonucleic acid                    |
| DNase I                 | Deoxyribonuclease I                      |
| DZ                      | Dark zone                                |
| E0771                   | Murine mammary cancer cell line          |

|                  |                                                        |
|------------------|--------------------------------------------------------|
| ECM              | Extracellular Matrix                                   |
| EDTA             | Ethylenediaminetetraacetic acid disodium salt solution |
| ELISA            | Enzyme-Linked Immunosorbent Assay                      |
| FBS              | Foetal Bovine Serum                                    |
| FBS-HI           | Foetal Bovine Serum Heat-Inactivated                   |
| FDA              | Food and Drug Administration                           |
| FDCs             | Follicular dendritic cells                             |
| FITC             | Fluorescein Isothiocyanate                             |
| g                | Grams                                                  |
| G418             | Geneticin aminoglycoside antibiotic                    |
| GC               | Germinal centre                                        |
| GFP              | Green Fluorescent Protein                              |
| h                | Hour                                                   |
| H&E              | Haematoxylin and Eosin                                 |
| H <sub>2</sub> O | Water                                                  |
| HEK293           | Human embryonic kidney cells                           |
| HLA              | Human Leukocyte Antigen                                |
| i.p.             | Intraperitoneal                                        |
| ICOSL            | Inducible costimulatory ligand                         |
| Ifng             | Interferon Gamma                                       |
| Ig               | Immunoglobulin                                         |
| IgV              | Immunoglobulin variable genes                          |
| IL               | Interleukin                                            |
| kb               | Kilobases                                              |
| kDa              | Kilodaltons                                            |
| Klf8             | Kruppel-like factor 8                                  |
| L                | Litres                                                 |
| LB               | Luria Broth                                            |
| LLC1             | Murine Lewis lung carcinoma cell line                  |
| LPS              | Lipopolysaccharide                                     |

|                 |                                             |
|-----------------|---------------------------------------------|
| LZ              | Light zone                                  |
| M               | Molar                                       |
| MECA32          | Pan-endothelial cell antigen-antibody clone |
| mg              | Milligram                                   |
| mGFP            | Membrane green fluorescent protein          |
| MHC             | Major Histocompatibility Complex            |
| min             | Minute(s)                                   |
| ml              | Millilitres                                 |
| mM              | Millimolar                                  |
| mm <sup>3</sup> | Cubic millimetre                            |
| MOPS            | 3-(N-morpholino) propane sulfonic acid      |
| mRNA            | Messenger ribonucleic acid                  |
| MW              | Molecular weight                            |
| MZ              | Marginal zone                               |
| NaCl            | Sodium chloride                             |
| ng              | Nanogram                                    |
| NK              | Natural killer                              |
| nm              | Nanometre                                   |
| nM              | Nanomolar                                   |
| NMS             | Normal mouse serum                          |
| ns              | Non-significant                             |
| nt              | Nucleotides                                 |
| PBS             | Phosphate-Buffered Saline                   |
| PC              | Plasma cells                                |
| PCR             | Polymerase Chain Reaction                   |
| PD-1            | Programmed Cell Death Protein 1             |
| PD-L1           | Programmed Death-Ligand 1                   |
| PE              | Phycoerythrin                               |
| Pg              | Picogram                                    |
| PVDF            | Polyvinylidene Fluoride                     |

|          |                                                            |
|----------|------------------------------------------------------------|
| RNA      | Ribonucleic Acid                                           |
| ROBO4    | Roundabout Guidance Receptor 4                             |
| rpm      | Revolutions Per Minute                                     |
| RQ       | Relative expression                                        |
| RT       | Reverse Transcription                                      |
| s.c.     | Subcutaneous                                               |
| SD       | Standard deviation                                         |
| SDS-PAGE | Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis |
| SHM      | Somatic hypermutation                                      |
| SOC      | Super Optimal Medium with Catabolic Repressor              |
| TBE      | Tris borate EDTA                                           |
| TBS      | Tris-buffered saline                                       |
| TCA      | Trichloroacetic Acid                                       |
| TCR      | T Cell Receptor                                            |
| TEM      | Tumour endothelial marker                                  |
| Tfh      | Follicle T helper cells                                    |
| Tgfb1    | Transforming growth factor-beta                            |
| TIL      | Tumour-Infiltrating Lymphocyte                             |
| TLR      | Toll-like receptor                                         |
| TLS      | Tertiary lymphoid structures                               |
| TNBC     | Triple-Negative Breast Cancer                              |
| Treg     | Regulatory T Cell                                          |
| TT       | Tetanus toxoid                                             |
| TTc      | Fragment C of tetanus toxin                                |
| VEGF     | Vascular Endothelial Growth Factor                         |
| β-ME     | 2-Mercaptoethanol                                          |
| μg       | Micrograms                                                 |
| μL       | Microliters                                                |
| μM       | Micromolar                                                 |

## **Chapter 1: Introduction**

### **1.1 Immune System**

#### **1.1.1 Overview of the Immune System**

The immune system is a complex network of humoral factors, cells, tissues, and organs cooperating to defend the body against infections and diseases. It is generally divided into two main categories: innate and adaptive immunity.

Innate immunity serves as the body's first line of defence, responding to pathogens in a generalised manner (Marshall et al., 2018). This immune system component is active from birth, providing immediate yet non-specific protection against infection. Key elements of innate immunity include physical barriers such as the skin and mucous membranes, immune cells like monocytes/macrophages, neutrophils, natural killer (NK) cells, dendritic cells, endothelial cells, plasma protein and various cytokines that trigger inflammatory responses.

Adaptive immunity, or acquired immunity, involves a more targeted response to specific pathogens (Chi et al., 2024). Adaptive immunity develops in response to infection and vaccination. The primary components of adaptive immunity are lymphocytes, specifically T and B cells. These cells can recognise specific antigens via their unique receptors. While these antigens are often derived from pathogens, they can also include modified self-antigens, such as tumour-associated antigens, or even unmodified self-antigens in the context of autoimmunity. Additionally, adaptive immunity generates immunological memory, resulting in an enhanced response to repeated exposure to the same pathogen. This results in long-term effector functions such as increased T cell help, cytotoxicity, or specific antibodies.

In the context of cancer, the immune system plays a dual role in suppressing and promoting tumour growth and progression (Zamarron & Chen, 2011). This phenomenon is known as cancer immunoediting and encompasses three phases: elimination, equilibrium, and escape (Mittal et al., 2014). During the elimination phase, innate and adaptive immune responses collaborate to detect and destroy cancerous cells before they proliferate (Mittal et al., 2014). If specific tumour cells survive, they may enter the equilibrium phase, during which the immune system controls and inhibits their growth without completely eradicating them. Eventually, some tumour cells may adapt and become resistant to immune attacks, progressing to the escape phase, where they grow unchecked and evolve into clinically detectable tumours.

Beyond direct elimination, the interplay between immune cells and the tumour microenvironment is highly complex. While cytotoxic T cells (CD8<sup>+</sup> T cells) and Natural Killer (NK) cells are key players in anti-tumour immunity by directly killing cancer cells (Raskov et al., 2021; Wang et al., 2015), other immune subsets can unfortunately contribute to tumour progression. For instance, regulatory T cells (Tregs) and certain myeloid-derived suppressor cells (MDSCs) can actively suppress anti-tumour immune responses, allowing cancer cells to evade destruction (Goswami et al., 2022; Marvel & Gabrilovich, 2015). Similarly, tumour-associated macrophages (TAMs), depending on their polarization (M1 vs. M2), can either be tumoricidal or promote tumour growth, angiogenesis, and metastasis (Pan et al., 2020; Zhang et al., 2024). Even B cells, while primarily recognized for antibody production and antigen presentation (Hoffman et al., 2016), can also have dual roles, with some subsets like regulatory B cells (Bregs) contributing to immunosuppression (Fridman et al., 2023). This intricate balance of pro-

and anti-tumour activities within the immune system underscores the challenges and opportunities in developing effective immunotherapies.

### **1.1.2 B Cells**

#### **1.1.2.1 Overview of B Cells**

B cells are essential elements of the adaptive immune system (Chi et al., 2024). One main task is producing antibodies vital for protecting against pathogens. Antibodies are specialised adaptations of the B cell receptor (BCR), consisting of two heavy and light chains (Spiegelberg, 1989). Diversity in the immune system is generated through the recombination of gene segments known as V (variable), D (diversity), and J (joining) segments in B and T cell receptors (Christie et al., 2022; Schatz & Ji, 2011). This process, called V(D)J recombination, occurs during lymphocyte development and is mediated by the RAG1 and RAG2 enzymes, which introduce double-strand breaks at specific recombination signal sequences (Schatz & Ji, 2011). The segments are then randomly joined, with additional diversity introduced by nucleotide insertions and deletions at the junctions by terminal deoxynucleotidyl transferase (TdT) (Christie et al., 2022). In antibodies, the variable regions of heavy and light chains, shaped by this recombination, establish antigen specificity. In contrast, the constant regions of the heavy chains determine the antibody class and its immune functions (Zhang, Zhang, et al., 2022). This combination of diversity at junctions enables the immune system to identify a wide range of antigens, facilitating robust immune responses to pathogens.

Antibodies are classified into different isotypes based on the structure of their heavy chain's constant region, impacting their roles and interactions within the immune

system (Hoffman et al., 2016). For example, IgM is the first antibody to respond to infection, forming complexes that activate the complement system and initiate an inflammatory response. IgG, the predominant antibody in the blood, is essential for neutralisation and opsonisation, which boosts pathogen phagocytosis and complement system activation, which is crucial for immune defence.

Among IgG subclasses, IgG1 stands out due to its major isotype and significant role in the immune response. It is induced by Th2-type cytokines such as IL-4 and mediates various immune functions, including antibody-dependent cellular cytotoxicity (ADCC), opsonisation, neutralisation, and complement activation (Vidarsson et al., 2014). IgG1 is primarily involved in responses to protein antigens and is crucial for combating extracellular pathogens and toxins. Additionally, the adaptability of IgG1 and other antibodies in switching classes through class switch recombination enables B cells to tailor their responses to specific pathogens or environmental signals, enhancing the effectiveness of the immune system (Vidarsson et al., 2014).

Antibodies can prevent cells from being infected by pathogens through neutralisation, which blocks the pathogens' ability to bind to host cells (Lu et al., 2018). This function is essential for preventing viruses and bacteria from causing infections. Furthermore, antibodies assist in eliminating pathogens through mechanisms such as activating the classical complement pathway, leading to the lysis or phagocytosis of targeted organisms (Goldberg & Ackerman, 2020). Antibody-dependent cellular cytotoxicity (ADCC) is a crucial immune response in which antibodies bind to infected or cancerous cells, marking them for destruction. (Wang et al., 2015). Natural killer (NK) cells identify these tagged cells via the antibody's constant region of the Fc region and release cytotoxic agents to eliminate the targets.

B cells can be categorised into B-1 and B-2 cells (Hardy & Hayakawa, 2001). B-1 cells represent an innate-like population capable of producing natural antibody IgM early during infection without prior antigen sensitisation (Baumgarth et al., 2000). In contrast, B-2 cells, or conventional B cells, most B cells, develop from hematopoietic stem cells in the bone marrow and are responsible for the high-affinity antibody responses that occur in the later stages of an immune reaction (De Silva & Klein, 2015). These cells undergo a complex maturation process, involving interactions with T cells that provide essential signals for B cell activation and class switching.

The spleen plays a crucial role in B cell final maturation, with its functions organised into distinct regions that support various immune activities (Loder et al., 1999). The white pulp, where B cells reside, facilitates essential interactions between B and T cells, enhancing the adaptive immune response. Marginal Zone (MZ) B cells, located at the periphery of the white pulp, excel in capturing blood-borne antigens and initiating T-cell-independent immune responses (Siu et al., 2022).

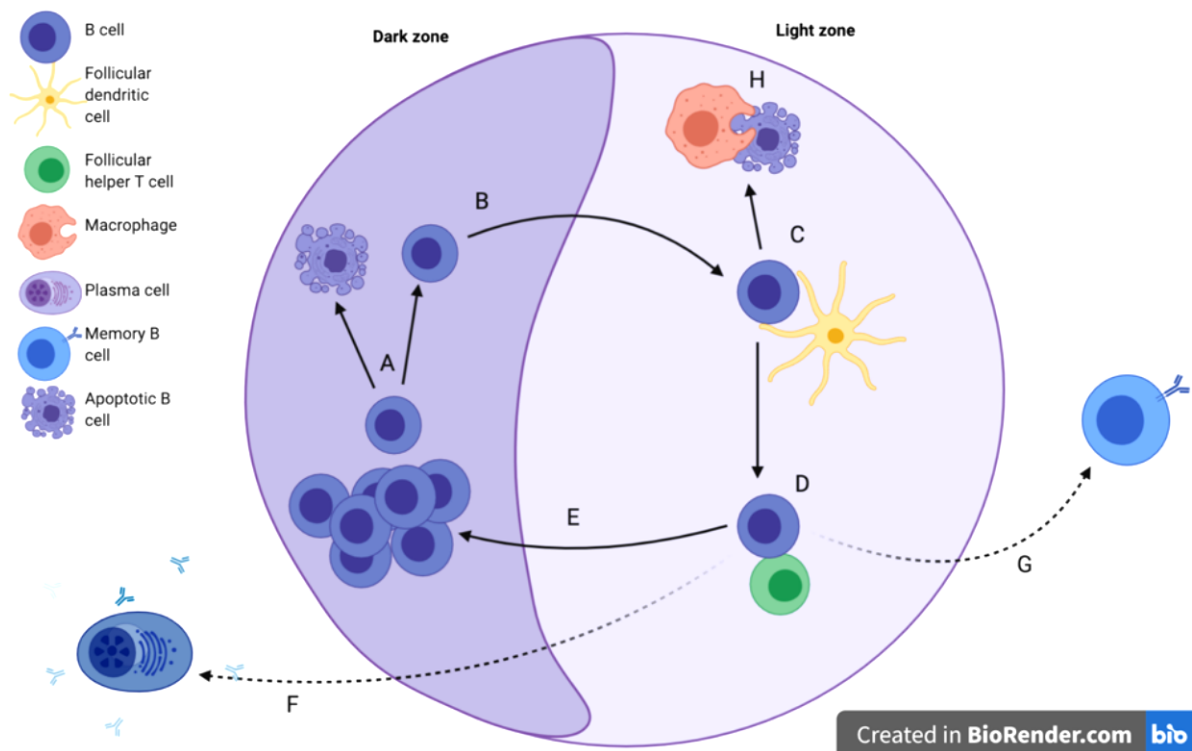
#### **1.1.2.2 B Cell Activation**

##### **1.1.2.2.1 T-cell-dependent response**

The germinal centre (GC) reaction is a well-organized process essential for the adaptive immune response, particularly by improving antibody specificity and efficacy through affinity maturation (Haberman et al., 2019; MacLennan, 1994). Antigen-activated B cells receive help from T helper cells specific for the same antigen at the B-T cell zone interface in the spleen/lymph node, leading to their proliferation. Part of these activated B cells differentiate into plasma cells in extrafollicular foci, which produce robust low-affinity antibodies. Part of the cells migrate back into the outer of

the follicle, where they undergo clonal expansion to form the germinal centre (Haberman et al., 2019). Mature germinal centres have two primary compartments: the dark zone (DZ) and the light zone (LZ) (Figure 1.1).

Centroblasts experience proliferation and somatic hypermutation (SHM) in the DZ, introducing point mutations in the immunoglobulin variable area (IgVDJ). This mutation is vital as it varies the binding affinity of B cell receptors (BCRs) to antigens (Haberman et al., 2019). Centrocytes move into LZ, called centrocytes. Follicular dendritic cells (FDCs) are strategically located within germinal centre (GC) light zones and play a critical role by presenting antigens in immune complexes. This setup allows centrocytes to be evaluated for their specificity by binding antigens, which is essential for their selection (Liu et al., 1996). Additionally, FDCs secrete CXCL13, a primary chemokine plus CXCL12 produced by reticular cells in DZ necessary for organising GCs into DZ and LZ, which is crucial for proper GC B-cell selection and mutation (Allen et al., 2004; Rodda et al., 2015).



**Figure 1.1 Germinal Centre Response.** Once B cells are activated, they enter the germinal centre to enhance their antibody affinity through a process called affinity maturation. (A) In the dark zone, these B cells multiply and undergo somatic hypermutation, introducing genetic alterations in their antibody variable region. Those acquiring harmful mutations are eliminated through apoptosis. (B) B cells retaining functional B cell receptors (BCRs) move to the light zone, where (C) they evaluate their newly altered BCRs by competing for antigen presented on follicular dendritic cells. (D) B cells with higher-affinity BCRs are selected based on how much antigen they can present to follicular helper T (T<sub>fh</sub>) cells and the extent of help they receive. (E) These positively selected B cells can then return to the dark zone, proliferating based on the degree of T cell help and undergo additional rounds of mutation and selection. (F) Some selected B cells eventually differentiate into antibody-producing plasma cells, while (G) others become memory B cells, ready for future immune responses. (H) Those that fail to gain selection signals in the light zone undergo apoptosis and are removed. *Adapted from (Hudson, 2022).*

The selection process in the light zone is highly competitive, with centrocytes competing for limited survival signals, such as IL-21 from follicular helper T cells (Tfh) (King et al., 2008). Centrocytes with high-affinity BCRs, able to bind, phagocytose and present more significant amounts of antigen to Tfh cells, are selected to get help from Tfh cells to differentiate into plasma cells or return to the dark zone for further proliferation and mutation. This process enhances their affinity through iterative rounds of somatic hypermutation and selection. Lower affinity B cells die through neglect.

Follicular helper T (Tfh) cells are vital throughout the germinal centre reaction, providing essential cytokines such as IL-21 and IL-4 while expressing co-stimulatory signals like CD40L and ICOSL (Bannard et al., 2013; Zhang et al., 2018). These interactions enhance the formation and maintenance of the germinal centre and assist the somatic hypermutation process. Plasma cells typically exit the germinal centre through the GC-T zone interface (Zhang et al., 2018). In contrast, memory B cells generally leave via the light zone toward the sub-capsular sinus in the lymph nodes (Zhang, Garcia-Ibanez, et al., 2022), poised to mount swift responses upon re-exposure to antigens (Weisel & Shlomchik, 2017). This intricate interplay and migration within the germinal centre ensure that only B cells with highly refined antigen specificity are selected for advanced immune functions. In the context of our conjugate vaccine strategy, this germinal centre reaction is paramount. By linking tumour endothelial markers (CLEC14A and ROBO4) to the immunogenic TTc carrier, we aim to overcome self-tolerance and trigger the T-cell help necessary to initiate and sustain robust germinal centre responses. This, in turn, leads to the generation of highly specific, class-switched antibodies capable of effectively targeting the tumour vasculature, thereby disrupting angiogenesis and inhibiting tumour growth.

#### **1.1.2.2.2 T-cell-independent response**

T cell-independent (TI) responses are immune reactions that occur without the assistance of T cells (Hjalmsdottir et al., 2024). They are classified into two types: TI type 1 and TI type 2. TI type 1 antigens are typically mitogenic, activating a broad range of B cells regardless of their specific antigen receptors (Vinuesa & Chang, 2013). This can lead to a polyclonal activation in which substances like lipopolysaccharide (LPS) from Gram-negative bacteria can directly stimulate B cells via toll-like receptors (e.g. TLR4) or other pattern recognition receptors (Alugupalli et al., 2004). TI-1 antigens act as adjuvants at smaller densities to enhance antigen-specific BCR activation. Consequently, these antigens stimulate B cell activation and the production of antibodies.

In contrast, TI type 2 (TI-2) antigens specifically activate B cells through extensive cross-linking of the B cell receptor (BCR) through repetitive epitopes on the antigen (McDonald et al., 2021). These antigens frequently include polysaccharides found on the surfaces of certain bacteria, such as those causing pneumonia. The immune response to TI-2 antigens primarily involves the production of IgM antibodies and is more reliant on the antigen's physical properties (McDonald et al., 2021). The response to TI type 2 antigens can lead to memory and class-switching (e.g., IgG3 in mice), although it is less robust than T-cell help responses.

#### **1.1.2.3 B Cells in Cancer**

B cells are a crucial component of the adaptive immune system, primarily recognised for their roles in antibody production and antigen presentation to CD4 T cells (Chi et al., 2024). In cancer, B cells take complex and dual roles within the tumour

microenvironment, contributing to both tumour-promoting inflammation and anti-tumour immune responses (Xue et al., 2024).

Within the tumour microenvironment (TME), B cells can influence tumour progression and patient outcomes through various mechanisms (Xue et al., 2024). They produce antibodies, especially IgG1, that bind to tumour antigens, facilitating the destruction of cancer cells via complement activation and antibody-dependent cellular cytotoxicity (ADCC) (Boyerinas et al., 2015; Carmi et al., 2015; Gilbert et al., 2011). This antibody production also aids in the opsonisation of tumour cells, enhancing their recognition by other immune cells (Sharonov et al., 2020). Additionally, B cells act as antigen-presenting cells that activate CD4 T cells, which bolster cytotoxic T cell responses against the tumour (Bruno et al., 2017; Rossetti et al., 2018). B cells also secrete a range of cytokines that modulate the immune response within the tumour (Xue et al., 2024). These cytokines can either support anti-tumour immunity or contribute to immunosuppression. Pro-inflammatory cytokines like IL-6 and TNF-alpha promote immunity (Helmink et al., 2020), while others like IL-10 or TGF-beta may potentially support tumour growth by hindering effective immune responses (Mirlekar, 2022). Typically, IL-10 or TGF-beta, mainly produced by regulatory B cells (Bregs), a specific subset of B cells, plays a vital role in maintaining immune tolerance and can reduce the efficacy of anti-tumour immunity (Huai et al., 2021).

#### **1.1.2.3.1 B Cells and Tertiary Lymphoid Structures (TLS)**

Tertiary lymphoid structures (TLS) represent a captivating connection between the adaptive immune response (e.g., activation of T and B cells) and chronic inflammation in the tumour microenvironment (Teillaud et al., 2024). TLS evolve from immature

clusters of infiltrating immune cells, particularly lymphocytes (T and B cells), along with associated stromal components, into organised centres that can exert significant anti-tumour effects. They exhibit a hierarchical structure that develops in response to the inflammatory signals (e.g., cytokines like TNF- $\alpha$ , IL-6, TGF- $\beta$ ) in tumours, which can either promote or inhibit cancer progression.

Follicular dendritic cells (FDCs) are essential in mature TLS, especially in the development of germinal centre reactions (Martinez-Riano et al., 2023). FDCs offer structural and functional support for B cell selection and survival, which is crucial for robust immune responses. In the germinal centres of TLS, FDCs perform several critical roles. One of their primary functions is to present antigens to GC B cells as antigen-antibody immune complexes (Fridman et al., 2023). These complexes are bound to the surface of FDCs via receptors such as the complement receptor CR2 (CD21), CR1 (CD35) and Fc receptor (Liu et al., 1997). This presentation is crucial as it maintains the antigens in a form that is easily accessible to B cells, thereby facilitating the selection process. During affinity maturation, B cells experience a selection process in which those expressing B cell receptors (BCRs) with specificity and a higher affinity for the antigen receive survival signals and proliferate (Nzula et al., 2003; Zhang et al., 2013). In comparison, those with lower affinity probably undergo apoptosis without help from Tfh cells. This switching and affinity maturation is mediated by activation-induced cytidine deaminase (AID) (Germain et al., 2014). It is crucial for adapting the immune response to different pathogens or tumour antigens, ensuring that the antibodies produced are most effective for neutralising or eliminating the antigen. In addition to their antigen presentation functions, FDCs secrete various factors vital for B cell survival and differentiation. BAFF is a crucial survival protein for B cells, promoting

their proliferation and preventing apoptosis (Rodriguez et al., 2021). It also supports the maturation of B cells into plasma cells.

In secondary lymphoid organs such as lymph nodes, CD4<sup>+</sup> T cells—particularly follicular helper T (Tfh) cells—play a central role in the formation and function of germinal centres (GCs). Tfh cells localise within B cell follicles, where they support B cell activation, proliferation, and affinity maturation. Through expression of key surface molecules like CD40 ligand (CD40L), which binds to CD40 on B cells, Tfh cells initiate class switch recombination and promote germinal centre formation (Kinker et al., 2023). Additionally, Tfh cells produce cytokines such as Interleukin-21 (IL-21) and Interleukin-4 (IL-4), which are essential for B cell survival, somatic hypermutation, and differentiation into high-affinity plasma and memory B cells (Gu-Trantien et al., 2013). IL-21 is particularly important for sustaining GC reactions and enhancing B cell maturation, while IL-4 supports immunoglobulin class switching, especially to IgG and IgE isotypes.

This specialised role of Tfh cells is recapitulated in tertiary lymphoid structures (TLS), which form in non-lymphoid tissues such as chronically inflamed organs or tumours (Pimenta & Barnes, 2014). Within TLS, CD4<sup>+</sup> T cells, and especially Tfh cells, similarly localise in B cell-rich zones, enabling direct and sustained interactions with B cells (Teillaud et al., 2024). These interactions mirror those in conventional GCs, facilitating B cell activation, class switch recombination, and differentiation into antibody-producing plasma cells within the tumour microenvironment. The presence of IL-21 and IL-4 in TLS supports local B cell maturation and contributes to the generation of tumour-reactive antibodies (Teillaud et al., 2024). Thus, Tfh–B cell interactions within

TLS are instrumental in shaping anti-tumour immunity by fostering local humoral responses and enhancing the immune system's ability to target tumour cells effectively.

High endothelial venules (HEVs) within TLS play a crucial role in facilitating the entry of lymphocytes, e.g. T cells and B cells, from circulation into the tumour microenvironment (Blanchard & Girard, 2021). These structures, characterised by their distinctive cuboidal endothelial cells, support the migration of lymphocytes through specialised molecular interactions (Blanchard & Girard, 2021). The lymphocyte entry process begins with rolling along the endothelium, mediated by selectins. L-selectin (CD62L) on lymphocytes, including B cells and T cells, binds to peripheral node addressin (PNAd) presented by HEVs (Michie et al., 1993; Rosen, 2004). This initial interaction slows the lymphocytes, establishing more stable adhesion. Following this, chemokines such as CCL21 secreted by HEVs activate integrins like LFA-1 on lymphocytes, then bind to CCR7, strengthening the LFA-1 activation and their binding to ICAM-1 on the endothelial cells (Shamri et al., 2005). This firm adhesion is essential for lymphocytes to withstand the blood flow during diapedesis, where they migrate between endothelial cells into the TLS.

In the context of vaccination, particularly with conjugate vaccines targeting self-antigens, the induction and maturation of TLS within the tumour microenvironment or draining lymph nodes can be crucial. By presenting vaccine antigens effectively to B cells and recruiting Tfh cell help, TLS provide an optimal environment for driving robust germinal centre reactions, affinity maturation, and class-switching (McDonald et al., 2021; Teillaud et al., 2024). This coordinated immune activity within TLS facilitates the generation of high-affinity, antigen-specific antibodies that are essential for mediating therapeutic anti-tumour effects, such as the destruction of tumour vasculature.

## **1.2 Introduction to Cancer**

### **1.2.1 Overview of Cancer**

Cancer remains a significant global health challenge, resulting in millions of fatalities each year. Cancer is characterised by the uncontrolled proliferation of abnormal cells and their capacity to metastasise to distant organs and tissues (Hanahan, 2022; Hanahan & Weinberg, 2011). The incidence, progression, and clinical outcomes of cancer show considerable variation across different populations and geographical regions, significantly influenced by genetic predispositions, environmental factors, and lifestyle behaviours (Wu et al., 2018). This underscores its complex and multifactorial nature.

A fundamental obstacle in advancing cancer research stems from its notable biological heterogeneity; each tumour often possesses distinct genetic mutations and unique molecular profiles (Wu et al., 2018). This inherent variability complicates diagnostic accuracy, the effectiveness of therapies, and the reliable prediction of patient prognosis. At the cellular level, cancer progression is driven by genetic alterations that disrupt critical cellular processes, including cell cycle regulation, apoptosis, and DNA repair pathways (Huang & Zhou, 2021). These disruptions directly contribute to tumorigenesis and the aggressive phenotypes observed clinically.

Understanding these intricate biological mechanisms is crucial for advancing therapeutic strategies. However, the complexities inherent in cancer biology continue to present substantial scientific and clinical challenges, necessitating ongoing research efforts and interdisciplinary collaboration to enhance patient outcomes.

### **1.2.2 Traditional Therapies**

The primary methods for treating cancer are surgery, chemotherapy, and radiation therapy. Surgery physically removes tumours but is constrained by the tumour's location and the presence of metastasis. Chemotherapy involves drugs like cisplatin and doxorubicin that target rapidly dividing cells but often harm healthy cells as well, causing side effects such as nausea, fatigue, hair loss, and immunosuppression (Anand et al., 2023). Radiation therapy employs high-energy particles such as X-rays or proton beams to eliminate cancer cells (Baskar et al., 2012). However, it can also affect nearby healthy tissues, potentially leading to skin irritation or damage to internal organs like the lungs and heart.

While these treatments are effective in many instances, they also have significant drawbacks. Non-specific toxicity can severely impact patients' quality of life through fatal side effects, including chronic pain, cognitive impairment, or reduced immunity. Furthermore, cancer cells often develop resistance to chemotherapy drugs like paclitaxel or methotrexate, diminishing treatment efficacy and increasing the likelihood of disease recurrence (Anand et al., 2023). Cancer recurrence after initial treatments, such as breast cancer recurrence following surgery or prostate cancer recurrence after radiation therapy, typically manifests as a more aggressive and treatment-resistant disease (Adams et al., 2023; Riggio et al., 2021). These limitations underscore the urgent need for more precise and enduring cancer treatments. The development of targeted therapies, such as trastuzumab for HER2-positive breast cancer (Early Breast Cancer Trialists' Collaborative, 2021), and immunotherapies, like checkpoint inhibitors (e.g., pembrolizumab for melanoma and lung cancer)(Dang et al., 2016), signifies promising advancements. These therapies focus on molecules crucial to cancer cell

progression or enhance the immune system's ability to recognise and attack cancer cells. Nonetheless, achieving lasting cures remains challenging, leading to ongoing research into the intricate interactions between cancer biology and treatment responses.

### **1.2.3 Immunotherapies in Cancer**

Targeted therapies represent a revolutionary advancement in cancer treatment, offering a far more precise therapeutic strategy compared to conventional methods such as chemotherapy and radiotherapy. These therapies are designed to interact with and disrupt molecular markers unique to cancer cells, often involving proteins or signalling pathways essential for tumour proliferation and survival. Targeted therapies significantly minimise collateral damage to healthy, non-malignant cells by selectively targeting these cancer-specific markers, thus considerably reducing treatment-related toxicities (Liu et al., 2024). Consequently, this specificity not only enhances the efficacy of the treatment concerning tumour regression but also markedly improves patient quality of life by minimising adverse side effects.

Furthermore, targeted approaches facilitate the personalisation of treatment, allowing therapeutic regimens tailored to individual patient profiles based on their tumours' genetic and molecular characteristics (Liu et al., 2024). This precision medicine paradigm offers distinct advantages, including the potential to overcome resistance mechanisms commonly encountered in traditional treatments by continually identifying and addressing new molecular targets as scientific understanding of cancer biology progresses (Liu et al., 2024).

Identifying and validating molecular targets forms the cornerstone of developing targeted cancer therapies. Recent decades have seen remarkable advancements in molecular biology and genetics, significantly reshaping our understanding of oncogenesis and tumour progression mechanisms. Advanced techniques such as genomic sequencing, transcriptomic analyses, and detailed characterisation of molecular signalling pathways have uncovered numerous potential therapeutic targets (Satam et al., 2023). These molecular targets often include specific proteins, enzymes, cellular receptors, or genetic mutations uniquely expressed or substantially upregulated within tumour cells.

Notably, the discovery and subsequent targeting of the HER2 receptor in specific breast cancer subtypes have changed therapy outcomes, illustrating the clinical impact of molecular-targeted therapies (Xu et al., 2024). Similarly, identifying activating mutations in the BRAF gene in melanoma has led to developing and applying inhibitors that selectively target these mutant proteins, significantly reducing tumour growth (Klein et al., 2013). Accurately detecting these molecular anomalies facilitates the creation of targeted therapeutic agents and improves patient stratification, ensuring that treatment is appropriately aligned with those most likely to benefit. Consequently, continuous advancements in identifying and targeting these molecular markers are expected to improve treatment outcomes and facilitate greater accessibility of personalised cancer therapy for patients.

Beyond small molecule inhibitors and antibodies that directly target cancer cells, immunotherapies represent a distinct and rapidly evolving class of targeted treatments that harness the body's own immune system to fight cancer.

A significant breakthrough in cancer immunotherapy has been the development of immune checkpoint inhibitors. These therapies block proteins called checkpoints that normally prevent immune cells from attacking healthy cells. By inhibiting these checkpoints, such as PD-1/PD-L1 (Programmed Cell Death Protein 1 / Programmed Death-Ligand 1) or CTLA-4, immune checkpoint inhibitors "release the brakes" on the immune system, allowing T cells to recognize and kill cancer cells more effectively (Dang et al., 2016). Examples include pembrolizumab for melanoma and lung cancer. Another revolutionary area involves adoptive cell therapies, specifically T-cell receptor (TCR) engineered T cells and Chimeric Antigen Receptor (CAR) T cell therapies (Sternier & Sternier, 2021). CAR T-cell therapy involves genetically engineering a patient's own T cells to express a CAR that recognizes a specific antigen on cancer cells (Sternier & Sternier, 2021). This engineered receptor allows the T cells to directly bind to and kill tumour cells, even those that do not present antigens via MHC molecules (Sternier & Sternier, 2021). For example, CAR T cell therapy targeting CLEC14A has demonstrated potential efficacy in oncological applications, with engineered T cells showing enhanced proliferation, immune functionality, and cytotoxic capabilities against CLEC14A-expressing cells *in vitro* and significantly reducing tumour growth and vascular density *in vivo* without harmful effects on non-malignant tissues (Zhuang et al., 2020). Similarly, anti-ROBO4 CAR T cells have significantly reduced tumour growth in cancer models (Hirobe et al., 2022).

TCR-engineered T cells involve isolating T cells and genetically modifying them to express a new TCR that recognizes specific cancer antigens presented by MHC molecules (Shafer et al., 2022). This approach aims to enhance the natural T cell response against tumours.

These cellular immunotherapies offer highly specific and potent anti-tumour effects by directly engaging the patient's immune cells against cancer. While promising, challenges such as high production costs can limit their accessibility, particularly in developing countries.

#### **1.2.4 Cancer Vaccines**

Cancer vaccines, a type of cancer immunotherapy, seek to harness the body's immune system to identify and fight cancer cells, offering a promising approach to cancer treatment. These vaccines are classified into protein/peptide vaccines, nucleic acid-based vaccines (e.g. DNA, mRNA), cell-based vaccines and virus vaccines, each employing different mechanisms to stimulate an immune response (Lin et al., 2022). The following will focus on mRNA, DNA, and protein vaccines.

mRNA vaccines introduce a specific messenger RNA (mRNA) segment into host cells, which encodes cancer antigens (tumour-specific antigen or tumour-associated antigen) (Sayour et al., 2024). The antigen-presenting cells (e.g. dendritic cells) then use their molecular machinery to translate this mRNA into the protein antigen. This protein is processed and displayed on the cell surface, bound to Major Histocompatibility Complex (MHC) molecules. This presentation mobilises the immune cells, e.g. CD8 T cells, to recognise and attack cancer cells that express this antigen. For instance, RO7198457 is an mRNA vaccine designed to target neo-epitopes selected for their potential immunogenicity in melanoma cancer patients (NCT03815058). This results in a better prognosis than chemotherapy alone for pancreatic cancer. Another clinic trials showed that mRNA neoantigen vaccine

combined with anti-PD-L1 immunotherapy or chemotherapy could induce substantial T cell activity, better clinic results than chemotherapy alone in pancreatic ductal adenocarcinomas (Rojas et al., 2023). mRNA vaccine can be customised for individual tumours, potentially offering a highly personalised treatment (Wang et al., 2024).

DNA vaccines introduce plasmids containing the DNA sequences that encode cancer antigens (Pagliari et al., 2023). The antigen-presenting cells take up these plasmids, producing the antigens and eliciting humoral and cellular immune responses. Like mRNA vaccines, DNA vaccines can target specific characteristics of an individual's tumour. However, they generally have lower efficacy than mRNA vaccines due to poor immunogenicity. Oncept, a DNA-based cancer vaccine for dogs, targets oral malignant melanoma by introducing a non-canine tyrosinase gene into a DNA plasmid (Pellin, 2022) This vaccine was the first therapeutic cancer vaccine approved by the USDA and received its full licence in 2010. However, the study hasn't shown definitive proof that the Oncept melanoma vaccine could improve human clinical outcomes.

Protein vaccines involve administering tumour-specific antigens (protein or small peptide) to the patient (Lin et al., 2022). These antigens are delivered directly in their protein form, often combined with adjuvants to enhance the immune response. Currently, this type of vaccine targeting viral protein has been offered for the prevention of cancer (e.g. vaccine for HPV and HBV). However, the therapeutic HPV vaccine remains to be explored. This protein vaccine is more traditional and can target shared cancer antigens, providing potentially safer and simplified manufacturing (Park et al., 2023; Pollet et al., 2021). Their enhanced safety profile arises from the absence of infectious agents or genetic material, minimizing risks of pathogenicity or unintended genetic alterations in the host (Park et al., 2023). Furthermore, the manufacturing

process for protein vaccines is generally more straightforward, relying on established methods for protein expression and purification, which allows for a high degree of control over product purity and consistency (Pollet et al., 2021). However, it may be less effective at inducing a cellular immune response than mRNA vaccines. Therefore, research has designed different delivery systems to increase the interaction of cancer vaccine components with the immune system. These modified cells are then reintroduced into the patient's body to stimulate an immune attack on cancer cells. Another strategy for different nanocarriers (e.g. virus-like particles) has been designed to deliver cancer-specific antigens to antigen-presenting cells (Pielenhofer et al., 2020).

### **1.3 Angiogenesis in Cancer**

Angiogenesis, forming new blood vessels from preexisting vasculature, is a fundamental process critical for physiological growth, wound healing, and pathological conditions such as cancer (Otrock et al., 2007).

Under normal physiological conditions, angiogenesis is tightly regulated by a delicate balance of molecular signals that promote (pro-angiogenic) and inhibit (anti-angiogenic) the growth of new vessels (Otrock et al., 2007). This regulatory system ensures that angiogenesis occurs only in necessary situations, such as wound healing, embryonic development, or the menstrual cycle.

Pro-angiogenic factors, including vascular endothelial growth factor (VEGF), activate receptors on endothelial cells, which line the inner surface of blood vessels (Ghalehbandi et al., 2023). This process initiates pathways that enhance endothelial

cell proliferation, migration, and survival. Other contributors, such as fibroblast growth factors (FGFs) and angiopoietins, support organising endothelial cells into new vascular structures (Farooq et al., 2021; Thomas & Augustin, 2009). In contrast, anti-angiogenic factors like angiostatin and endostatin, breakdown products of plasminogen and collagen XVIII, respectively, inhibit these processes to prevent excessive vessel formation, thereby maintaining vascular stability and function (Thomas & Augustin, 2009).

In cancerous conditions, the precise regulation of angiogenesis is disrupted, resulting in uncontrolled vascular growth, a hallmark of tumour progression (Hanahan, 2022; Hanahan & Weinberg, 2011). Cancer cells can exploit the angiogenic process to obtain a continuous supply of nutrients and oxygen for rapid growth and metastasis. They achieve this by overexpressing pro-angiogenic factors like VEGF, which is significantly elevated in many tumours (Ghalehbandi et al., 2023). The increased levels of VEGF overwhelm the natural anti-angiogenic mechanisms, promote the formation of new blood vessels that invade the tumour (Ghalehbandi et al., 2023).

Tumour vessels are often structurally and functionally abnormal. They can be leaky, tortuous, and poorly organised, contributing to chaotic blood flow and increasing the likelihood of tumour cell dissemination and metastasis (Lugano et al., 2020). Additionally, abnormal blood vessels in tumours create a hypoxic environment that can further drive angiogenesis and tumour progression through mechanisms such as the upregulation of hypoxia-inducible factor 1 (HIF-1), a key regulator of the response to low oxygen levels (Lugano et al., 2020).

Overall, anti-angiogenic therapy has significantly advanced cancer treatment; however, overcoming resistance and managing side effects remain crucial for its

ongoing success. While agents like bevacizumab (Avastin)(Ferrara et al., 2005), which targets VEGF-A, have improved outcomes in various solid tumours such as colorectal, lung, and renal cell carcinomas, their clinical benefit is often transient due to the development of adaptive resistance mechanisms by the tumour (Garcia et al., 2020). These resistance mechanisms can involve the upregulation of alternative pro-angiogenic pathways or the recruitment of pro-angiogenic immune cells within the tumour microenvironment (Yang et al., 2025). Furthermore, anti-angiogenic therapies are associated with a distinct spectrum of side effects, including hypertension, proteinuria, thrombosis, and impaired wound healing, which necessitate careful patient monitoring and management to ensure continued treatment and maintain quality of life (Yang et al., 2025). Future strategies include combination therapies, multi-targeted drugs, and exploring well-characterised angiogenic markers such as ROBO4 and CLEC14a. These approaches aim to make treatments more effective, personalised, and resilient to adaptive mechanisms.

### **1.3.1 Vascular Targeting in Cancer**

Vascular targeting in cancer is a therapeutic approach that aims to inhibit tumour growth by disrupting the blood supply to the tumour (Siemann & Horsman, 2009). Unlike traditional chemotherapy, which directly targets cancer cells, vascular targeting strategies focus on the endothelial cells that form tumour blood vessels (Yang et al., 2025). These endothelial cells are generally more genetically stable than cancer cells, making them less prone to developing drug resistance (Morimoto et al., 2024). By cutting off the tumour's access to oxygen and nutrients, these therapies can starve the tumour and prevent its growth and metastasis (Morimoto et al., 2024). This strategy

includes approaches like inhibiting angiogenesis (formation of new blood vessels) or directly destroying existing tumour vasculature.

#### **1.3.1.1 Roundabout Guidance Receptor 4 (ROBO4)**

Roundabout Guidance Receptor 4 (ROBO4) is an endothelial-specific receptor within the Roundabout receptor family (Park et al., 2003). It primarily regulates angiogenesis and maintains vascular stability and integrity (Dai et al., 2019).

ROBO4, first described by Roy Bicknell's group in 2002 as 'Magic Roundabout', is an endothelial-specific receptor gene (Huminiecki & Bicknell, 2000). Structurally distinct from other Robo receptors, ROBO4 comprises two immunoglobulin (Ig)-like domains, two fibronectin type-III repeats, a transmembrane region, and an intracellular domain featuring conserved signalling motifs (Yadav & Narayan, 2014).

ROBO4 signalling is complex, involving multiple ligands and downstream effectors. ROBO4 primarily interacts with the Slit glycoproteins (Slit2 and Slit3) and the UNC5B receptor to mediate its effects on vascular integrity. The binding of Slit2 to ROBO4 activates signalling cascades that stabilise vascular networks by reducing vascular endothelial growth factor (VEGF)-mediated endothelial permeability and migration (Jones et al., 2008). Additionally, ROBO4 interacts with the UNC5B receptor, acting as a ligand or co-receptor. This interaction competes with VEGF receptor-2 (VEGFR2) signalling by inhibiting Src kinase phosphorylation, thereby preserving vascular integrity and counteracting VEGF-induced angiogenesis and vessel permeability (Koch et al., 2011).

#### **1.3.1.1.1 Role of ROBO4 in Angiogenesis and Tumour Progression**

ROBO4 expression is dynamically regulated during both physiological and pathological angiogenesis. Under normal physiological conditions, ROBO4 helps stabilise blood vessels (Jones et al., 2008); however, its expression patterns become more complex during pathological states. ROBO4 is frequently upregulated in tumour vasculature, such as breast cancer, melanoma, glioma, colorectal cancer, non-small cell lung cancer, pancreatic cancer, and bladder carcinoma (Dai et al., 2019). Increased ROBO4 expression in these contexts correlates with enhanced endothelial survival, altered vascular permeability, and survival signalling pathways, such as PI3K/Akt (Dai et al., 2019). This upregulation of ROBO4 in tumour vessels actively supports the hypoxic tumour microenvironment by promoting the survival of endothelial cells and maintaining altered vascular integrity within these low-oxygen conditions. This, in turn, helps the tumour adapt to and even benefit from the harsh, oxygen-deprived environment, which is often characteristic of rapidly growing tumours.

VEGF regulates ROBO4 expression (Gong et al., 2019), inflammatory cytokines (e.g., Tnf, IL-1 $\beta$ ), and hypoxia-induced factors (HIFs) (Tanaka et al., 2017). The Notch and TGF- $\beta$  pathways, shear stress and tumour-secreted factors like FGFs also contribute to ROBO4 upregulation, supporting tumour angiogenesis (Adzraku et al., 2024).

#### **1.3.1.1.2 Therapeutic Potential of ROBO4**

Preclinical evidence supports ROBO4 as an effective target for modulating pathological angiogenesis, critical for tumour growth and metastasis. Soluble ROBO4 that combines ROBO4's extracellular domain with the Fc fragment of human IgG inhibits angiogenesis in rodent subcutaneous models, inhibits VEGF-stimulated

endothelial cell migration, proliferation, and inhibits tube formation in rat aortic assay (Suchting et al., 2005). The study further presented that ROBO4-Fc or ROBO4-Fc conjugated foreign carrier protein significantly reduces tumour vascularisation and suppresses the growth in Lewis lung carcinoma by inhibiting the VEGF signalling pathway (Zhuang et al., 2015).

Another promising strategy involves antibody-drug conjugates (ADCs) targeting ROBO4 (Yoshikawa et al., 2013). Due to the selective expression of ROBO4 on tumour endothelial cells, these ADCs preferentially accumulate in tumour tissue, delivering cytotoxic agents specifically to the tumour vasculature. Compared to ADCs that target VEGFR, which may lead to systemic toxicity owing to broader expression profiles, ROBO4-targeted ADCs demonstrate superior specificity and reduced off-target effects (Yoshikawa et al., 2013). These ADCs effectively disrupt the tumour blood supply and significantly decrease tumour growth and metastasis in B16BL6 murine melanoma (Yoshikawa et al., 2013).

These therapeutic approaches targeting ROBO4 collectively underscore its promising role in inhibiting tumour angiogenesis and highlight ROBO4 as a desirable candidate for clinical translation into cancer therapy.

#### **1.3.1.2 C-type Lectin Domain Family 14 Member A (CLEC14A)**

CLEC14A (C-type Lectin Domain Family 14 Member A) is a transmembrane protein primarily expressed by endothelial cells (Zelensky & Gready, 2005). Roy Bicknell's group first identified the discovery of CLEC14A as a tumour endothelial marker (Mura et al., 2012). They discovered that CLEC14A is significantly upregulated in tumour endothelial cells compared to normal tissues. Further studies confirmed its tumour

specificity through comprehensive transcriptomic analyses of 5,332 RNA expression profiles across various healthy and cancerous tissues, including breast, lung, renal cell carcinoma, ovarian, and prostate cancers (Robinson et al., 2020).

CLEC14A is a transmembrane protein cleaved by RHBDL2 (Rhomoid Like 2), releasing its ectodomain into the extracellular space (Noy et al., 2016). These two portions, membrane-bound and extracellular, have opposing effects. The membrane-bound domain enhances filopodia elongation in terminal endothelial cells, thereby facilitating sprouting angiogenesis (Ki et al., 2013; Noy et al., 2016). Conversely, the cleaved ectodomain released extracellularly competes with multimerin-2 (MMRN2), disrupting VEGFA/VEGFR interactions and inhibiting angiogenesis (Noy et al., 2016).

#### **1.3.1.2.1 Role of CLEC14A in Angiogenesis and Tumour Progression**

CLEC14A is a vital endothelial receptor that significantly contributes to angiogenesis by promoting vascular remodelling and sprouting essential for forming new vessels (Mura et al., 2012). Its primary biological role involves mediating cell-cell contacts and interactions with the extracellular matrix (ECM) (Noy et al., 2016). CLEC14A specifically binds to ECM components such as MMRN2, a large glycoprotein involved in vascular development, facilitating the maturation and stability of vascular networks under physiological and pathological conditions (Noy et al., 2016; Zanivan et al., 2013). Furthermore, CLEC14A is involved in cytoskeletal rearrangement, influencing endothelial cell morphology and migration, further enhancing angiogenic sprouting (Noy et al., 2016). CLEC14A is highly expressed in tumour-associated vasculature and is linked to increased angiogenic activity and tumour progression (Robinson et al., 2020). Its presence leads to irregular, permeable vessels typical of aggressive

tumours. This abnormal vascular development promotes primary tumour growth and contributes to metastasis by generating structurally weak vessels.

#### **1.3.1.2.2 Therapeutic Potential of CLEC14A**

The significant increase in CLEC14A expression in tumour blood vessels compared to healthy ones suggests the potential function of CLEC14A in anti-angiogenic therapies. The research focused on the C-type lectin-like domain (CTLD) of CLEC14A has shown that it is vital for regulating endothelial cell movement and forming cellular extensions during the development of new blood vessels (Ki et al., 2013). Using phage display technology, scientists have created specific human antibodies targeting the CTLDs in human and mouse CLEC14A (Ki et al., 2013). These antibodies effectively inhibit movement and network formation of endothelial cells without compromising cell health or normal function. These studies indicate that CLEC14A-CTLD is a promising target for anti-angiogenic therapy and a potential new avenue for cancer treatment.

Another investigation evaluated the potential benefits of inhibiting the CLEC14A-MMRN2 interaction (Noy et al., 2015). Employing a monoclonal antibody to disrupt this interaction significantly hindered Lewis lung carcinoma (LLC) tumour growth and diminished the formation of new blood vessels in the tumour.

CAR T cell therapy targeting CLEC14A has demonstrated potential efficacy in oncological applications (Zhuang et al., 2020). This research involved engineering T cells with B cell receptors that recognise CLEC14A, which is called CLEC14A-specific CAR T cells. In vitro, these modified T cells exhibited enhanced proliferation, immune functionality, and cytotoxic capabilities against cells expressing CLEC14A. In vivo, CLEC14A-specific CAR-T cells significantly reduced tumour growth and vascular

density within the mouse tumours, such as Rip-Tag2, murine pancreatic ductal adenocarcinoma, and Lewis lung carcinoma (LLC). Importantly, these treatments proved safe and had no harmful effects on non-malignant tissues, providing the therapeutic potential of CLEC14A.

#### **1.4 Conjugate Cancer Vaccine Strategy**

Conjugate vaccine, a strategy of protein vaccines, attaches bacterial polysaccharides (weak antigen) to a protein carrier (potent antigen, e.g. diphtheria toxin) to significantly improve immune response in children (Khan, 2006). This innovation transforms a T-cell-independent polysaccharides vaccine to a T-cell-dependent one, essential for creating durable immunity (Rappuoli, 2018; Rappuoli et al., 2019)(Figure 1.2). This is achieved by covalently linking the polysaccharide (the weak antigen) to a highly immunogenic protein carrier (Figure 1.2). Upon administration, antigen-presenting cells (APCs), such as dendritic cells and macrophages, efficiently process the carrier protein alongside the polysaccharide antigen. The carrier-derived peptides are then presented on MHC class II molecules, leading to the activation of vital CD4<sup>+</sup> T cells. These activated T helper cells, particularly T follicular helper (T<sub>fh</sub>) cells, provide essential signals (e.g., through CD40-CD40L interactions and cytokine secretion like IL-21) to polysaccharide-specific B cells that have internalised the conjugate antigen. This crucial T-cell help drives the B cells to proliferate, form germinal centres, undergo somatic hypermutation and affinity maturation, and finally differentiate into long-lived plasma cells producing high-affinity, isotype-switched antibodies (such as IgG). This process is essential for creating durable immunity and generating memory B cells, ensuring a rapid and robust recall response upon subsequent antigen exposure. Such

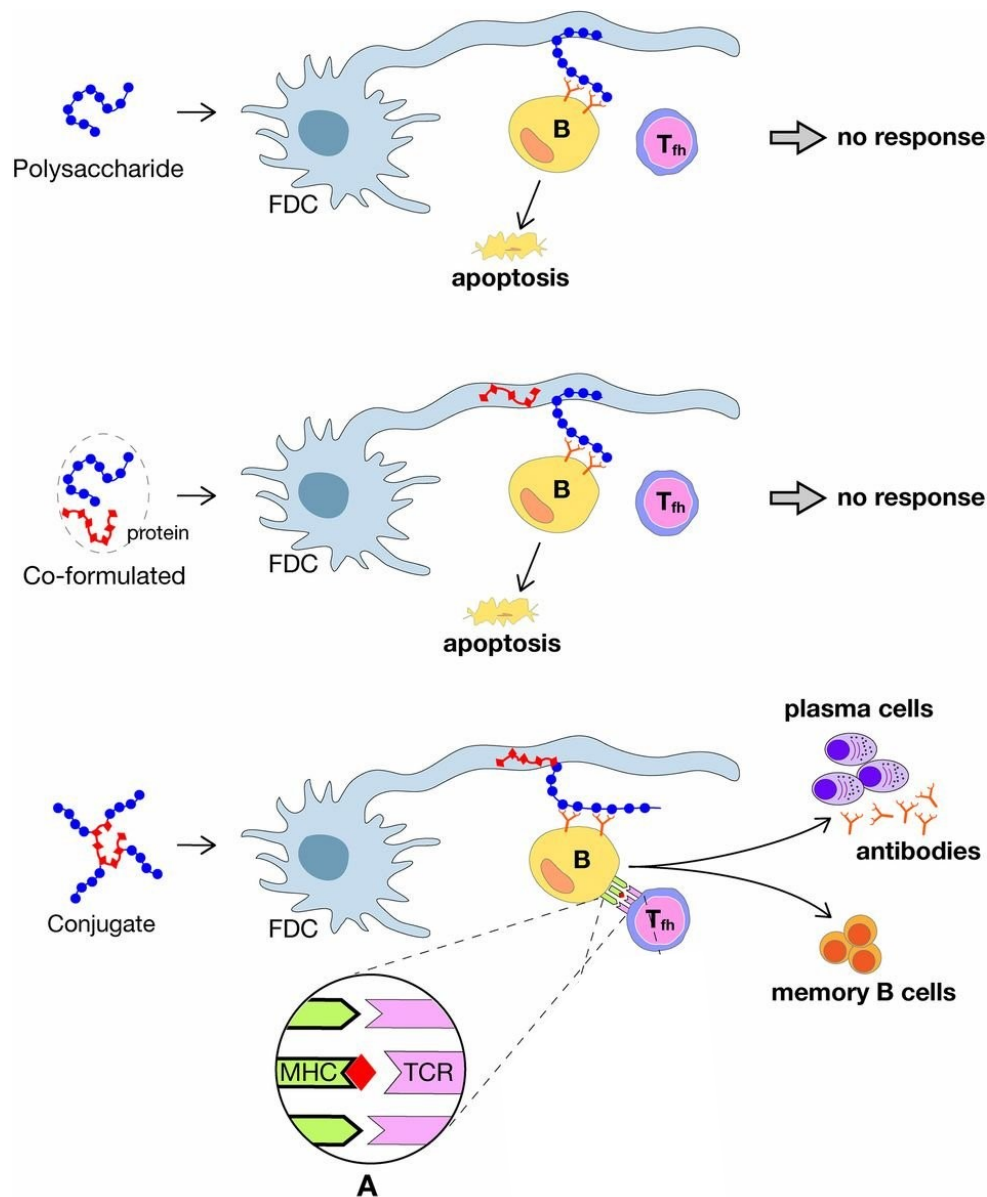
vaccines have played a vital role in reducing the prevalence of bacterial diseases such as *Haemophilus influenzae type b* (Hib disease) and *Streptococcus pneumonia* (pneumococcal disease), particularly in populations with underdeveloped or compromised immune systems (Bilukha et al., 2005).

Conjugate vaccines optimise innate and adaptive immune responses to T-independent antigens such as bacterial capsular polysaccharides (Figure 1.2) (Rappuoli, 2018). Upon administration of conjugate protein, antigen-presenting cells (APCs), such as dendritic cells and macrophages, efficiently catch and process the carrier protein with the polysaccharide antigen and then present peptides on MHC molecules, triggering vital CD4 T-cell activation (Rappuoli, 2018). This T-cell activation is essential for supporting polysaccharide-specific B-cell responses. These B cells bind the conjugate antigen via their B cell receptors, internalise it, and present carrier-derived peptides to the activated T helper cells. These B cells proliferate and form germinal centres, then finally mature into long-lived plasma cells that produce high-affinity, isotype-switched antibodies and develop memory cells, ensuring prolonged immunity.

In recent years, conjugated vaccine strategy has been used in cancer immunotherapy to target tumour-associated self-antigens, which also suffer from sub-optimal T cell help. This challenge arises because self-antigens, by definition, are naturally present in the body, leading to immune tolerance mechanisms that prevent robust immune responses against them (Fan et al., 2023). Consequently, self-antigen-specific B cells struggle to receive adequate T cell help, resulting in restricted autoantibody secretion and weak immune activation against the tumour. For example, recombinant protein Epidermal Growth Factor (EGF) chemically conjugated with carrier protein diphtheria toxin mutant CRM197, plus adjuvant Montanide ISA 51 VG, was tested in advanced

solid tumours in a clinical trial phase in China. The result showed that the EGF-CRM197 vaccine can induce a good immune response targeting EGF in patients, was safe and well tolerated (Xiong et al., 2021). Another very similar conjugate vaccine, the CIMAvax-EGF vaccine, developed in Cuba, has proved to be safe and effective in Non-small cell lung cancer, particularly in patients with high baseline serum EGF level (Flores Vega et al., 2023). Here, recombinant EGF is chemically conjugated to recombinant P64k derived from *Neisseria meningitidis*. The clinical trial data has shown that the CIMAvax-EGF vaccine could induce a high level of neutralising antibodies against EGF, which block the EGF-EGFR interaction. Recent clinical trial data combining CIMAvax-EGF with nivolumab (anti-PD-1 immune checkpoint inhibitor) showed the combination therapy is safe and can induce a higher titre of good anti-EGF antibody response than CIMAvax-EGF alone.

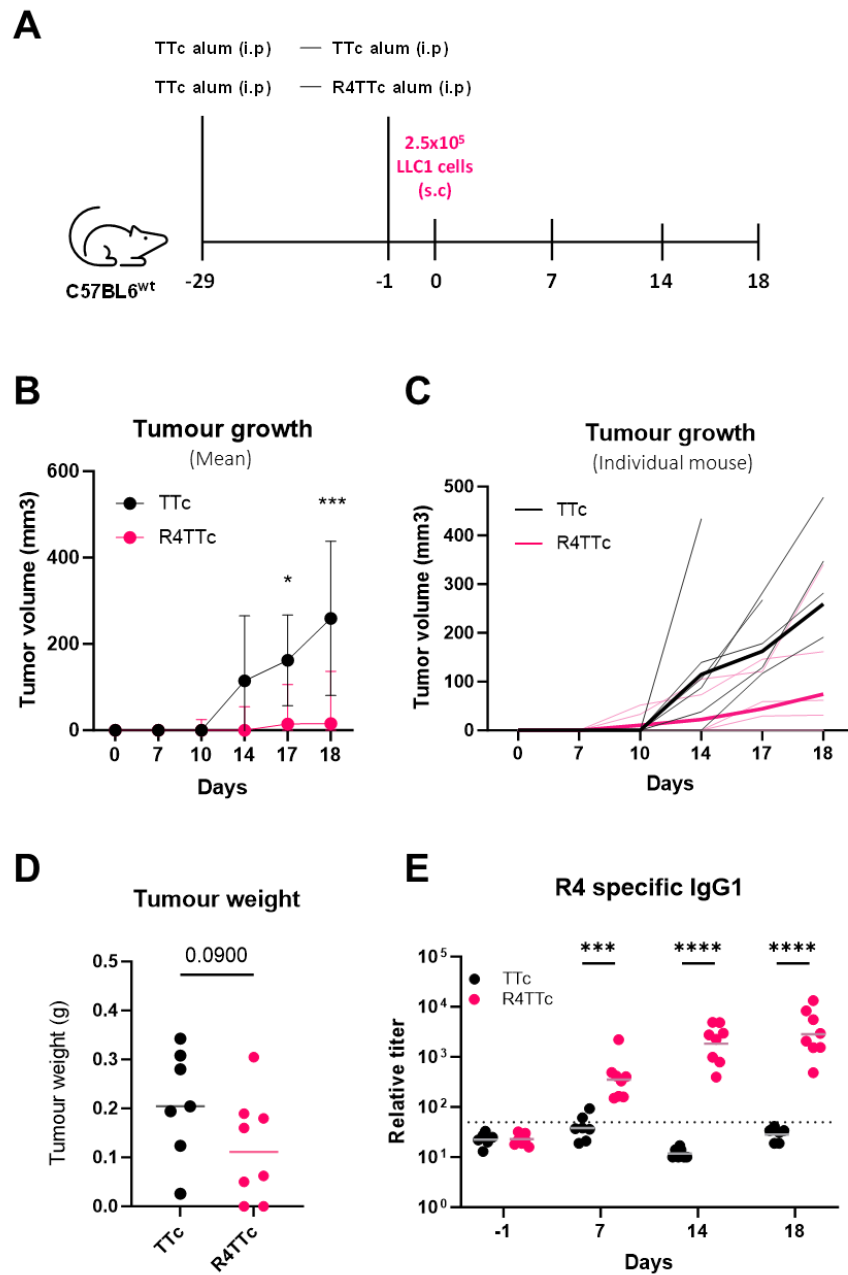
Our lab developed a conjugate vaccine that can induce high titres of antibodies specific to ROBO4. By chemically linking the extracellular domain of ROBO4 to a common foreign vaccine carrier, chicken gamma globulin, autoreactive B cells can recruit CD4+ T cells to help specific for the carrier. This protocol can efficiently induce autoreactive anti-tumour vessel antibodies, resulting in tumour suppression and delayed tumour growth in the Lewis Lung Carcinoma cell (LLC1) transplant model (Zhuang et al., 2015). Our lab recently started translating this vaccine protocol to carrier proteins used in human vaccination.



**Figure 1.2** Illustration of the interactions between polysaccharide and conjugate vaccines with follicular dendritic cells (FDC), B cells, and T follicular helper (T<sub>fh</sub>) cells in germinal centres (GCs). At the top, the diagram features a polysaccharide vaccine by itself. The middle section displays a polysaccharide combined with a protein. At the bottom, a conjugate vaccine is shown. Circle A represents how conjugate vaccines activate the T-cell receptor (TCR). In Circle A, the peptide binds to the MHC molecule, acting as the TCR-recognised antigen. *Adapted from Rappuoli (2018).*

TTc, a non-toxic fragment C of tetanus toxin, was selected as a carrier protein. While conjugate vaccines traditionally link weak polysaccharide antigens to carrier proteins to enhance immunogenicity, our project employs a protein-protein conjugation strategy. TTc has been often utilised as a carrier in conjugate vaccines due to its ability to elicit robust CD4<sup>+</sup> T-cell responses (Bayart et al., 2022). Moreover, it is safe to use in humans (Xu et al., 2021), and given the high global coverage of tetanus toxoid (TT) vaccination, with many countries achieving over 80% vaccination rates in infants and women of reproductive age (Organization, n.d.), there should be widespread pre-existing T cell memory (Wang et al., 2021) which will guarantee efficient and rapid recruitment of T cell help in patients. TT has expanded from conventional vaccines to cancer immunotherapy, where it is chemically linked to antigens like HER2, showing CD4<sup>+</sup> T cell induction supporting the production of anti-Her2 antibodies in mice, leading to the reduction of breast tumours in mice (Chotprakaikiat et al., 2016). The effectiveness of TTc in eliciting strong immune responses against previously weakly immunogenic antigens highlights its pivotal role in vaccine design.

In our lab, ROBO4 genetically linked to TTc has been successfully engineered, with recombinant protein vaccine having the advantage of defined composition (Carrier-priming boost strategy to target ROBO4 self-antigen) (Escobar-Riquelme, 2024). This vaccine effectively bypassed self-tolerance and stimulated the production of ROBO4-specific IgG1 antibodies, significantly impeding tumour angiogenesis and growth in LLC1 tumours in mice (Figure 1.3).



**Figure 1.3 Suppressed tumour growth following ROBO4TtC injection.** (A) Diagram of the immunisation protocol: Mice received a TtC alum prime, followed by a booster with either ROBO4TtC alum or TtC alum four weeks later. One day post-boost, LLC1 cells were subcutaneously injected into the right flank. (B-C) Tumour growth curves displayed as mean values and individual mouse data were analysed using two-way ANOVA with mixed-effects analysis and Šídák's multiple comparisons. (D) Tumour weight was assessed using an unpaired Mann-Whitney test. (E) ROBO4-specific IgG1 antibody levels in serum measured by ELISA. Statistical significance was determined by two-way ANOVA with mixed-effects analysis and Šídák's multiple comparisons. \*\*\*\*,  $p < 0.0001$ ; \*\*\*,  $p < 0.001$ ; \*,  $p < 0.05$ . Each symbol represents one mouse. *Figure adapted from Escobar-Riquelme (2024).*

## 1.5 Thesis Aim

Cancer vaccination presents a cost-effective strategy for cancer immunotherapy. Directly targeting tumour cells poses challenges due to their high mutagenicity and heterogeneity, often necessitating personalised treatments that can be expensive and less accessible for clinical application. Targeting angiogenesis is a promising alternative. Tumour endothelial markers are advantageous targets as they are expressed by endothelium, which is not transformed tissue and, therefore, genetically stable, exposed to the immune system, and permit broad applicability across various tumour types and diverse HLA (Human Leukocyte Antigen) backgrounds with simplified manufacturing and off-the-shelf availability. However, these markers are frequently self-antigens with low immunogenicity, failing to elicit robust immune responses due to insufficient T-cell support. Our lab's research indicates that a conjugated vaccine, utilizing TTc as a carrier, can provide rapid immunization to mice against self-antigens (Robo4 etc.) (Figure 1.3) (Escobar-Riquelme, 2024).

This vaccine protocol should be widely usable for any cancer-associated cell surface-expressed autoantigen. Therefore, one project aim is to develop a vaccine targeting the tumour endothelial marker: CLEC14A. Then, the immune response of CLEC14A will be analysed.

The ROBO4-TTc cancer vaccine permits broad applicability across various tumour types if the tumour upregulates ROBO4. Therefore, another project aim is to evaluate the efficacy of the ROBO4TTc vaccines in a breast cancer model.

## **1.6 Hypothesis and Objectives**

### **Hypothesis**

We hypothesise that a conjugate vaccine formed by linking the extracellular region of mouse CLEC14A to fragment C of tetanus toxin (CLEC14A-TTc) will induce a specific antibody response targeting CLEC14A. Furthermore, we hypothesise that the ROBO4-TTc vaccine will inhibit tumour growth in a mouse model of breast cancer.

### **Objectives**

The specific objectives of this thesis are as follows:

- i. To design, produce, and purify the genetically linked CLEC14A-TTc protein.
- ii. To assess the generation of CLEC14A-specific antibodies following immunisation with the CLEC14A-TTc.
- iii. To evaluate the generation of ROBO4-specific antibodies and their impact on tumour growth in breast cancer.
- iv. To investigate the mechanism of the ROBO4TTc vaccine in breast tumour growth.

## **Chapter 2: Methodology**

### **2.1 Development of expression plasmids for CLEC14A, ROBO4, and TTc**

#### **2.1.1 Plasmids**

The sequence of the extracellular region of mouse CLEC14A (C14a) was primarily sourced from Roy Bicknell's lab at the University of Birmingham. The TTc sequence was generously provided by Dr Natalia Savelyeva from the University of Southampton. To enhance the secretion of the proteins in the culture media, an Fc-tag was added to CLEC14A and TTc. A "GSSGSSGSS" peptide sequence was employed as a linker to connect the CLEC14A and Fc, or TTc and Fc sequences. The CLEC14A-Fc sequence was designed to include restriction enzyme sites, such as NheI and ApaI. The CLEC14A-TTc sequence was codon-optimised for mammalian production and obtained in the standard pUCIDT vector containing an ampicillin-resistance cassette (Integrated DNA Technologies IDT).

The mammalian expression vector pcDNA3.1-mGFP, obtained from Dr Michael Price at the University of Birmingham, was chosen as the backbone vector for subcloning. The pcDNA3.1 backbone vector was created by excising the membrane green fluorescent protein (mGFP) sequence using NheI and ApaI restriction enzymes, resulting in sticky ends. The CLEC14A-Fc sequence was extracted from the pUCIDT vector, digested with the same restriction enzymes, and ligated into the pcDNA3.1 backbone vector. Several methods confirmed successful ligation, including restriction enzyme profiling, PCR (Polymerase Chain Reaction), and Sanger sequencing.

Dr Fernanda Nicole Escobar-Riquelme from the University of Birmingham, UK, kindly provided the pcDNA3.1 TTc-Fc plasmid (Escobar-Riquelme, 2024). The pcDNA3.1

CLEC14a-TTc-Fc plasmid was created by inserting the CLEC14A sequence into the pcDNA3.1 TTc-Fc vector using PCR cloning. Overlapping primers were used to amplify the CLEC14A sequence and the pcDNA3.1 TTc-Fc plasmid. The purified PCR products were cloned together using HIFI DNA assembly following DNA gel electrophoresis and gel extraction. All plasmids described in this thesis are listed in Table 2-1, with the complete DNA and protein sequences provided in the Appendix A.

*Table 2-1 Descriptions of commercial and constructed plasmids.*

| Plasmid name                   | Description                                                                                                                                                                                                                                                                  | Source                                                             |
|--------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------|
| <b>pcDNA3.1 mGFP</b>           | The pcDNA3.1 mammalian expression vector contains mGFP (membrane GFP) under the CMV promoter. mGFP is flanked by Apal and NheI restriction enzymes. The pcDNA3.1 plasmid also includes Ampicillin and Neomycin resistance genes for bacterial and eukaryotic cell selection. | Dr Michael Price (University of Birmingham, UK)                    |
| <b>pcDNA3.1 TTc-Fc</b>         | The pcDNA3.1 plasmid contains the TTc-Fc sequence.                                                                                                                                                                                                                           | Dr Fernanda Nicole Escobar-Riquelme (University of Birmingham, UK) |
| <b>pUCIDT CLEC14A-Fc</b>       | The commercial pUCIDT vector comprises codon-optimised sequences for mouse CLEC14A (extracellular domain) and human Fc, flanked by Apal and NheI restriction enzymes. Bacterial resistance: ampicillin.                                                                      | IDT                                                                |
| <b>pcDNA3.1 CLEC14A-Fc</b>     | The CLEC14A-Fc sequence extracted from the pUCIDT CLEC14A-Fc plasmid was introduced into the pcDNA3.1 backbone vector using restriction enzymes (NheI and Apal) for subcloning.                                                                                              | Constructed in house                                               |
| <b>pcDNA3.1 CLEC14A-TTc-Fc</b> | The CLEC14A sequence plus linker extracted from the pcDNA3.1 CLEC14A-Fc plasmid was inserted into the pcDNA3.1 TTc-Fc vector using PCR cloning and HIFI DNA assembly.                                                                                                        | Constructed in house                                               |

### 2.1.2 DNA/RNA handling and quantification

Recognising the sensitivity of DNA and RNA, the tests were conducted in RNase and DNase-free settings, using sterile filter tips. Nuclease-free water (Promega P1193) was used for DNA, and RNase-free water (Thermo Fisher A57775) for RNA. DNA samples were maintained at -20°C, and RNA samples were kept at -80°C. The NanoDrop 2000 spectrophotometer (Thermo Scientific) was used to conduct quantitative analysis at 260 nm.

### 2.1.3 Enzyme restriction assays

Restriction enzymes were used for DNA ligase and plasmid profiling. The High Fidelity (HF) Restriction Enzymes Apal-HF, NheI-HF, Scal-HF, and BamHI-HF (NEB, New England Biolabs) used in the studies are shown in Table 2-2. Following NEB's guidelines, a master mix was created by mixing the DNA sample, rCutSmart buffer, and restriction enzymes. One unit of enzyme was used to digest 500 ng or 1oneµg of DNA. The total volume was adjusted to 25 µL using nuclease-free water (Promega P1193). The reaction mixture was incubated at 37°C for 15 minutes. Finally, the reactions were stopped by heating the samples to 65°C or 80°C for 20 minutes. The digested DNA samples were stored at -20°C for further use.

*Table 2-2 Restriction enzymes are used for subcloning and digesting DNA.*

| Enzyme   | Catalog number | Digestion Buffer | Digestion Temperature | Inactivation Temperature | Company |
|----------|----------------|------------------|-----------------------|--------------------------|---------|
| Apal-HF  | R0114          | rCutSmart        | 25°C                  | 65°C                     | NEB     |
| NheI-HF  | R3131          | rCutSmart        | 37°C                  | 80°C                     | NEB     |
| Scal-HF  | R0157          | rCutSmart        | 37°C                  | 65°C                     | NEB     |
| BamHI-HF | R0136          | rCutSmart        | 37°C                  | -                        | NEB     |

#### **2.1.4 Agarose gel electrophoresis**

Electrophoresis DNA gels were prepared using 1% w/v agarose (Invitrogen) in Tris-borate-EDTA (TBE) buffer (Thermo Scientific 15581044). The solution was heated to dissolve the agarose, cooled to approximately 50°C, and mixed with SYBR Safe DNA Gel Stain (Thermo Scientific S33102). Gel Loading Dye, Purple (6x) (New England Biolabs 87024) was combined with the DNA samples in a 1:5 ratio. The Quick-Load Purple 1kb DNA Ladder (NEB N0552) was used to determine the size of the DNA. The gel was run at 100 Volts for 50-60 minutes and visualised using a UV transilluminator. The images were recorded using the GeneGenius Gel Imaging System (Syngene). Finally, the images were analysed using Image Acquisition software (Syngene).

#### **2.1.5 Bacteria culture and transformation**

Bacterial cultures were grown in 20 g/L Luria Broth (LB) medium (Sigma L3022) and 37 g/L LB agar (Sigma L2897), supplemented with 100 µg/mL Carbenicillin disodium salt (Thermo Scientific J61949-03). NEB 5-alpha competent E. coli (NEB C2987H) was employed to amplify the plasmids following the manufacturer's instructions. Competent bacteria were thawed on ice for 10 minutes. 1 pg to 100 ng of plasmid DNA was added, and the mixture was gently flicked. After incubation on ice for 30 minutes, the mixture was heat-shocked at 42°C for 30 seconds and then returned to ice for 5 minutes. To facilitate recovery, a super optimum medium with a catabolic repressor (SOC) medium (NEB B9020) was added, and the bacteria were incubated at 37°C for 1 hour with shaking at 300 rpm. The transformed bacteria were spread onto LB-agar plates and cultured overnight at 37°C for approximately 16 hours. The following day, a single

colony was selected and cultured in 3-5 mL of LB medium at 37°C with shaking at 200 rpm for 2-3 hours. Subsequently, 1-2.5 mL of this pre-culture was mixed with 5 or 25 mL of warm LB medium and cultured overnight at 200 rpm for 12-16 hours. The culture was used to purify plasmids and prepare bacterial stocks for long-term preservation.

For bacterial stocks, the LB overnight culture was mixed with sterile glycerol (Sigma G5516) to a final concentration of 25% v/v glycerol and then stored at -80°C. To prevent melting during recovery, the stocks were transported on dry ice. Under sterile conditions, a small quantity of the stock was scraped with a pipette tip, resuspended in a pre-warmed LB medium, and cultured overnight as previously described.

#### **2.1.6 Plasmid purification**

The QIAprep Spin Miniprep Kit (Qiagen 27104) or the Plasmid Plus Midi Kit (Qiagen 12943) was used for plasmid purification, following the manufacturer's guidelines. Initially, bacterial overnight cultures were centrifuged at over 6000 x g for 15 minutes at 4°C. All subsequent centrifugation procedures were performed at 13,000 RPM using an Eppendorf Centrifuge 5810R (Eppendorf UK Limited). The culture pellet was resuspended in Buffer P1 for plasmid DNA purification, followed by the addition of Buffer P2 for lysis. Subsequently, Buffer N3 was included in the mixture and centrifuged. The supernatant was then transferred to a QIAprep 2.0 spin column and centrifuged for 60 seconds. The column was washed with Buffer PE and centrifuged once more. Finally, Buffer EB or nuclease-free water (Promega P1193) was added to the membrane within the column. After centrifugation, the plasmid was eluted and stored at -20°C.

### **2.1.7 Gel extraction**

The DNA of interest was extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen 28704). Briefly, a transilluminator was employed to visualise and isolate the targeted DNA fragment. The gel slice was weighed and dissolved in Buffer QG for 10 minutes at 50°C to eliminate impurities such as proteins and salts. Subsequently, DNA was precipitated with isopropanol and transferred to a QIAquick spin column. Following centrifugation, the column was washed with Buffer PE. Finally, nuclease-free water (Promega P1193) or Buffer EB (10 mM Tris-Cl, pH 8.5) was utilised to elute the DNA from the column.

### **2.1.8 DNA ligation**

T4 DNA ligase 5 Weiss U/μL (Thermo Scientific EL0014) was employed following the manufacturer's guidelines for DNA ligation. The ligation reaction was prepared using T4 DNA Ligase Buffer, vector and insert DNA, T4 DNA Ligase, and nuclease-free water (Promega P1193). Various linear vector-to-insert DNA ratios (1:1, 1:3, 1:5) were assessed to optimise the ligation. The mixture was incubated at room temperature for 10 minutes before being heat-inactivated at 65°C for 10 minutes. Finally, the mixture was placed on ice for bacterial transformation.

### **2.1.9 Sanger sequencing**

Sanger sequencing at Source BioScience (Nottingham, UK) was employed to confirm the cloning. Primers were dispatched to the company at 3.2 pmol/μL, and plasmids were sent at a concentration of 100 ng/μL, adhering to the company's specifications. SnapGene Software v1.1.3 (GSL Biotech LLC) was employed to analyse the sequencing results in Gene Bank Sequence format.

## **2.2 Constructing the CLEC14A-TTc-Fc expression vector**

### **2.3 DNA assembly strategy**

Following the manufacturer's guidelines, the NEBuilder HiFi DNA Assembly Cloning Kit (NEB E5520S) was used for DNA assembly (CLEC14A and pcDNA3.1 TTc-Fc). First, the NEBuilder Assembly Tool version 2.8.2 (NEB; <https://nebuilder.neb.com/>) was employed to design 20-nucleotide overlapping primers for the upper and lower strands. To optimise amplification, primers of 15 and 18 nucleotides were tested (Table 2-3).

The High-Fidelity DNA Polymerase reagent (NEB M0491S) and dNTPs mix (Invitrogen 10297018) were used for PCR amplification. The manufacturer's protocol was followed to prepare a 25 μL reaction mixture containing 5 μL of 5X Q5 Reaction Buffer, 0.5 μL of 10 mM dNTPs, 1.25 μL of 10 μM forward and reverse primers, 0.25 μL of Q5 High-Fidelity DNA Polymerase, 5 μL of 5X Q5 High GC Enhancer, and 10 ng of DNA. The volume was topped up to 25 μL with nuclease-free water (Promega P1193). The samples were transferred to a Bio-Rad C1000

thermocycler and subjected to thermocycling (The thermocycling conditions described in Table 2-4). After gel electrophoresis, the PCR products were extracted and quantified.

The total volume was adjusted to 20  $\mu$ L using nuclease-free water (Promega; P1193). The assembly reaction mixture was created with a 1:2 vector-to-insert ratio, including an additional 10  $\mu$ L of NEBuilder HiFi DNA Assembly Master Mix. The linear vector was typically used at a 0.03-0.2 pmol concentration. Various controls were implemented, including negative controls with either only insert or only vector, while positive controls were sourced from the kit. The reaction mixtures were incubated at 50°C for 15 minutes. Subsequently, the previously described method transformed 2  $\mu$ L of the assembled products or controls into NEB 5-alpha competent E. coli cells. Finally, individual colonies were assessed for cloning confirmation via Sanger sequencing.

*Table 2-3 Oligonucleotides are used in DNA assembly. Lowercase letters are employed to indicate overhangs. The nucleotides included in the overhangs were excluded from the calculation of melting  $T_m$  at <https://tmcalculator.neb.com>.*

|          | Primer     | Sequence (5' to 3')                   | $T_m$ | $T_a$ | Product |
|----------|------------|---------------------------------------|-------|-------|---------|
| <b>1</b> | pcTTcFc.F3 | ctctggcgggaGGATGGTCTTGTATTATCTTC      | 56    | 57    | 7495bp  |
|          | pcTTcFc.R3 | cgggccggccCATGGTGGCAAGCTTG            | 60    |       |         |
|          | CLEC14A.F5 | tgccaccatgGGCCGGCCCGCCTTG             | 73    | 72    | 1434bp  |
|          | CLEC14A.R5 | aagaccatccTCCGCCAGAGCCGCC             | 71    |       |         |
| <b>2</b> | pcTTcFc.F4 | ctctggcgggaGGATGGTCTTGTATTATCTTCTTCTG | 62    | 63    | 7495bp  |
|          | pcTTcFc.R4 | cgggccggccCATGGTGGCAAGCTTGCTAG        | 65    |       |         |
|          | CLEC14A.F6 | tgccaccatgGGCCGGCCCGCCTTGGCC          | 81    | 72    | 1434bp  |
|          | CLEC14A.R6 | aagaccatccTCCGCCAGAGCCGCCGCC          | 79    |       |         |

*Table 2-4 Thermocycling conditions for PCR using Q5 high-fidelity DNA polymerase. The annealing temperature varied across reactions based on the melting temperature of the primers.*

| Step                 | Temperature | Time                                                    |
|----------------------|-------------|---------------------------------------------------------|
| Initial denaturation | 98°C        | 30 s                                                    |
| 30-34 cycles         | 98°C        | 10 s                                                    |
|                      | 50-72°C*    | 30 s                                                    |
|                      | 72°C        | 3 mins                                                  |
| Final Extension      | 72°C        | 20-30 s per kb<br>40-50 s per kb<br>(for long products) |
| Hold                 | 4-10°C      | ∞                                                       |

## 2.4 Cell culture conditions and procedures

HEK293 cells (ATCC CRL-1573) were used to produce recombinant proteins. The cells were cultured in Complete Minimum Essential Medium (MEM) supplemented with sodium bicarbonate and Earle's salts (Sigma M2279). The complete medium was prepared by adding 10% (v/v) heat-inactivated foetal bovine serum (FBS-HI) (Sigma F9665), 1 mM sodium pyruvate (Sigma-Aldrich S8636-100mL), 2 mM L-glutamine (Gibco 25030081), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco 15140-122). Stable cell lines were selected by incorporating 500 µg/mL of G418 disulfate salt (Sigma-Aldrich A1720-5G) into the complete MEM medium, which was subsequently maintained with 300 µg/mL G418 disulfate salt.

The oestrogen receptor  $\alpha$  negative (ER $\alpha$ -) model for C57BL/6 mice, the EO771 cell line (ATCC CRL-3461), was maintained in complete Dulbecco's Modified Eagle

Medium (DMEM ATCC 30-2002), supplemented with 20 mM HEPES (Thermo Fisher Scientific 15630-080) and 10% foetal bovine serum (FBS) (Gibco 10270106).

The 4T1 cell line (CLS 300300, Cytion, Germany) is a triple-negative breast cancer (TNBC) model for BALB/c mice. It was grown in RPMI 1640 basal media (Gibco 21875034) supplemented with 10% FBS (Gibco 10270106).

All cell culture media were filtered using a 0.22 µm vacuum filtration system (Corning BC503 or Millipore S2HVU05RE) to ensure a sterile environment. They were stored at 4°C for no more than a month. Subsequently, the cells were cultured at 37°C with 5% CO<sub>2</sub> in a humidified incubator (Thermo Scientific BB15). All cell culture activities were conducted using a strict aseptic technique.

To achieve optimal cell handling, we strictly followed the supplier's guidelines. Each cryopreserved vial was stored in vaporised liquid nitrogen. Throughout the thawing process, we maintained strict aseptic conditions. The vial was quickly thawed for 1-2 minutes in a 37°C water bath. Next, the thawed cell suspension was placed into 9 mL of pre-warmed complete medium. After centrifugation, the pellet was resuspended in 1 mL of complete medium. We then added 1 mL of the cell suspension to a pre-incubated flask, which was cultured at 37°C in a humidified 5% CO<sub>2</sub> incubator (Thermo Scientific BB15).

Once the cells reached 70–90% confluency, they were sub-cultured twice to thrice weekly. The sub-culturing process began with discarding the spent medium and gently rinsing the cells with warm PBS (Sigma D8537) to remove residual serum. 3 mL of 0.05% Trypsin-EDTA 1X (Gibco 25300-054) was added to detach the cells from the flask. After 5–10 minutes incubating at 37°C, the trypsin activity was neutralised with a

complete medium. Subsequently, the cells were centrifuged and resuspended in the desired volume. Cell counts were conducted using a disposable cell counting slide (Biosigma BVS100).

For long-term preservation, the cells were combined with 10% DMSO (Sigma-Aldrich D2650) and stored in Cryovial tubes (Greiner Bio-One 123277). A CoolCell container (Corning 432004) facilitated controlled freezing overnight at  $-1^{\circ}\text{C}$  per minute, using a freezer set to  $-80^{\circ}\text{C}$ . Subsequently, the cryovials were transferred to liquid nitrogen storage.

## **2.5 Protein expression, detection, and purification**

### **2.5.1 Transient transfection of HEK293 cells**

HEK293 cells were transiently transfected using Lipofectamine 3000 (Invitrogen L3000-008) for analysing protein expression. The day before transfection,  $6-8 \times 10^5$  cells were seeded in each well of a 6-well plate with complete Minimum Essential Medium (MEM) supplemented with Earle's salts and sodium bicarbonate (Sigma M2279). The following day, the culture medium was replaced with Opti-MEM reduced serum medium (Gibco 31985047) and incubated for one hour at  $37^{\circ}\text{C}$ .

For the Lipofectamine transfection, the manufacturer's instructions were followed. The first tube was prepared with 5  $\mu\text{L}$  of Lipofectamine 3000 (Invitrogen L3000-008) in 250  $\mu\text{L}$  of Opti-MEM, while the second tube contained 2.5  $\mu\text{g}$  of plasmid DNA in 250  $\mu\text{L}$  of Opti-MEM. The tubes were subsequently mixed with a pipette and incubated at room

temperature for 15 minutes. Finally, the mixture was added to the HEK293 cells, and the plate was gently spun to ensure even distribution.

Some cells were transfected with the pcDNA3.1 mGFP plasmid as a positive control for transfection efficiency. GFP expression in transfected cells was visualised at 24, 48, and 72 hours post-transfection using an EVOS FL Imaging System (Thermo Fisher WYG88).

To confirm the production of CLEC14A-Fc and CLEC14A-TTc-Fc proteins, the culture medium was collected and centrifuged 24, 48, and 72 hours post-transfection. The supernatants were subsequently stored at -20°C for future analysis.

### **2.5.2 Deoxycholate (DOC)-trichloroacetic acid (TCA) protein precipitation**

The deoxycholate (DOC)-trichloroacetic acid (TCA) technique is a method for protein precipitation suitable for low-abundance proteins (1 µg/ml). Initially, 100 µL of a 0.15% DOC solution (SIGMA-ALDRICH D6750-10G) was added to 1 mL of the protein sample and mixed thoroughly using a vortex. Subsequently, 100 µL of 100% TCA (SIGMA-ALDRICH T6399-500G) was introduced and vortexed. The sample was then placed on ice for 30 minutes to facilitate protein precipitation. Following this, centrifugation was performed at 10,000 x g for 5-15 minutes at 4°C, after which the supernatant was discarded. The pellet was washed with cold acetone (typically 500 µL for each wash) to remove residual TCA before being centrifuged again. Lastly, the pellet was resuspended in 20-30 µL of double distilled water and stored at -20°C for further analysis.

### 2.5.3 Structure prediction of the recombinant proteins

The predicted three-dimensional (3D) structures of the extracellular domains of mouse CLEC14A and ROBO4, as well as the CLEC14A-TTc-Fc and ROBO4-TTc-Fc fusion proteins, were generated using the AlphaFold2-based prediction tool available at AlphaFoldServer.com. Amino acid sequences for each construct, including the extracellular domains of CLEC14A and ROBO4, the full-length CLEC14A-TTc and ROBO4-TTc fusions, and the Fc-tagged versions, were individually submitted in FASTA format. The resulting models yielded a detailed visualisation of domain folding and the spatial arrangement of linker and fusion regions. Web links to the predicted structures are listed in the Table 2-5.

*Table 2-5 Web links to the predicted structures of the recombinant proteins*

| <b>Recombinant protein</b> | <b>Web link</b>                                                                                                   |
|----------------------------|-------------------------------------------------------------------------------------------------------------------|
| <b>CLEC14A</b>             | <a href="https://alphafoldserver.com/fold/883eb8b21f60ac3">https://alphafoldserver.com/fold/883eb8b21f60ac3</a>   |
| <b>CLEC14A-TTc-Fc</b>      | <a href="https://alphafoldserver.com/fold/2c0e6966d76ffdc8">https://alphafoldserver.com/fold/2c0e6966d76ffdc8</a> |
| <b>ROBO4</b>               | <a href="https://alphafoldserver.com/fold/5bed012462229249">https://alphafoldserver.com/fold/5bed012462229249</a> |
| <b>ROBO4-TTc-Fc</b>        | <a href="https://alphafoldserver.com/fold/634cb7438f61b2a3">https://alphafoldserver.com/fold/634cb7438f61b2a3</a> |
| <b>TTc</b>                 | <a href="https://alphafoldserver.com/fold/22fe1e5abd8a9d03">https://alphafoldserver.com/fold/22fe1e5abd8a9d03</a> |
| <b>Fc</b>                  | <a href="https://alphafoldserver.com/fold/3d1b83144154ad07">https://alphafoldserver.com/fold/3d1b83144154ad07</a> |

#### **2.5.4 SDS-PAGE (denaturing conditions)**

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method for separating proteins by molecular size. A cocktail was prepared using a protein sample, 25% NuPAGE® LDS sample buffer (Invitrogen NP0007), and 10% NuPAGE® sample reducing agent (Invitrogen NP0009), topped up with distilled water to reach a total volume. The cocktail was then incubated at 70°C for 10 minutes.

Pre-cast NuPAGE® 4-12% gradient bis-tris gels (Invitrogen NP0321 or NP0335) were used for SDS-PAGE. A prime-step pre-stained broad protein ladder (BioLegend 773301), ranging from 6.5-270 kDa, was loaded into the first well to provide a protein size reference. The protein samples were subsequently loaded into the remaining wells.

The gel was run at 200 volts for 35-45 minutes under reducing conditions. The running buffer was prepared with 5% NuPAGE® MOPS SOS running buffer (20X) (Novex NP0001), mixed with 95% distilled water and 0.275% NuPAGE® Antioxidant (Invitrogen NP0005). This combination creates a reducing environment in the gel, facilitating protein separation based on molecular size.

#### **2.5.5 Coomassie blue staining**

SimplyBlue SafeStain (Invitrogen LC6060) visualises protein bands on polyacrylamide gels. Initially, the gel was washed three times with distilled water, followed by dyeing it with SimplyBlue for one hour and subjecting it to another wash. To enhance image clarity, the gel was incubated overnight at 4°C in distilled water. Finally, the gel was imaged using a ChemiDoc imaging system (Bio-Rad).

### 2.5.6 Western blot

A Western blot device (LiCor 929-97305) transferred proteins from a polyacrylamide gel to an Immobilon-P PVDF Membrane (Merck IPVH08100). The PVDF membrane was initially activated by soaking it in 50% methanol for 10 to 20 seconds. The excess alcohol was removed by transferring the membrane to distilled water (dH<sub>2</sub>O) for 10 to 20 seconds. A 1X transfer buffer was prepared using 95% dH<sub>2</sub>O and 5% NuPAGE Transfer Buffer 20X (Novex NP0006). The membrane was then allowed to equilibrate for 15 to 20 minutes in the 1X transfer buffer. Two pre-wetted sponges (Invitrogen EI9052), filter paper (Merck IBFP0785C), the gel, the activated membrane, additional filter paper, and three more pre-wetted sponges were layered to create the transfer sandwich. This sandwich was positioned into the XCell SureLock Mini-Cell gel tank (Invitrogen EI0001), and the entire module was securely fastened. The 1X transfer buffer was poured into the inner chamber of the blot module until the blotting sandwich was completely submerged. Half of the tank's outer chamber was filled with distilled water. Finally, the transfer was carried out for 2 hours at 30 volts.

Before immunoblotting, the membrane was blocked with 5% w/v dried skim milk (Marvel 806051) in Tris-buffered saline (TBS) for one hour at room temperature on a shaking platform. The TBS was prepared using 150 mM Tris-HCl (Sigma T3253), 45 mM Tris-base (Sigma T1503), and 150 mM NaCl (Melford Biolaboratories S0520). Subsequently, the membrane was rinsed three times with the washing buffer (0.1% Tween-20 (Sigma P1379) in PBS) for five to ten minutes at room temperature. The primary antibody was then incubated with the membrane at 4°C overnight on a shaking platform (Table 2-6). The following day, the membrane was rinsed three times with the washing buffer for five to ten minutes at room temperature. Afterwards, the membrane

was incubated with the secondary antibody for one hour at room temperature on a shaking platform (Table 2-6). Finally, the membrane was rinsed three times with the washing buffer for five to ten minutes. The gel was imaged using the ChemiDoc imaging system (Bio-Rad). For analysis, Bio-Rad's Image Lab Standard Edition v6.0.1 software was employed. Commercially available his-tagged mouse CLEC14A protein (SinoBiological 50304-T16) and mouse ROBO4 (SinoBiological 51081-T16) were positive controls.

*Table 2-6 Primary and secondary antibodies were used for Western blot analysis.*

| Antibody                                  | Catalogue | Dilution Factor | Supplier       |
|-------------------------------------------|-----------|-----------------|----------------|
| Anti-CLEC14A Antibody, Rabbit Polyclonal  | 50304-T16 | 5000            | SinoBiological |
| Anti-ROBO4 Antibody, Rabbit Polyclonal    | 51081-T16 | 5000            | SinoBiological |
| Monoclonal Anti-Human IgG (Fc specific)   | I6260-2ML | 10000           | Sigma-Aldrich  |
| Mouse Monoclonal Anti-Tetanus Toxin       | ab278064  | 500             | Abcam          |
| Donkey Anti-Rabbit IgG H&L (IRDye® 680RD) | ab216779  | 15000           | Abcam          |
| Goat anti-Mouse IgG H&L (IRDye® 800CW)    | ab216772  | 15000           | Abcam          |

### **2.5.7 Stable transfection of HEK293 cells**

HEK293 cells were stably transfected to enhance the production of CLEC14A-Fc and CLEC14A-TTc-Fc proteins. First, 100 µg of plasmid was linearised for 2.5 hours at 37°C using Scal-HF (NEB R3122) restriction enzyme, and then the reaction was heat-inactivated for 20 minutes at 80°C. The plasmid was precipitated by adding 2.5 volumes of isopropanol (Fisher Chemical, 10284250) and 10% of the final volume of sodium acetate buffer solution (Sigma-Aldrich S7899). After centrifugation at 13,000 RPM for 5 minutes, the supernatant was discarded. The pellet was washed with 70%

ethanol and centrifuged again. After discarding the supernatant, the pellet was air-dried for 10-15 minutes under sterile conditions. Finally, the pellet was resuspended in 100  $\mu$ L of PBS (Sigma D8537). After protein quantification using BCA or Nanodrop, the samples were stored at -20°C.

The pcDNA3.1 CLEC14A-Fc/CLEC14A-TTc-Fc plasmids were linearised and sterilised before being transfected into HEK293 cells. The G418 resistance cassette was included in the pcDNA3.1 expression plasmid. To select the transfected cells, the medium was changed two days post-transfection, and the cells were treated with 500  $\mu$ g/mL of the antibiotic G418 disulphate salt (Sigma-Aldrich A1720-5G). The cells were cultured until they reached 70–80% confluence. The stable transfected cells were stored in cryovials in liquid nitrogen.

ROBO4-TTc-Fc and TTc-Fc stably transfected cells were kindly supplied by Dr Fernanda Nicole Escobar-Riquelme at the University of Birmingham (Escobar-Riquelme, 2024).

#### **2.5.8 Protein production and purification**

For scaled-up protein production,  $8\text{--}10 \times 10^6$  HEK293 stably transfected cells were seeded into 150cm<sup>2</sup> TC Flasks (Corning BC302) in a complete MEM medium containing 300  $\mu$ g/mL G418 disulfate salt (Sigma-Aldrich A1720-5G) and incubated in a humidified incubator with 5% CO<sub>2</sub> at 37°C. Two days later, 4-5 mL of fresh complete medium was added to each flask. 2 days later, the mediums were collected, centrifuged, filtered and stored at -20°C.

The Fc-tagged proteins were purified using a HiTrap Protein A HP column (GE Healthcare Cytiva 17040203). This column uses the specific interaction between Protein A and the Fc region of antibodies/protein. The bound proteins were eluted with 0.1 M citric acid (Melford C3450) at pH 2.6-2.8, which disrupts the Protein A-Fc binding. The eluted protein was dialysed in PBS using a Slide-A-Lyzer dialysis cassette with a 10K MW cut-off (Thermo Scientific 66455) at 4°C overnight. This step removes the salts and small contaminants from the proteins. The dialysed protein was filtered and stored at 4°C. Finally, the purified protein was quantified using a NanoDrop spectrophotometer (Thermo Scientific NanoDrop 2000), and SDS-PAGE and Western blot confirmed the purity of the protein.

## **2.6 Animal work**

### **2.6.1 Mice**

Female C57BL6 and BALB/c mice, aged 8 to 10 weeks, were acquired from Charles River Laboratories. The animals were housed in specific pathogen-free conditions at the Biomedical Services Unit (BMSU) at the University of Birmingham. All procedures have been approved by the University of Birmingham Ethics Committee AWERB (Animal Welfare Ethical Review Board) and are conducted under the UK Home Office Project licence: PP3338444 and PP8702596.

### **2.6.2 Immunisation**

The antigens TTc, CLEC14A-TTc-Fc, and ROBO4-TTc-Fc proteins were precipitated using aluminium potassium sulphate (alum). Initially, the antigen was mixed in a 1:1 ratio with a 9% w/v solution of aluminium potassium sulphate (Fisher Chemical 7784-24-9). Subsequently, the pH was adjusted to 6.5-7 using 10 M sodium hydroxide (Sigma S5881-1KG). The mixture was incubated for 1 hour at room temperature on a rotator. The antigen was washed twice with PBS (Sigma D8537). Finally, the pellet was resuspended in PBS at 40-50 µg per 200 µL for intraperitoneal (i.p.) injection per mouse.

Mice were initially primed with TTc protein in alum to elicit a TTc-specific CD4 memory response. After four weeks, the mice were administered ROBO4-TTc or CLEC14A-TTc in alum. Two weeks later, the mice received a boost with a second ROBO4-TTc-Fc or CLEC14A-TTc-Fc in alum. This priming-boost injection protocol was optimised beforehand (Escobar-Riquelme, 2024).

### **2.6.3 Triple-negative breast cancer (TNBC) mice models**

Tumour cell transplantation was conducted by the technicians at the Biomedical Services Unit (BMSU) of the University of Birmingham. One day after the second boost, tumour cells were implanted into the fourth mammary fat pad of the mice. The number of transplanted tumour cells varied by animal model:  $2 \times 10^4$  4T1 tumour cells for BALB/c mice and  $1 \times 10^5$  EO771 tumour cells for C57BL/6 mice. Following the implantation, the mice were monitored daily and weighed bi-weekly. Tumour measurements using callipers were recorded every 3-4 days. The equation for calculating tumour volume

was: tumour volume (mm<sup>3</sup>) = (height x width<sup>2</sup>)/2. (Elizondo et al., 2020; Yoshimura et al., 2001). Three to four weeks after implantation, the mice were culled through an overdose of anaesthetic before cardiac bleeding and tissue were harvested.

## **2.7 Flow cytometry**

### **2.7.1 Tissue processing**

R10 medium was created by combining RPMI 1640 basal medium (Gibco 21875034) with 10% FBS (Gibco 10270106). A spleen half was meticulously mashed and filtered through a 70 µm cell strainer (Falcon 352350) to obtain a single cell suspension using R10 medium. The cells were then centrifuged into a 15-50 mL centrifuge tube (Corning 430791). After removing the supernatant, the pellet was resuspended in 1 mL of ACK Lysing Buffer (Gibco A10492-01) to lyse blood cells and incubated for 4 minutes on ice. To halt the reaction, 5 mL of R10 medium was added, followed by centrifugation. Lastly, the pellet was resuspended in 1-2 mL of fresh R10 medium on ice until needed.

For lymph node cell preparation, the entire lymph node was crushed using needles and filtered through a 70 µm cell strainer (Falcon 352350) with R10 medium. The resulting cell pellet was resuspended in R10 medium.

The tumour was cleaned and weighed, then chopped into small pieces with scissors. These pieces were placed in 1 mL of digestion buffer containing RPMI1640, 1% Collagenase D at 1 mg/mL (Roche 11088866001), and 0.1% Deoxyribonuclease I (DNase I, Sigma DN25). The tube was set in a shaking heat block at 35°C and 700 rpm for 20 minutes. After digestion, the mixture was filtered into a 15-50 mL Falcon tube. Following the addition of the R10 medium, the mixture was centrifuged, the

supernatant was discarded, and the pellet was resuspended in the required volume of the R10 medium.

### **2.7.2 Surface and intracellular cell staining**

Cell staining followed the protocol by Brandi et al. (2023). Centrifugation was performed at 400 x g for 5 minutes at 4°C. A 96-well round-bottom plate (Corning 3799) was seeded with  $1-1.5 \times 10^6$  cells per well. After centrifugation, the supernatant was discarded.

The cells were then resuspended in 50 µL of CD16/CD32 monoclonal antibody (1:200 diluted in PBS; Invitrogen 14-0161-82) for blocking and incubated for 15 minutes at 4°C. Each well received a wash of 200 µL of PBS (Sigma D8537), followed by centrifugation and supernatant disposal.

Each well was then resuspended in 50 µL of viability dye containing CD8α-Biotin diluted in PBS for both biotin and viability staining. After a 15-minute incubation at 4°C, a wash with 200 µL of PBS containing 2% heat-inactivated foetal bovine serum (FBS-HI; Sigma F9665) was performed, followed by centrifugation and removing the supernatant. Then, 50 µL of Streptavidin-AF350 in PBS with 2% FBS-HI was added to each well, which was incubated for 15 minutes at 4°C and then washed again.

Next, the cells were incubated for 20 minutes at 4°C with 50 µL of surface antibody master mix in Brilliant Stain Buffer (BD Biosciences 563794) to stain surface markers, followed by two washes with PBS/2% FBS-HI, centrifugation, and supernatant disposal. Permeabilisation and fixation were performed using the Foxp3/Transcription

factor staining buffer set (eBioscience 00-5523-00). After adding 70  $\mu$ L of Fix/Perm Buffer to each well, the cells were incubated at room temperature in the dark for 20 minutes. They were washed with 200  $\mu$ L of Perm/Wash buffer, centrifuged, and the supernatant removed. Each well received 50  $\mu$ L of intracellular antibody master mix in Perm/Wash buffer at 4°C for 20 minutes (Table 2-7). After two washes with Perm/Wash buffer, centrifugation, and supernatant disposal, the cells were resuspended in 100–200  $\mu$ L of PBS/2% FBS-HI in each well and kept at 4°C until acquisition.

Acquisitions were performed using a Sony ID7000 spectral cytometer. UltraComp eBeads™ compensation beads (Invitrogen 01-2222-42) were used for unmixing with single-colour controls. Data analysis was carried out using FlowJo v10.10.0 software, while statistical testing and data visualisation were performed with GraphPad Prism version 10.0.0.

**Table 2-7 Spectral cytometry antibodies.**

| Target             | Fluorochrome   | Supplier       | Catalogue   | Clone       | Dilution Factor | Staining condition |
|--------------------|----------------|----------------|-------------|-------------|-----------------|--------------------|
| BTLA (CD272)       | PE-Fire700     | BioLegend      | 139127      | 6A6         | 100             | Extracellular      |
| CD11b              | SparkYG593     | BioLegend      | 101281      | M1/70       | 400             | Extracellular      |
| CD11c              | SparkBlue550   | BioLegend      | 117365      | N418        | 200             | Extracellular      |
| CD127              | PE-Cy5         | ThermoFisher   | 15-1271-83  | A7R34       | 100             | Extracellular      |
| CD160              | PE             | BioLegend      | 143003      | 7H1         | 50              | Extracellular      |
| CD169              | AF647          | BioLegend      | 141720      | C068C2      | 400             | Extracellular      |
| CD19               | SparkNIR685    | BioLegend      | 115567      | 6D5         | 200             | Extracellular      |
| CD206              | PE-Cy7         | BioLegend      | 141719 25   | C068C2      | 200             | Extracellular      |
| CD25               | BV480          | BD Biosciences | 566202      | PC61        | 400             | Extracellular      |
| CD27               | BUV496         | BD Biosciences | 741094      | LG.3A10     | 100             | Extracellular      |
| CD3                | FITC           | BioLegend      | 100203      | 17A2        | 400             | Intracellular      |
| CD39               | BUV395         | BD Biosciences | 567264      | Y23-1185    | 100             | Extracellular      |
| CD4                | SparkBlue574   | BioLegend      | 100489      | GK1.5       | 400             | Extracellular      |
| CD44               | AF700          | BioLegend      | 103026      | IM7         | 200             | Extracellular      |
| CD45               | SparkViolet538 | BioLegend      | 103179      | 30-F11      | 100             | Extracellular      |
| CD62L              | BV570          | BioLegend      | 104433      | MEL-14      | 100             | Extracellular      |
| CD69               | BUV615         | BD Biosciences | 751593      | H1.2F3      | 100             | Extracellular      |
| CD8 $\alpha$       | Biotin         | BioLeg.        | 100703      | 53          | 200             | Extracellular      |
| CTLA-4 (CD152)     | BV421          | BioLegend      | 106312      | UC10-4B9    | 50              | Intracellular      |
| CXCR3              | BV650          | BioLegend      | 126531      | CXCR-3173   | 50              | Extracellular      |
| F4/80              | PE-Fire640     | BioLegend      | 157319      | QA17A29     | 25              | Extracellular      |
| FoxP3              | Vio R667       | Miltenyi       | 130-111-682 | REA788      | 50              | Intracellular      |
| Granzyme B         | Pacific Blue   | BioLegend      | 515408      | GB11        | 100             | Intracellular      |
| ICOS-L (CD275)     | VioBright B515 | Miltenyi       | 130-116-450 | REA990      | 50              | Extracellular      |
| Ki67               | BUV805         | ThermoFisher   | 368-5698-82 | SolA15      | 100             | Intracellular      |
| KLRG-1             | APC-eF780      | ThermoFisher   | 47-5893-82  | 2F1         | 200             | Extracellular      |
| LAG-3 (CD223)      | BUV737         | BD Biosciences | 741820      | C9B7W       | 25              | Extracellular      |
| Ly6C               | BV785          | BioLegend      | 128041      | HK1.4       | 200             | Extracellular      |
| Ly6G               | PerCP          | BioLegend      | 127653      | 1A8         | 25              | Extracellular      |
| MHC II             | PE-Fire810     | BioLegend      | 107667      | M5/114.15.2 | 400             | Extracellular      |
| NK1.1              | APC/Fire810    | BioLegend      | 156519      | S17016D     | 200             | Extracellular      |
| PD-1 (CD279)       | BV605          | BioLegend      | 135220      | 29F.1A12    | 100             | Extracellular      |
| PD-L1 (CD274)      | PerCP-eF710    | ThermoFisher   | 46-5982-80  | MIH5        | 100             | Extracellular      |
| Perforin           | APC            | BioLegend      | 154304      | S16009A     | 100             | Intracellular      |
| PVR (CD155)        | BUV661         | BD Biosciences | 749991      | TX56        | 100             | Extracellular      |
| Streptavidin       | AF350          | Thermo         | S11249      | 6.7         | 200             | Extracellular      |
| TCR $\gamma\delta$ | BUV563         | BD Biosciences | 748993      | GL3         | 100             | Extracellular      |
| TIGIT              | PE-Dazzle594   | BioLegend      | 142110      | 1G9         | 25              | Extracellular      |
| TIM-3 (CD366)      | BV711          | BioLegend      | 119727      | RMT3-23     | 100             | Extracellular      |
| Viability          | Zombie NIR     | BioLegend      | 423106      | N/A         | 1600            | Intracellular      |
| Viability          | eFlour 780     | BioLegend      | 65-0865-14  | N/A         | 1000            | Intracellular      |
| XCR1               | BV510          | BioLegend      | 148218      | ZET         | 50              | Extracellular      |

## **2.8 Enzyme-linked immunosorbent assay (ELISA)**

For blood processing, samples were collected and incubated at 37°C for 1 hour. Following centrifugation at 10000 RPM for 8-10 minutes, sera were collected and stored at -20°C for later analysis.

ELISA plates (Thermo Fisher 442404) were coated with 100 µL of coating buffer containing 1 µg/mL of either His-tagged ROBO4 or CLEC14A protein (SinoBiological 51081-MOBH / 50304-T16) and incubated at 4°C overnight. To prepare the coating buffer, 1.95 grams of sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>, BDH 4808670H) and 2.9 grams of sodium bicarbonate (NaHCO<sub>3</sub>, Sigma S5761) were dissolved in 1L of distilled water (dH<sub>2</sub>O).

The following day, plates were washed three times with 200 µL of washing buffer containing 0.05% Tween 20 (Sigma P1379) in PBS tablets (Gibco 18912-014) dissolved in dH<sub>2</sub>O. To prevent nonspecific binding, the wells were incubated with 200 µL of blocking buffer containing 1% w/v bovine serum albumin (BSA) (Sigma A7906) in PBS for 1–2 hours at room temperature. After discarding the supernatant, 150 µL of diluted mouse serum samples were added to each well. The plates were then incubated for 1 hour at 37°C. The serial serum dilutions were prepared in a blocking buffer starting at 1:50, followed by triple dilutions. After three washes, 100 µL per well of AP-conjugated goat anti-mouse IgG, IgG1 or IgM antibodies were mixed with blocking buffer and incubated for 1 hour at 37°C. After three washes, 100 µL of p-nitrophenyl phosphate (Sigma N2770) dissolved in dH<sub>2</sub>O was added, and the plates were incubated for a further hour at 37°C. Absorbance was measured at 405 nm on a

SpectraMax ABS Plus plate reader for 30 minutes to 2 hours. For antibodies used in this thesis, see Table 2-8.

ELISA results were analysed by plotting absorbances on GraphPad Prism software (version 10.1.0) and fitting them to a dilution curve on the X-axis. Relative titres were determined as the dilution at which the curve intersected a defined threshold. If the curves were not above the threshold, titres were calculated using extrapolation and labelled as below the detection level. Relative titres were represented on a logarithmic scale, and statistical analyses were performed using GraphPad Prism. An unpaired t-test or Mann-Whitney test was employed to compare the two groups. Comparisons involving more than two groups were assessed using two-way ANOVA with mixed-effects analysis, followed by Sidak's multiple comparisons test. Statistical significance was determined as follows: not significant (ns)  $p > 0.05$ ; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ; \*\*\*\* $p \leq 0.0001$ .

*Table 2-8 Antibodies were used for ELISA.*

| <b>Antibody</b>                     | <b>Dilution</b> | <b>Supplier</b> | <b>Catalogue</b> |
|-------------------------------------|-----------------|-----------------|------------------|
| Goat Anti-Mouse IgG, Human ads-AP   | 1/1000          | SouthernBiotech | 1030-04          |
| Goat Anti-Mouse IgG1, Human ads-AP  | 1/1000          | SouthernBiotech | 1070-04          |
| Goat Anti-Mouse IgG2a, Human ads-AP | 1/1000          | SouthernBiotech | 1080-04          |
| Goat Anti-Mouse IgG2b, Human ads-AP | 1/1000          | SouthernBiotech | 1090-04          |
| Goat Anti-Mouse IgM, Human ads-AP   | 1/2000          | SouthernBiotech | 1020-04          |

## **2.9 Gene expression by Real-time quantitative PCR (RT-qPCR)**

### **2.9.1 RNA extraction**

RNA extraction was performed using frozen cells (approximately  $1-1.5 \times 10^6$ ) and tissue sections (6-7  $\mu\text{m}$  thick) with the RNeasy Mini Kit (Qiagen 74104), following the manufacturer's recommendations. All centrifugation steps were conducted at 10,000 RPM at 20–25°C. The frozen tissue/cells were initially lysed in 350  $\mu\text{L}$  of Buffer RLT containing 1% 2-Mercaptoethanol ( $\beta$ -ME). The lysate was then pipetted into a QIAshredder spin column (Qiagen 79654) positioned in a 2 mL collection tube and centrifuged for 2 minutes. Next, pipetting added 350  $\mu\text{L}$  of 70% ethanol to the cleared lysate. Up to 700  $\mu\text{L}$  of the sample was transferred to an RNeasy spin column in a 2 mL collection tube and centrifuged for 15 seconds. Afterwards, the column was washed with 350  $\mu\text{L}$  of Buffer RW1 and centrifuged for 15 seconds. The RNase-Free DNase set (Qiagen 79254) was utilised for the DNase digestion. A DNase I mix (10  $\mu\text{L}$  DNase I stock solution mixed with 70  $\mu\text{L}$  Buffer RDD) was added to the membrane and incubated for 15 minutes at room temperature. Subsequently, the column was washed with 350  $\mu\text{L}$  of Buffer RW1 and centrifuged again for 15 seconds. The column was rinsed twice with 500  $\mu\text{L}$  of Buffer RPE and centrifuged for 15 seconds. The column was placed in a new 2 mL collection tube to eliminate residual liquids and centrifuged for 1 minute. Finally, the column was transferred to a new 1.5 mL centrifuge tube, and 30–50  $\mu\text{L}$  of RNase-free water was added to the membrane. After centrifugation, the eluted RNA was either immediately used for reverse transcription into cDNA or stored at -80°C.

### 2.9.2 Reverse transcription

The High-Capacity cDNA reverse transcription kit (Applied Biosystems 4368814) was used to reverse-transcribe RNA samples into cDNA. A 10  $\mu$ L RNA sample was mixed with 10  $\mu$ L of reverse transcription reaction mix (see Table 2-9), and thermocycling was performed under the conditions outlined in Table 2-10.

*Table 2-9 Reverse transcription reaction mix components.*

| Component                         | Volume ( $\mu$ l) |
|-----------------------------------|-------------------|
| 10x RT Buffer                     | 2                 |
| 25X dNTP mix (100 uM)             | 0.8               |
| 10x RT random primers             | 2                 |
| MultiScribe Reverse transcriptase | 1                 |
| RNase inhibitor                   | 0                 |
| Nuclease free water               | 4.2               |
| Total                             | 10                |

*Table 2-10 Thermocycling conditions for reverse transcription.*

| Step                  | Temperature ( $^{\circ}$ C) | Time (minutes) |
|-----------------------|-----------------------------|----------------|
| Primer Incubation     | 25                          | 10             |
| Reverse Transcription | 37                          | 120            |
| Enzyme Inactivation   | 85                          | 5              |
| Infinite hold         | 4                           | $\infty$       |

### 2.9.3 Real-time quantitative PCR (RT-qPCR)

TaqMan assays and cDNA templates were defrosted on ice. A 1:10 dilution of TaqMan Assays was made for the reaction mix in TaqMan Universal Master Mix II, No. UNG (Applied Biosystems 444040), as shown in the Table 2-11. 5  $\mu$ L of the reaction mixture and 1  $\mu$ L of frozen cDNA template (about 1-100 ng) were loaded into each well in MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems 4309849). The assays

were processed on a QuantStudio 5 system (Thermo Fisher). Thermal cycling conditions for RT-qPCR are shown in Table 2-12.

To minimise the effects of differences in cDNA quantities and provide a more precise representation of gene expression alterations among samples, the comparative threshold cycle (Ct) values were normalised using the *β2-microglobulin* (*β2m*) reference gene. Relative quantification was determined using the  $2^{-\Delta\Delta C_t}$  technique (Xiayu Rao, 2013), with the tumour cell lines as the reference for these calculations. The unpaired Mann-Whitney test was used for statistical analysis to compare the groups on GraphPad Prism software (version 10.1.0).

Table 2-11 Primers and probes used for qRT-PCR.

| Target gene      | Fluorescent dye | Primer sequence / TaqMan assay ID                                                        |
|------------------|-----------------|------------------------------------------------------------------------------------------|
| Mouse ROBO4      | FAM             | Mm00452963_m1                                                                            |
| Mouse CLEC14A    | FAM             | Mm00482102_s1                                                                            |
| β2-microglobulin | Cy5             | F:CATACGCCTGCAGAGTTAAGCA<br>R:ATCACATGTCTCGATCCCAGTAGA<br>Probe: CAGTATGGCCGAGCCCAAGACCG |
| IFN-γ            | FAM             | F:TCTTCTTGGATATCTGGAGGAACTG<br>R:GAGATAATCTGGCTCTGCAGGATT<br>Probe: TTCATGTCACCATCCTT    |

Table 2-12 Thermal cycling conditions for RT-qPCR.

| Step             | Temperature | Time       |
|------------------|-------------|------------|
| Hold             | 50°C        | 2 minutes  |
|                  | 95°C        | 10 minutes |
| PCR (40x cycles) | 95°C        | 15 second  |
|                  | 60°C        | 1 minute   |

## **2.10 Histology**

### **2.10.1 Frozen tissue sectioning (Cryosectioning)**

Microscopic examination was conducted using the frozen section (cryosectioning) technique. Snap-frozen tissues (e.g., tumour, spleen, placenta) were sectioned at 6–7  $\mu\text{m}$  with a cryostat (Leica CM1950). The sections were placed on SuperFrost Plus™ Adhesion microscope slides (Epredia J1800AMNZ) and air-dried for 30 minutes. Subsequently, the sections were fixed in cold acetone (VWR 67-64-1) at 4°C for 20 minutes. Finally, the sections were air-dried for another 20 minutes at room temperature and stored at -20°C.

### **2.10.2 Immunofluorescence (IF) labelling for frozen sections**

Immunofluorescence (IF) labelling detects and visualises protein locations within cells and tissues. Frozen sections were thawed at room temperature for 20 minutes. An ImmunoPen (Millipore 402176-1EA) was used to draw around the section on slides to create hydrophobic barriers and air-dried for 10 minutes at room temperature. The slides were then washed in a washing buffer (PBS, Gibco 18912-014) for 10 minutes to rehydrate the sections. The sections were incubated with a blocking buffer containing 10% v/v normal horse serum in a dilution buffer (1% w/v BSA in PBS) for 20 to 30 minutes to block free Fc regions. Primary antibodies were prepared in the dilution buffer and incubated for 1 hour at room temperature in a dark humidity box. After discarding the primary antibodies, the slides were washed in the washing buffer for 10 minutes. Secondary antibodies were added, and the sections were incubated for 1

hour at room temperature in a dark humidity box (see Table 2-12). After washing, the nuclei were stained with 0.1 µg/mL of 4',6-diamidino-2-phenylindole, DAPI (Sigma D9542) in PBS for 1-2 minutes and rewashed. The sections were coated with ProLong Diamond Antifade Mountant (Invitrogen P36970).

Sections were scanned using a slide scanner (Zeiss Axio Scan Z1), and the images were processed on Zeiss Zen Blue edition software. ImageJ, QuPath, and CellProfiler were used for quantification, and data visualisation was done on GraphPad Prism (version 10.1.0).

### **2.10.3 Multiplex imaging**

The MACSima™ imaging system (Miltenyi Biotec) uses a cyclic immunofluorescence approach where fluorochrome-conjugated antibodies bind to the tissue, are imaged, and then erased via photobleaching (Kinkhabwala et al., 2022). The antibodies are removed, and new ones are introduced, repeating the cycle to visualise 20+ biomarkers while preserving spatial resolution. First, tissue sections were prepared for multiplex imaging. Previously frozen tumour, spleen, and lymph node tissues were sectioned to a 6–7 µm thickness and fixed in 4% paraformaldehyde (PFA) for 10 minutes at room temperature. Following fixation, sections were washed three times with phosphate-buffered saline (PBS). To block non-specific Fc receptor binding, sections were incubated with 10% normal mouse serum in MACSima running buffer (Miltenyi Biotec 130-121-565) and washed thrice in PBS. For nuclear staining, sections were incubated with DAPI at a dilution of 1:7 in the running buffer for 10 minutes, followed by three washes with PBS. Before staining, all antibodies were centrifuged at

10,000 rpm to remove free fluorochromes. The antibodies used for multiplex imaging are listed in Table 2-13. Data acquisition and analysis were conducted using MACS® iQ View Analysis Software.

### ***Data Analysis Workflow with MACS iQ View***

Following high-resolution cell segmentation in MACS iQ View, data were exported as CSV files and analysed using GraphPad Prism (version 9.4.0). For each region of interest (ROI), TIFF-format images—including all stains and autofluorescence—were imported into MACS iQ View software (Version 1.2.2). The segmentation process used the platform's dedicated pipeline, using nuclear and cytoplasmic markers to delineate individual cells.

Nuclei were identified using the “Advanced Morphology for Tissue” setting, which relies on DAPI staining. Parameters such as Minimum/Maximum Diameter, Detection Sensitivity, Separation Force, and Smoothing Filter Sigma were fine-tuned based on DAPI signal intensity and tissue characteristics (see Table 2-14 for details). To define cytoplasmic regions, the “Constrained Donut” approach was applied, using automatically selected constraint channels. Detection Sensitivity and Donut Width settings were customised for each ROI, depending on fluorescence signal strength (see Table 2-14). A detailed workflow graph is provided in the Appendix B.

*Table 2-13 Antibodies used for immunofluorescence staining and multiplex imaging*

| Target                      | Conjugate       | Supplier               | Catalogue   | Clone    | Dilution Factor |
|-----------------------------|-----------------|------------------------|-------------|----------|-----------------|
| CD31                        | FITC            | eBioscience            | 11-0311-85  | 390      | 100             |
| B220                        | PE              | BD Bioscience          | 553090      | RA3-6B2  | 100             |
| CD4                         | APC             | Invitrogen             | 17-0041-82  | GK1.5    | 100             |
| CD11b                       | APC             | eBioscience            | 17-0112-83  | M1/70    | 100             |
| CD11c                       | APC             | eBioscience            | 17-0114-82  | N418     | 100             |
| Ki67                        | Rabbit          | Abcam                  | AB15580     | -        | 100             |
| Ly6G/C                      | FITC            | BioLegend              | 108406      | RB6-8C5  | 100             |
| CD45.2                      | PE              | eBioscience            | 12-0454-82  | 104      | 100             |
| PNAd                        | Alexa Flour 647 | BioLegend              | 120808      | MECA-79  | 100             |
| CD19                        | APC             | BioLegend              | 152410      | 1D3/CD19 | 100             |
| CD206                       | Biotin          | BioLegend              | 141714      | C068C2   | 100             |
| Streptavidin                | PE              | BioLegend              | 405203      | -        | 100             |
| F4/80                       | APC             | BioLegend              | 123116      | BM8      | 100             |
| IgG1                        | APC             | BD Bioscience          | 550874      | X56      | 100             |
| CD276 (PD-1)                | Alexa Flour 647 | BioLegend              | 109118      | RMP1-30  | 100             |
| CD138                       | PE              | BioLegend              | 142504      | 281-2    | 100             |
| IgM                         | PE              | BioLegend              | 406508      | RMM-1    | 100             |
| CD3                         | FITC            | BioLegend              | 100203      | 17A2     | 100             |
| DAPI                        | BV421           | Miltenyi Biotech       | 130-127-575 | -        | 100             |
| Panendothelial Cell Antigen | Alexa Flour 488 | BioLegend              | 120506      | MECA-32  | 100             |
| Anti-rabbit                 | Alexa Flour 647 | Jackson ImmunoResearch | 711-605-152 | -        | 100             |

*Table 2-14 Custom segmentation settings used in MACS iQ View*

| Nucleus                        |                      |                           |                           |                      |                        |
|--------------------------------|----------------------|---------------------------|---------------------------|----------------------|------------------------|
| Method                         | Reference channel    | Detection sensitivity [%] | Min/max Diameter [pixel]  | Separation force [%] | Smoothing filter Sigma |
| Advanced Morphology for Tissue | DAPI                 | 50                        | 35-100                    | 50                   | 3,0                    |
| Cytoplasm                      |                      |                           |                           |                      |                        |
| Method                         | Constrained channels |                           | Detection sensitivity [%] | Donut width [pixel]  |                        |
| Constrained Donut              | Automatic            |                           | 100                       | 100                  |                        |

## **2.11 Statistical analysis techniques**

All statistical analyses were conducted using GraphPad Prism (version 9.4.0), and specific tests are detailed in the corresponding figure legends. Statistical tests were applied to log-transformed values when the data were presented on a logarithmic scale.

Depending on the data distribution, a two-tailed t-test or the Mann-Whitney test was used to compare the two groups. Comparisons involving three or more groups were analysed using one-way ANOVA followed by Tukey's multiple comparisons test. Two-way ANOVA with Tukey's multiple comparisons test was applied in cases with multiple categories across two or more groups. A mixed-effects model with Šídák's multiple comparisons test was utilised if the data were matched. Correlation analyses were performed using nonlinear regression (curve fitting).

## **Chapter 3: CLEC14A-TTc conjugate vaccine induces CLEC14A-specific antibody response**

### **3.1 Introduction**

Angiogenesis describes the development of new blood vessels, a critical hallmark of cancer that plays a pivotal role in tumour progression. (Hanahan, 2022; Hanahan & Weinberg, 2011). Tumour angiogenesis supports the rapid growth of malignant cells by supplying oxygen and nutrients. As a result, anti-angiogenic therapies have gained significant attention in cancer research and therapeutic development. In the tumour microenvironment, hypoxia and low shear stress drive unique molecular changes in endothelial cells, leading to the expression of tumour endothelial markers (TEMs). Given their absence in healthy tissues, TEMs are promising therapeutic targets.

C-type lectin family 14 member A (CLEC14A) is a transmembrane protein involved in angiogenesis. Its extracellular region consists of five main domains: a signal peptide (SP), a C-type lectin domain (CTLD), a sushi domain (SD), an epidermal growth factor-like domain (EGFLD), and a mucin-like region (MLR) (Figure 3.8) (Khan et al., 2019; Mura et al., 2012). It is highly expressed in tumour endothelial cells but absent in healthy tissues (Mura et al., 2012; Zelensky & Gready, 2005). Mura et al. (2012) demonstrated that CLEC14A expression is upregulated under low shear stress using human umbilical endothelial cells (HUVECs) *in vitro*. Further studies have elucidated the functional roles of CLEC14A in tumour angiogenesis. Ki et al. (2013) and Noy et al. (2016) suggested that CLEC14A promotes filopodia formation, aiding the progression of sprouting angiogenesis. Notably, studies in a Lewis lung carcinoma (LLC) mouse model demonstrated that inhibiting CLEC14A/MMRN2 binding using an

anti-CLEC14A monoclonal antibody led to reduced tumour angiogenesis, and consequently restricted tumour growth (Khan, 2016; Noy et al., 2016). Significantly, CLEC14A deficiency does not impact vessel formation or structure during embryonic development, further supporting its potential as a selective therapeutic target.

Nevertheless, a key limitation in targeting CLEC14A for therapeutic purposes stems from its nature as a self-antigen. As a self-antigen, CLEC14A is naturally present in the body (albeit with limited expression in healthy tissues), meaning the immune system has developed immune tolerance to it (Robinson et al., 2020). This tolerance prevents the body from mounting a strong immune response against its own components. Consequently, when CLEC14A is presented as an antigen, it typically has low immunogenicity, failing to sufficiently activate T cells, which are essential for generating a robust, long-lasting antibody response. This insufficient T-cell help leads to restricted autoantibody secretion and weak immune activation, hindering its effectiveness as a standalone vaccine target (Bonasia et al., 2021). A well-established strategy to enhance immune responses against poorly immunogenic antigens is using conjugate vaccines. This approach has been successfully employed in bacterial vaccines, such as those targeting *Streptococcus pneumoniae* and *Neisseria meningitidis*, by linking a weak antigen to a highly immunogenic carrier protein (Peltola, 2000).

The carrier protein enhances immunity by eliciting a strong CD4<sup>+</sup> T cell response (Rappuoli, 2018). It provides peptide epitopes that are efficiently processed and presented by antigen-presenting cells (APCs) via MHC class II molecules, leading to the activation, proliferation, and differentiation of naïve CD4<sup>+</sup> T cells into helper T cell subsets, mainly T follicular helper (T<sub>fh</sub>) cells (Rappuoli, 2018). T<sub>fh</sub> cells play a critical role in the germinal centre reaction, delivering essential signals to B cells through

CD40-CD40L interactions and cytokine secretion (e.g., IL-21) (Zhuang et al., 2024). This support promotes B cell survival, somatic hypermutation, and affinity maturation while facilitating class-switch recombination to generate high-affinity antibodies. Furthermore, the generation of memory CD4<sup>+</sup> T cells ensures a rapid and robust recall response upon subsequent antigen exposure, reinforcing long-term immunity. Through these mechanisms, conjugate vaccines effectively enhance antigen-specific antibody production and establish durable immune memory (Escobar-Riquelme, 2024; Zhuang et al., 2015).

Tetanus toxin fragment C (TTc), a non-toxic fragment of tetanus toxin, is one of the most widely used carrier proteins in conjugate vaccines. TTc is well-established in human vaccination programs and has been shown to induce robust CD4<sup>+</sup> T cell responses (Xu et al., 2021). It has been successfully conjugated to weakly immunogenic antigens, such as HER2, leading to enhanced immune responses *in vivo* breast cancer models (Chotprakaikiat et al., 2016). Additionally, TTc has been used in polysaccharide-conjugate vaccines, effectively inducing specific immune responses against weakly immunogenic polysaccharides (Baker et al., 2007; McCarthy et al., 2013).

Additionally, recent studies have explored alternative immunotherapeutic strategies targeting CLEC14A. Zhuang et al. (2020) engineered CLEC14A-specific CAR T cells, demonstrating their ability to prevent tumour development in preclinical models, including LLC and pancreatic cancer. Notably, no adverse effects were observed in these studies, further underscoring CLEC14A's potential as a target for cancer immunotherapy.

In this study, we hypothesised that the conjugation of CLEC14A with TTc would induce its immunogenicity, leading to a robust anti-CLEC14A immune response and tumour growth inhibition.

### **3.2 Chapter Aims**

In this chapter, my study aims to produce a genetically linked conjugated vaccine between the extracellular domain of CLEC14A and TTc (CLEC14A-TTc) and to assess the resulting antibody response.

The objectives of this chapter are as follows:

- i) Confirm the expression of CLEC14A on mouse tumour tissues (4T1 and E0771).
- ii) Design and construct a genetic vector for mammalian expression of genetically conjugated CLEC14A-TTc protein.
- iii) Produce and purify the CLEC14A-TTc recombinant protein for vaccination.
- iv) Assess the generation of CLEC14A-specific antibodies after immunisation with the CLEC14A-TTc.

### 3.3 Results

#### 3.3.1 CLEC14A is expressed in several mouse tumour models (4T1, E0771 and LLC1)

While CLEC14A is a well-characterized tumour endothelial marker, it was crucial to confirm its expression specifically within the tumour models used in this study (4T1, E0771, and LLC1). Therefore, qRT-PCR was employed to measure the mRNA expression of *Clec14a* in these tumour tissues, as well as in placenta (as a positive control) (Figure 3.1). The 4T1 and E0771 models represent triple-negative breast cancer (TNBC), while the Lewis lung carcinoma (LLC1) model serves as a model for lung cancer research. Here, LLC1 tumour cells were injected subcutaneously (s.c.) into the right flank of C57BL/6 mice, whereas 4T1 and E0771 tumour cells were s.c. transplanted into the fourth mammary fat pad. Tumours were collected on day 24. *Clec14a* is expressed on the vessels of mature human and mouse placental tissues due to extensive new blood vessel development (Robinson et al., 2020; Zhuang et al., 2020). The placenta (provided by Professor Graham Anderson, University of Birmingham) was used as a positive control. Frozen tumour tissues and placenta samples were cryo-sectioned at 6–7  $\mu\text{m}$ . Tumour cell lines were snap-frozen at a number of  $1\text{--}2 \times 10^6$  cells.

cDNA was prepared from the tumour cell lines and tissues, and qRT-PCR assessed mRNA expression. Initially, primers and probes of *Clec14a* and *B2m* ( $\beta 2$ -microglobulin, housekeeping gene) were tested for multiplex in qRT-PCR using placental cDNA (Figure 3.1A). Placental cDNA was diluted at 1:1, 1:3, 1:9, and 1:27 ratios and examined by qRT-PCR in singleplex and multiplex analyses for *Clec14a* and *B2m*

expression. The assay run in parallel produced the same level of signals as the different primer pairs run individually. The value of the difference in Ct (delta Ct,  $\Delta Ct$ ) was similar across all different template dilutions (Figure 3.1A). The lines for singleplex and multiplex assays overlap, showing that primers and probes of *Clec14a* and *B2m* can be used in multiplex.

Next, the mRNA expression of *Clec14a* in cell lines, tumour tissues, and placenta was quantified (Figure 3.1B). Each graph represents data from a specific tumour model (4T1, E0771, and LLC1). The tumour cell line data points correspond to different passages of the same cells, while the tumour and placenta data points correspond to individual mice. Data were normalised using the low mRNA expression levels of *Clec14a* in the cell lines as a reference to calculate relative quantification (RQ,  $2^{-\Delta\Delta Ct}$ ). As expected, the placenta exhibited high *Clec14a* mRNA expression compared to the cell lines. Tumour tissues (particularly 4T1 tumours) showed significantly higher *Clec14a* mRNA expression. These results confirm *Clec14a* mRNA expression in the 4T1, E0771, and LLC1 tumours, indicating its presence primarily on the tumour endothelial cells within these models.

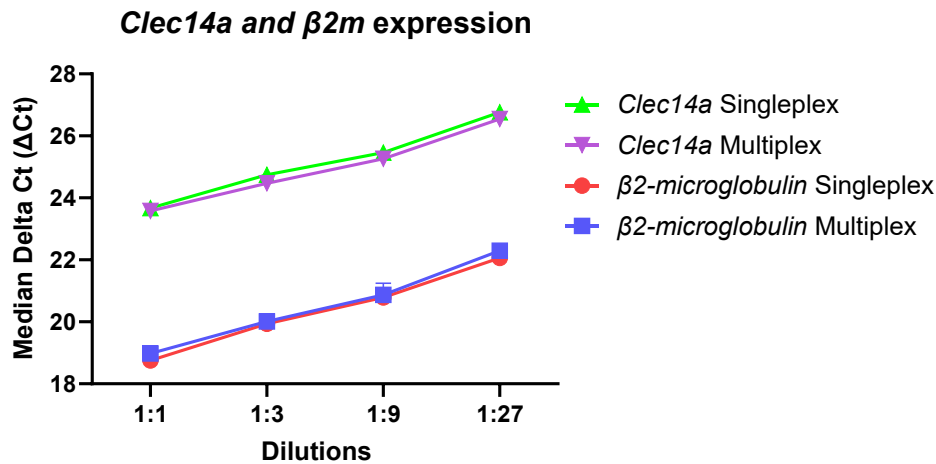
To confirm the gene expression data, we next sought to determine whether *Clec14a* expression was also elevated at the protein level in tumour models. Immunofluorescent staining further validated CLEC14A protein expression in tumour models and healthy tissues. Figure 3.2 illustrates nuclear staining with DAPI (blue), the pan-endothelial cell antigen-antibody (clone: MECA32) to identify endothelial cells (green) in embryonic and adult mouse tissue, and rabbit anti-CLEC14A antibody or rabbit IgG isotype control (red) was used to identify CLEC14A. The placenta, which undergoes extensive angiogenesis, was used as a positive control (Zhuang et al., 2020). In Figure 3.3, the

placenta showed strong CLEC14A (red) and MECA32 (green) expression and co-localisation (yellow). In contrast, healthy adult mouse pancreatic tissue showed no CLEC14A expression, with only MECA32 expression detected Figure 3.4.

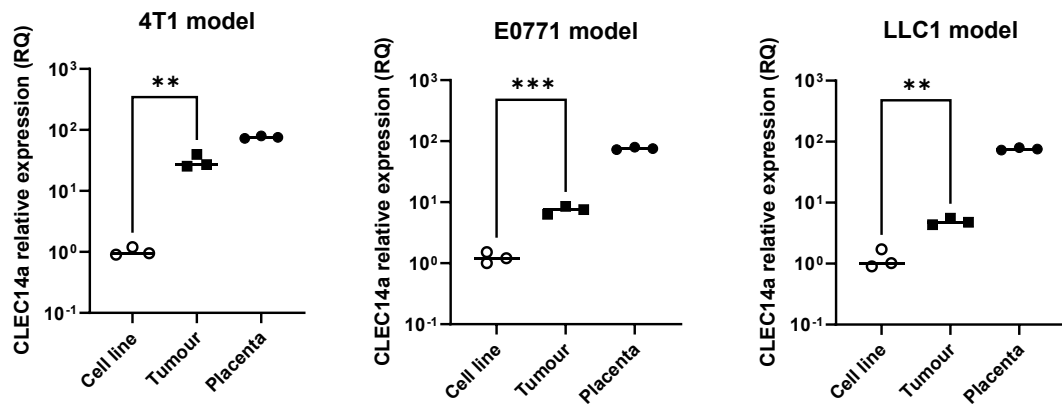
Tumour sections from 4T1, E0771, and LLC1 models (Figure 3.2, Figure 3.5, Figure 3.6 and Figure 3.7) exhibited high CLEC14A expression relative to the control tissue, with a partial co-localisation with MECA32. This partial co-localisation is significant as CLEC14A may be expressed on a subset of endothelial cells not exclusively marked by MECA-32, such as tip cells or those within tumour-induced abnormal vasculature, reflecting differences in vascular maturation or angiogenic state. Additionally, isotype control images for all tissues tested showed no signal in the red channel, confirming specificity. These results support CLEC14A expression at the protein and mRNA levels in 4T1, E0771, and LLC1 tumours.

In conclusion, the consistent expression of CLEC14A in tumour endothelial cells (developing vessels), coupled with its absence in healthy adult tissues (Mura et al., 2012), underscores its high potential as a selective therapeutic target. This differential expression provides a crucial window for specific anti-angiogenic interventions, making CLEC14A an attractive candidate for novel cancer treatments.

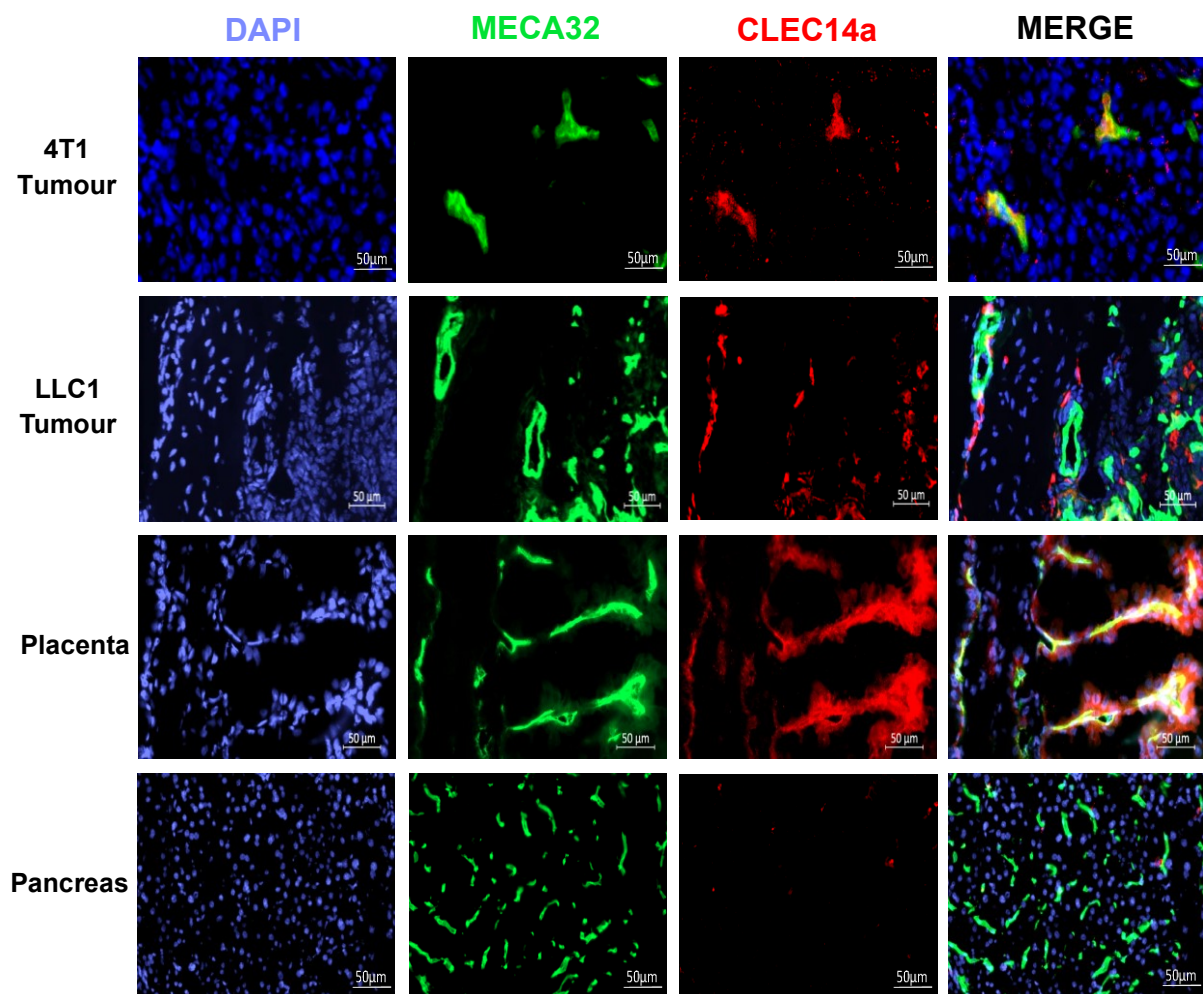
**A**



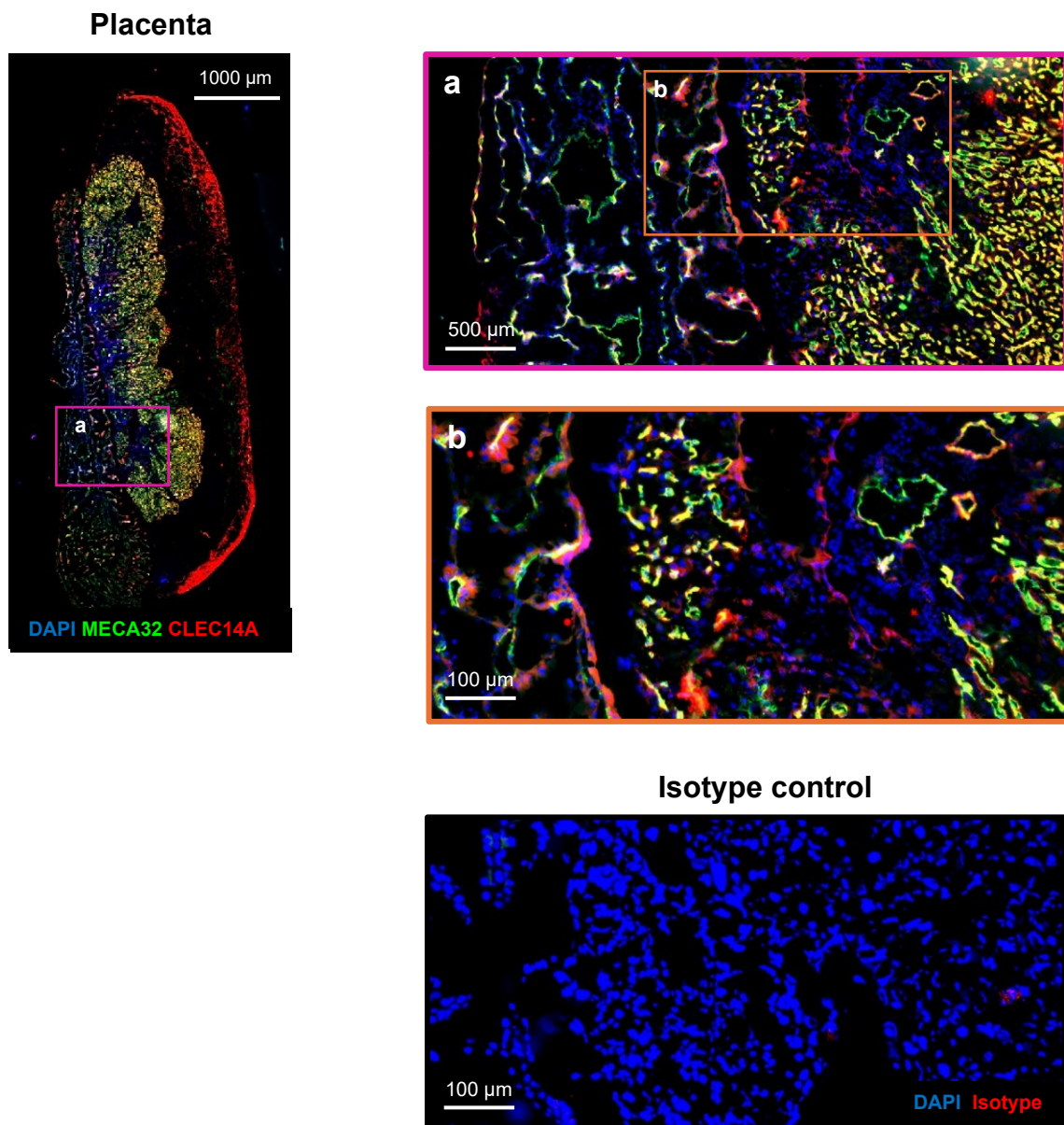
**B**



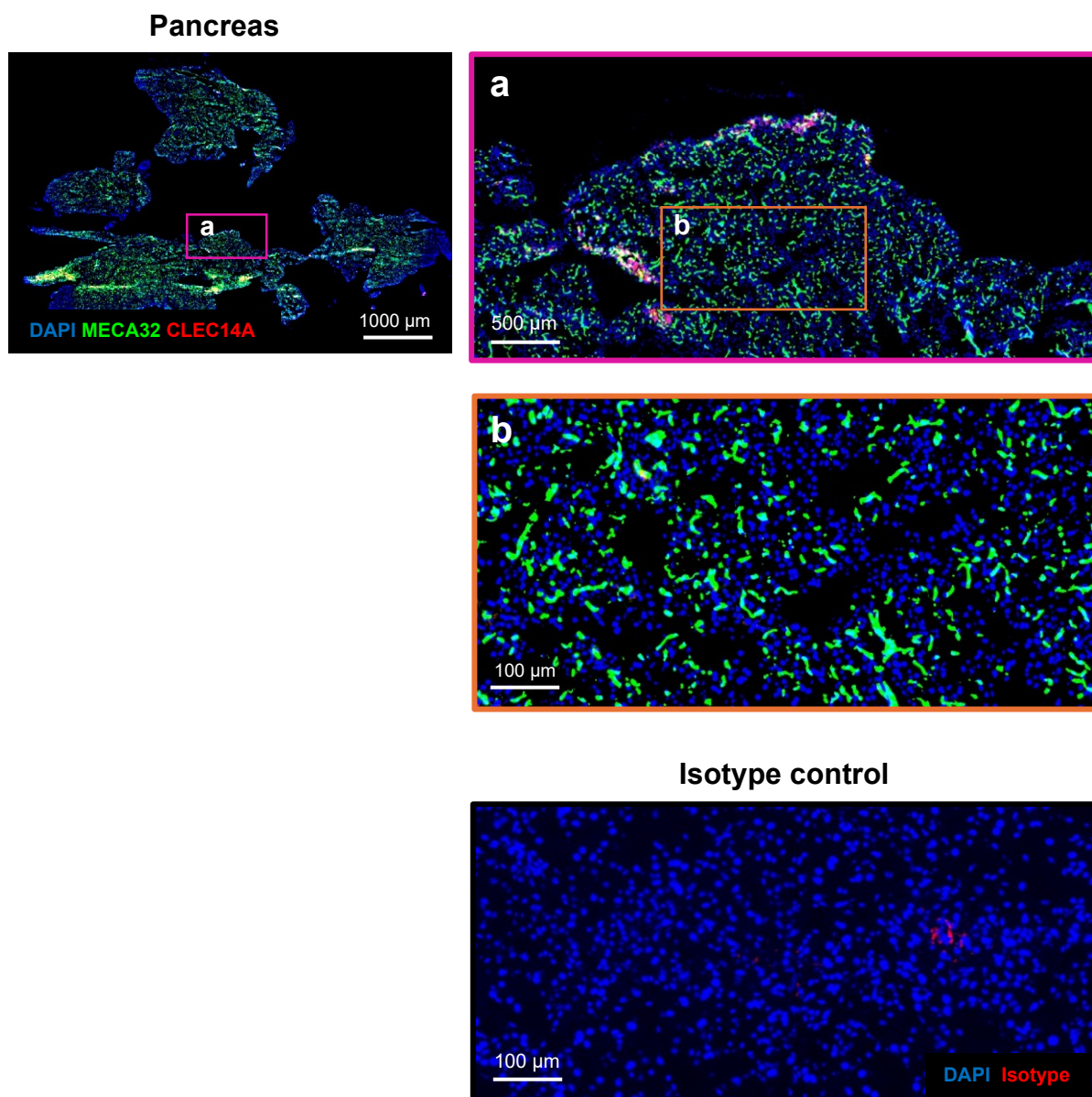
**Figure 3.1 *Clec14a* expression analysis by qRT-PCR.** (A) Multiplex qRT-PCR assay setup, demonstrating the standardisation of *Clec14a* expression relative to the housekeeping gene *B2m*. (B) Comparison of *Clec14a* relative expression between 4T1, E0771, and LLC1 tumours and their respective cell lines, normalised to *B2m*. Tumours were collected on day 24 after transplantation. Statistical tests were performed by unpaired t test (\*\*\*\*,  $p < 0.0001$ ; \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ; ns  $\geq 0.05$ ).



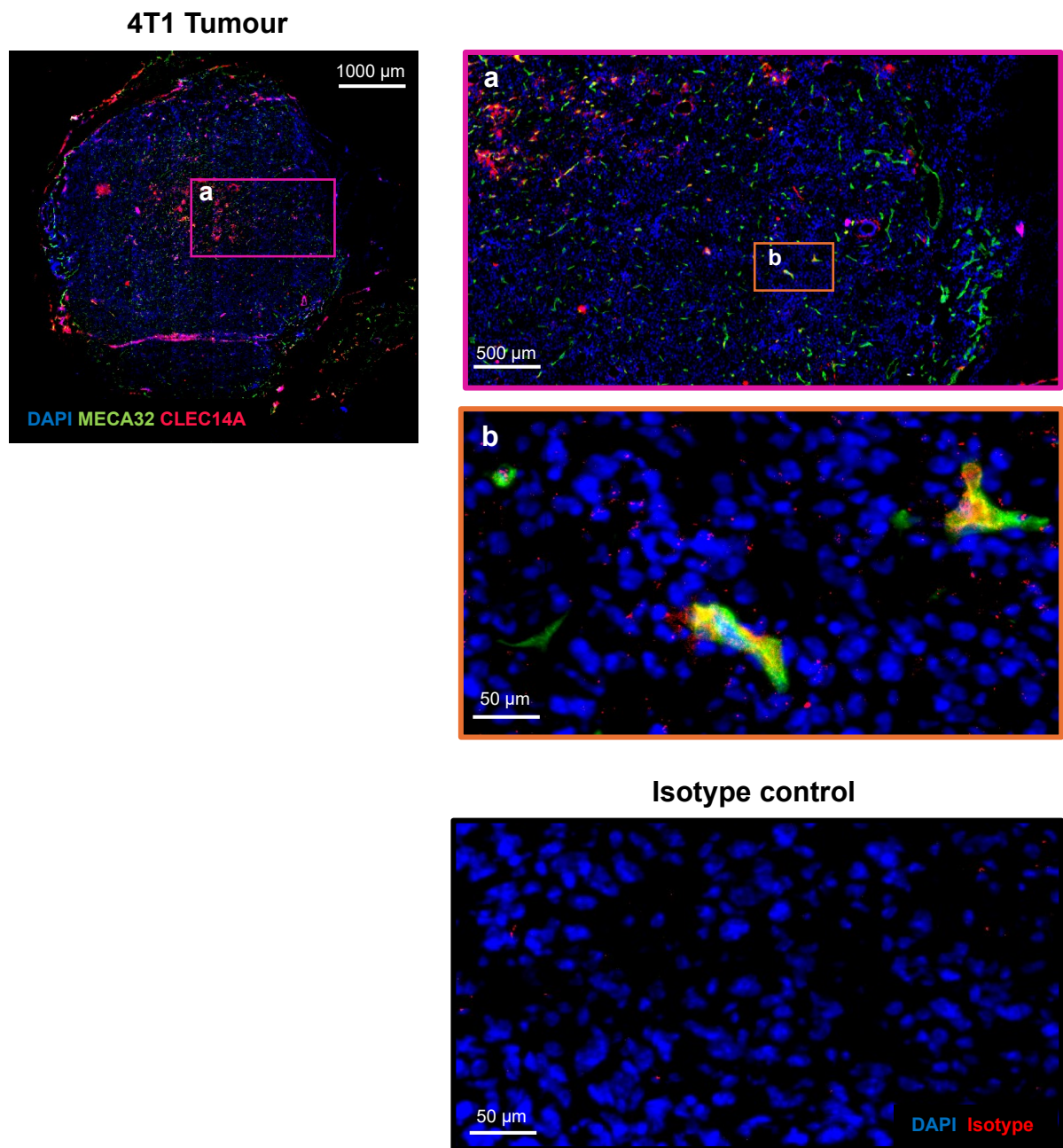
**Figure 3.2 Immunofluorescent imaging of CLEC14A in mouse tumour and healthy tissues.** Representative images showing CLEC14A expression in 4T1 and Lewis lung carcinoma (LLC1) tumours and placenta and pancreas tissues. Green, MECA32 staining for endothelial cells. Red, CLEC14A staining. Blue, DAPI for nuclei staining. Scale bars: 50  $\mu$ m.



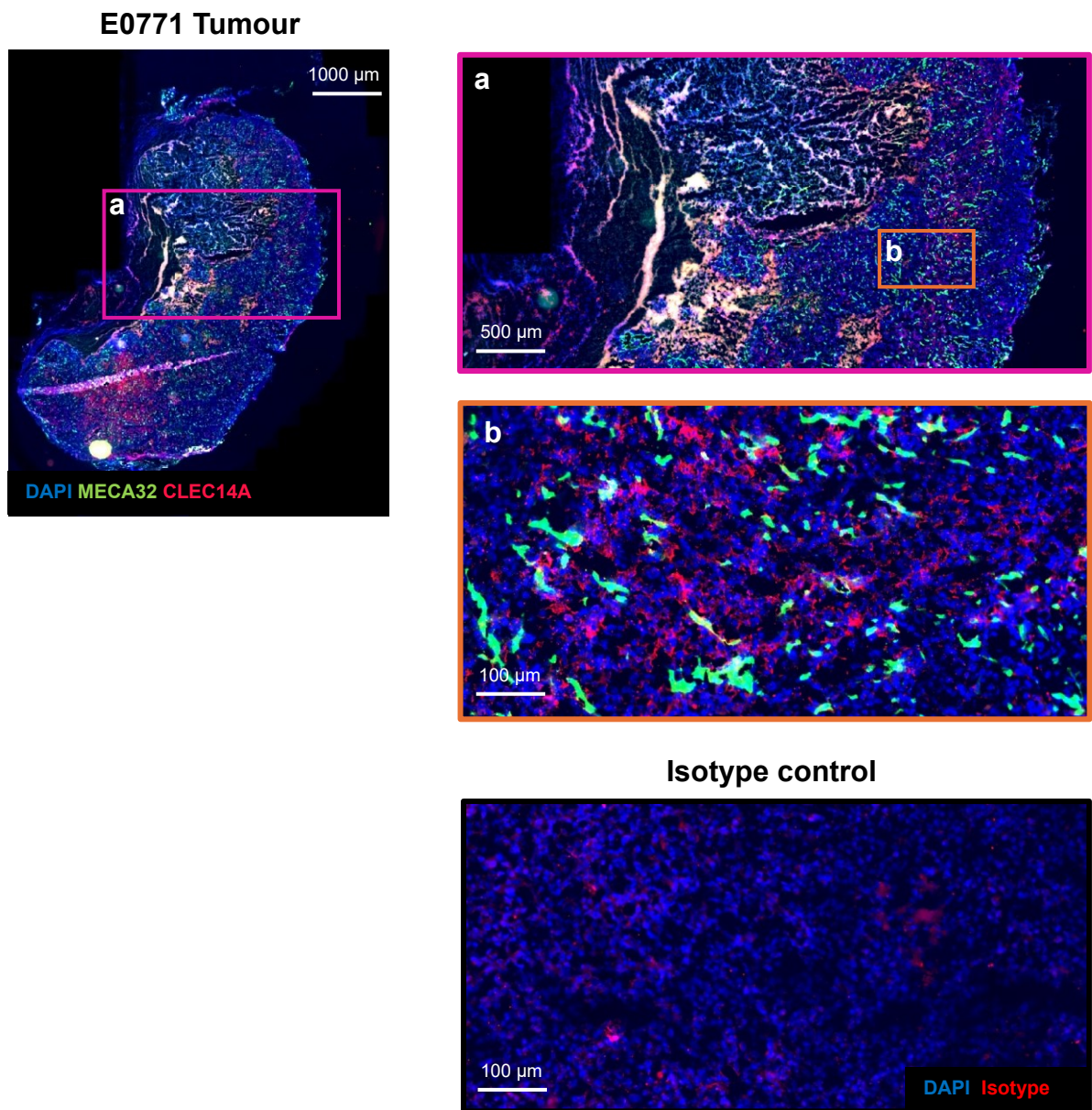
**Figure 3.3 Immunofluorescent staining of CLEC14A expression in placenta.** Representative images of CLEC14A (red) and MECA32 (green) staining to identify endothelial cells, with DAPI (blue) for nuclear staining. Isotype control showing MECA32 (green) and Rabbit Isotype IgG (red) staining on the same section. Scale bars: 1000 µm, 500 µm, and 100 µm.



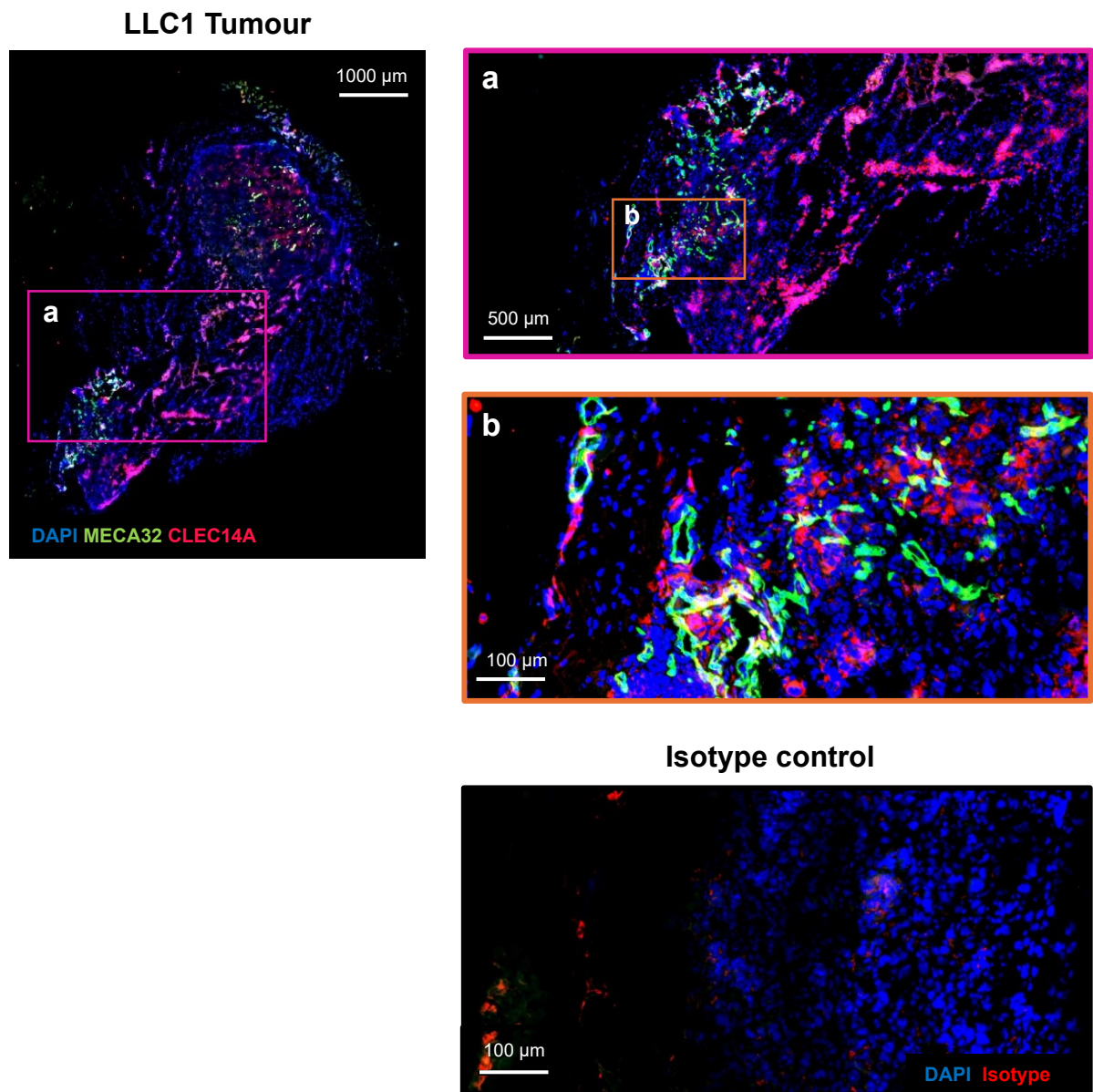
**Figure 3.4 Overview immunofluorescent staining of CLEC14A expression in pancreas.** Representative images of CLEC14A (red) and MECA32 (green) staining to identify endothelial cells, with DAPI (blue) for nuclear staining. Isotype control showing MECA32 (green) and Rabbit Isotype IgG (red) staining on the same section. Scale bars: 1000  $\mu\text{m}$ , 500  $\mu\text{m}$ , and 100  $\mu\text{m}$ .



**Figure 3.5 Immunofluorescent staining of CLEC14A expression in 4T1 tumour.** Representative images of CLEC14A (red) and MECA32 (green) staining to identify endothelial cells, with DAPI (blue) for nuclear staining. Isotype control showing MECA32 (green) and Rabbit Isotype IgG (red) staining on the same section. Scale bars: 1000 µm, 500 µm, and 50 µm.



**Figure 3.6 Immunofluorescent staining of CLEC14A expression in E0771 tumour.** Representative images of CLEC14A (red) and MECA32 (green) staining to identify endothelial cells, with DAPI (blue) for nuclear staining. Isotype control showing MECA32 (green) and Rabbit Isotype IgG (red) staining on the same section. Scale bars: 1000 µm, 500 µm, and 100 µm.



**Figure 3.7 Immunofluorescent staining of CLEC14A expression in LLC1 tumour.** Representative images of CLEC14A (red) and MECA32 (green) staining to identify endothelial cells, with DAPI (blue) for nuclear staining. Isotype control showing MECA32 (green) and Rabbit Isotype IgG (red) staining on the same section. Scale bars: 1000  $\mu\text{m}$ , 500  $\mu\text{m}$ , and 100  $\mu\text{m}$ .

### **3.3.2 CLEC14A-TTc vaccine production**

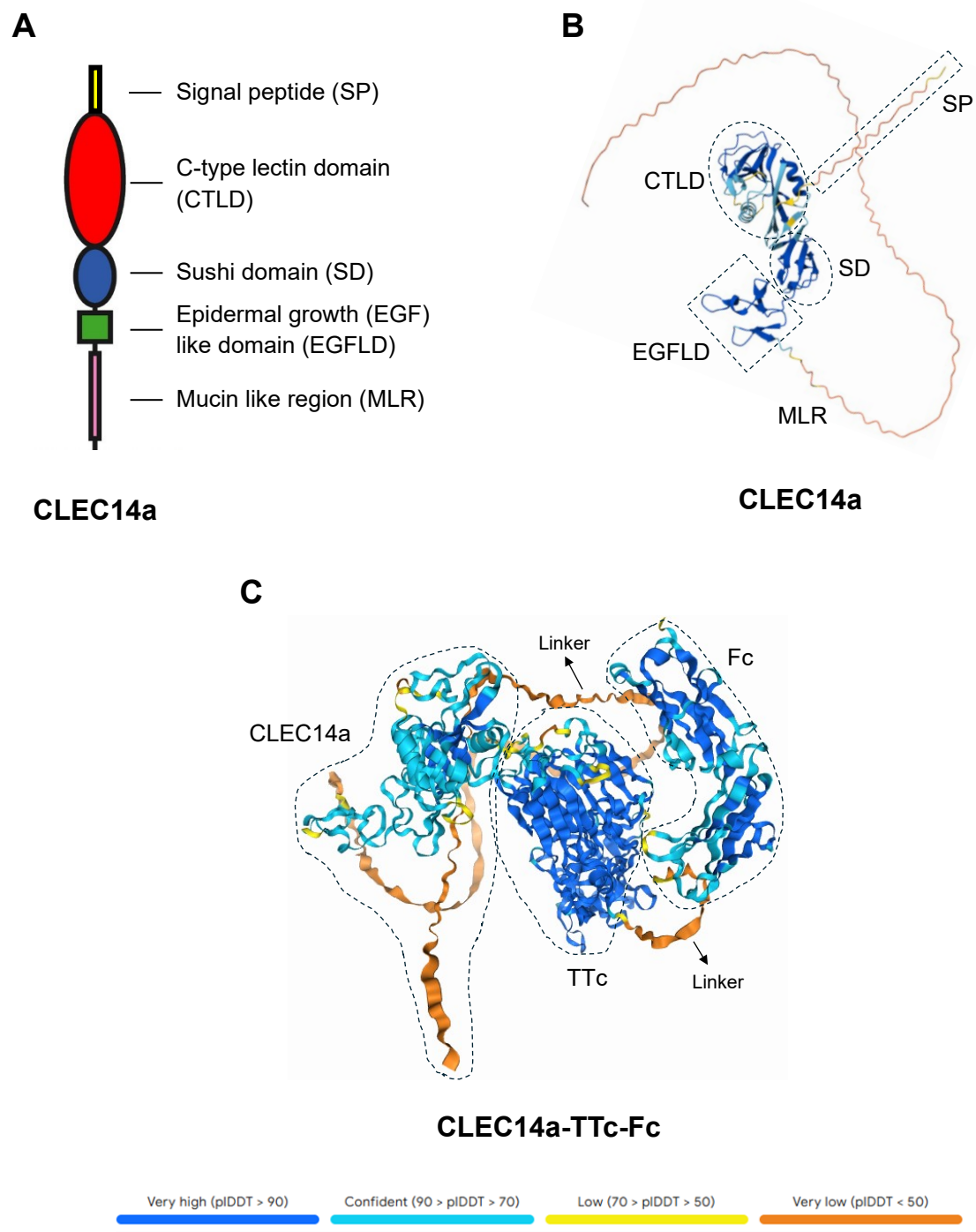
#### **3.3.2.1 Subcloning to obtain pcDNA3.1 CLEC14A-TTc**

To construct the CLEC14A-TTc vaccine, the extracellular domain of the mouse CLEC14A protein was genetically linked to Tetanus Toxin Fragment C (TTc) from the heavy chain of *Clostridium tetani* for expression in a mammalian system (Figure 3.8). First, the DNA sequences were codon-optimized for mammalian expression. A human Fc tag was added using a GSS linker to facilitate recombinant protein detection and purification. Additionally, restriction enzyme sites (NheI and Apal) were incorporated into the sequences to enable subcloning into the pcDNA3.1 mammalian expression vector. The CLEC14A-Fc sequence was synthesised in a standard plasmid (pUCIDT) containing an ampicillin resistance gene obtained from Integrated DNA Technologies (IDT). The pUCIDT-AMP GoldenGate CLEC14A-Fc and pcDNA3.1-mEGFP plasmids were amplified in *E. coli* 5-alpha Competent Cells through chemical transformation.

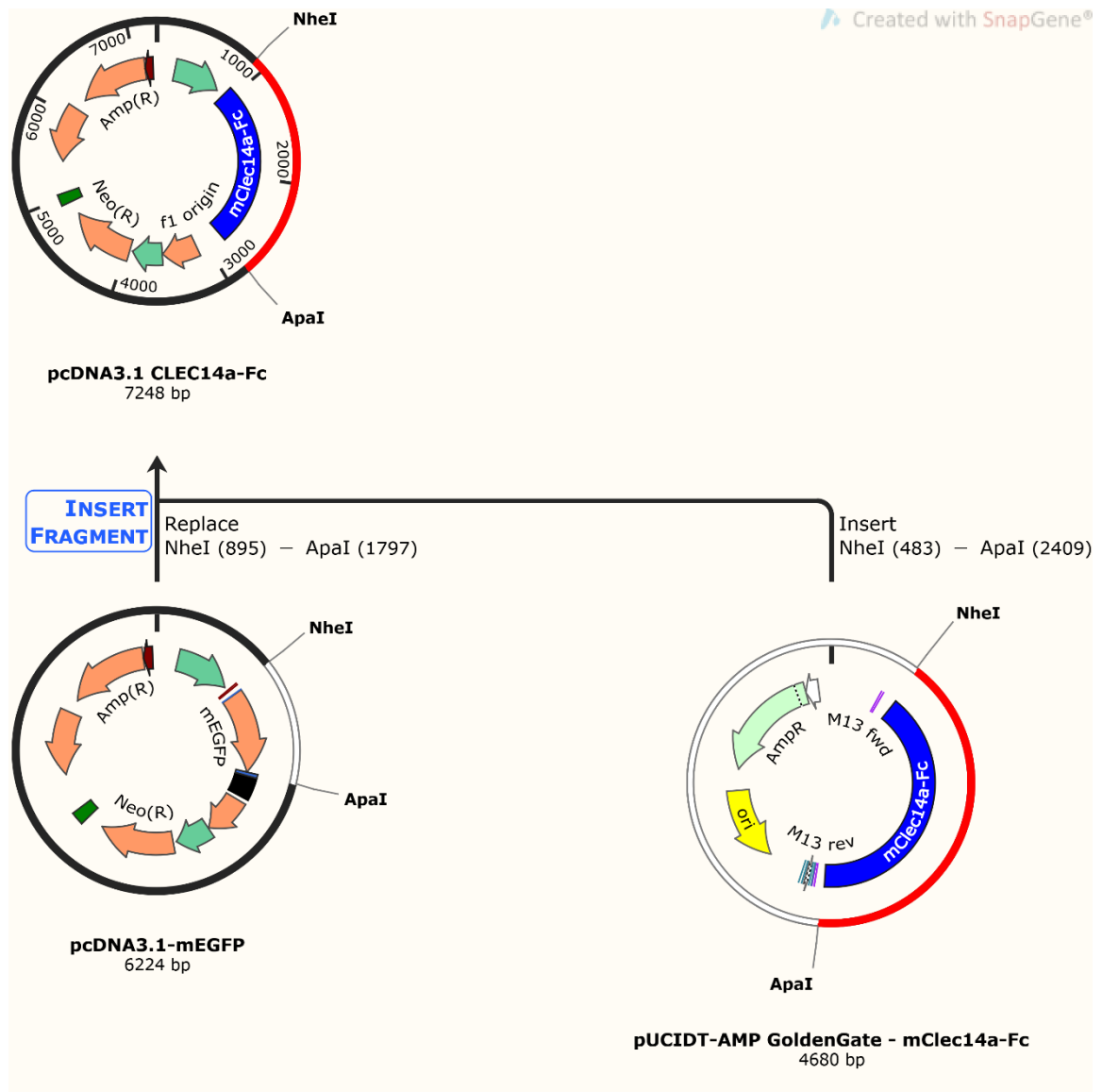
The subcloning strategy for inserting CLEC14A-Fc into the pcDNA3.1 plasmid, illustrated in Figure 3.9, was designed using SnapGene. The insert vector, pUCIDT-AMP GoldenGate CLEC14A-Fc, was digested with NheI, Apal, and Scal to enhance cloning efficiency. In contrast, the recipient vector, pcDNA3.1-mEGFP, was digested only with NheI and Apal. Agarose gel electrophoresis confirmed plasmid digestion for both constructs (Figure 3.10). Initial digestion of the insert vector with only NheI and Apal resulted in two bands: the CLEC14A-Fc sequence (1924 bp) and the vector backbone (2754 bp), which proved difficult to separate due to their similar sizes (data not shown). To resolve this, an additional enzyme, Scal, was used to cut the vector backbone further, resulting in three distinct bands: the CLEC14A-Fc sequence (1924 bp) indicated by a red arrow (Figure 3.10C) and vector backbone fragments of 990 bp

and 1764 bp (Figure 3.10D). Although the sizes of the CLEC14A-Fc fragment and one vector backbone fragment (1764 bp) were close, their different sticky ends—NheI-ApaI for CLEC14A-Fc and ApaI-ScaI for the backbone fragment—prevented unwanted ligation. The recipient vector digestion released the mGFP sequence (900 bp) and left the pcDNA3.1 backbone with NheI and ApaI sticky ends (5322 bp), as shown in the right panel of Figure 3.10. Large-scale restriction digestions, shown in Figure 3.10C and Figure 3.10D, followed by purification and quantification, prepared the CLEC14A-Fc fragment for ligation into the pcDNA3.1 vector, as indicated by the blue arrow in Figure 5D.

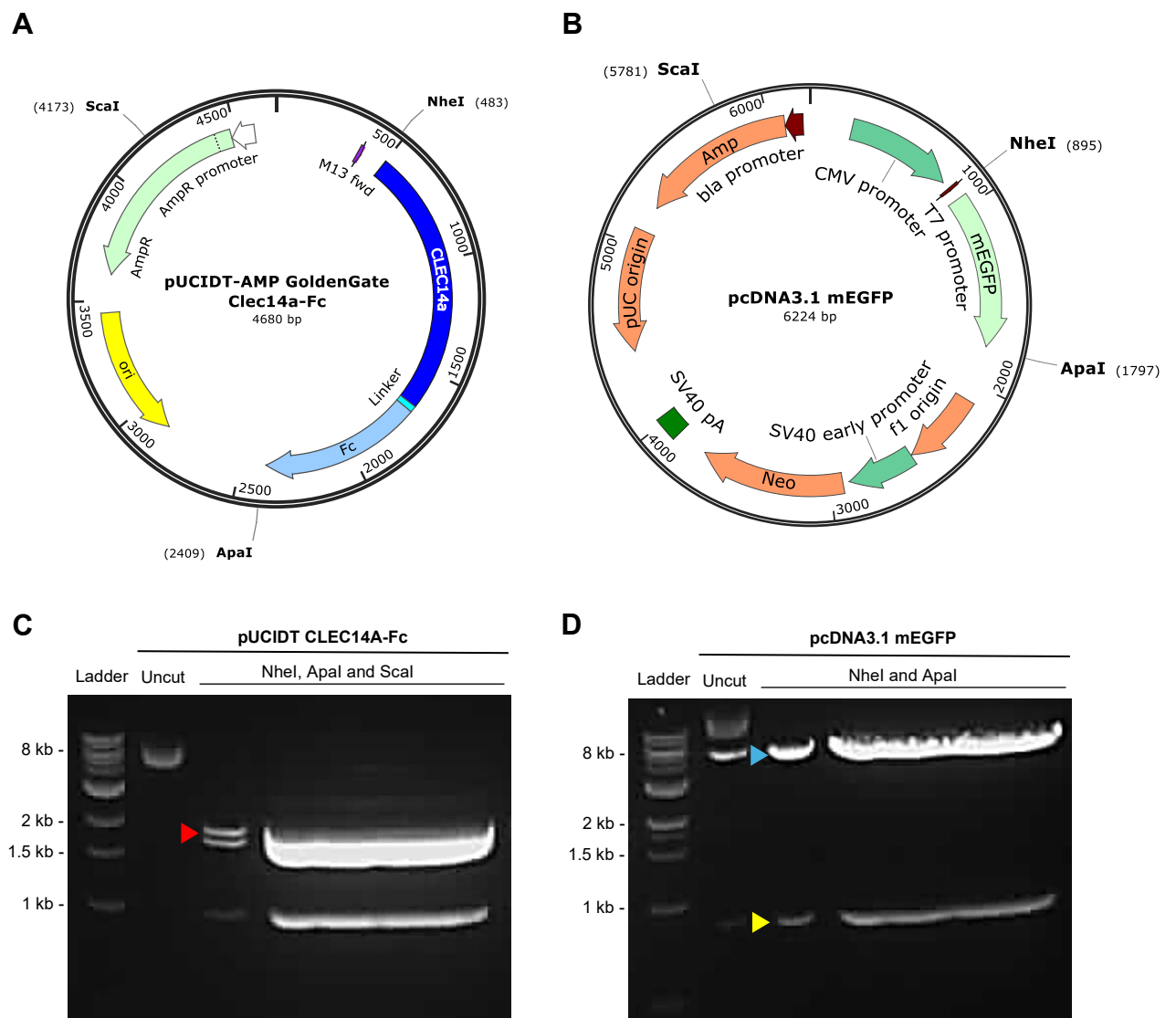
The newly constructed pcDNA3.1 CLEC14A-Fc plasmid (Figure 3.11A) was transformed into *E. coli* 5-alpha chemically competent cells and plated on LB agar containing ampicillin. Colonies were isolated after incubation at 37°C, and plasmid DNA was extracted from a single colony. To confirm successful ligation, restriction digestion was performed using NheI, ApaI, and BamHI. Agarose gel electrophoresis results (Figure 3.11B) presents the expected digestion patterns of pUCIDT CLEC14A-Fc, pcDNA3.1 mEGFP, and pcDNA3.1 CLEC14A-Fc (Figure 3.11C, left to right). Digestion with NheI and ApaI confirmed the presence of CLEC14A-Fc (1924 bp, red arrow) and the pcDNA3.1 backbone (5322 bp, blue arrow), validating the successful cloning of CLEC14A-Fc into the expression vector.



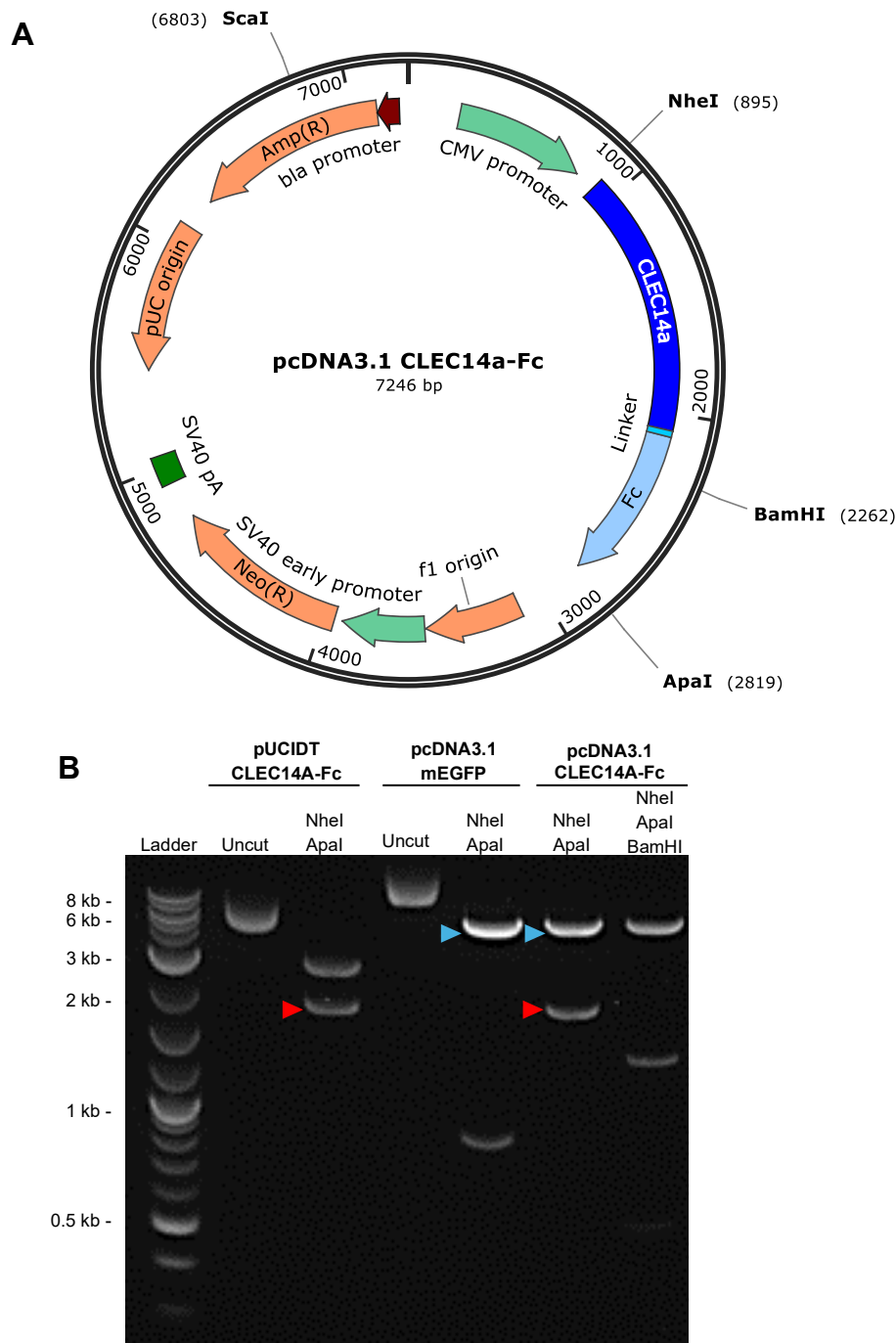
**Figure 3.8 Structure of the extracellular domain of CLEC14A and CLEC14A-TTc-Fc proteins.** (A) CLEC14A receptor structure, adapted from Mura et al. (2012) and Khan et al. (2019). The extracellular domain of CLEC14A comprises five main domains: signal peptide (SP), C-type lectin domain (CTLTD), sushi domain (SD), epidermal growth (EGF) like domain (EGFLD) and mucin-like region (MLR). (B and C) Predicted 3D structures of CLEC14A and CLEC14A-TTc-Fc generated using AlphaFold. The images illustrate the predicted folding and linker positioning by displaying the detailed spatial arrangements of each domain and the fusion constructs.



**Figure 3.9 Schematic representation of the subcloning strategy for the pcDNA3.1 CLEC14A-Fc vector.** The source plasmids, pcDNA3.1-mEGFP and pUCIDT-AMP GoldenGate-mCLEC14A-Fc, were digested with NheI and ApaI restriction enzymes. The CLEC14A-Fc sequence was then ligated into the pcDNA3.1 vector, producing the final pcDNA3.1 CLEC14A-Fc plasmid, as visualised using SnapGene software.



**Figure 3.10 Restriction enzyme cloning of the pcDNA3.1 CLEC14A-Fc vector.** Plasmid map of A) pUCIDT-AMP GoldenGate CLEC14A-Fc and B) pcDNA3.1 mEGFP created using SnapGene. DNA gel electrophoresis of C) pUCIDT-AMP GoldenGate mCLEC14A-Fc and D) pcDNA3.1 mEGFP plasmids digested with NheI and ApaI, showing the release of the CLEC14A-Fc sequence (1924 bp) indicated by the red arrow and mGFP sequence (900 bp) marked by yellow. As indicated by the blue arrow, the backbone pcDNA3.1 vector (5322 bp) was gel-purified for subcloning.



**Figure 3.11 Verification of restriction enzyme cloning of the pcDNA3.1 CLEC14A-Fc vector.** A) Plasmid map of pcDNA3.1 CLEC14A-Fc, generated using SnapGene. B) DNA gel electrophoresis of restriction enzyme digests and PCR analysis of the pcDNA3.1 CLEC14A-Fc vector. CLEC14A-Fc and pcDNA3.1 sequences were indicated with red and blue arrows, respectively.

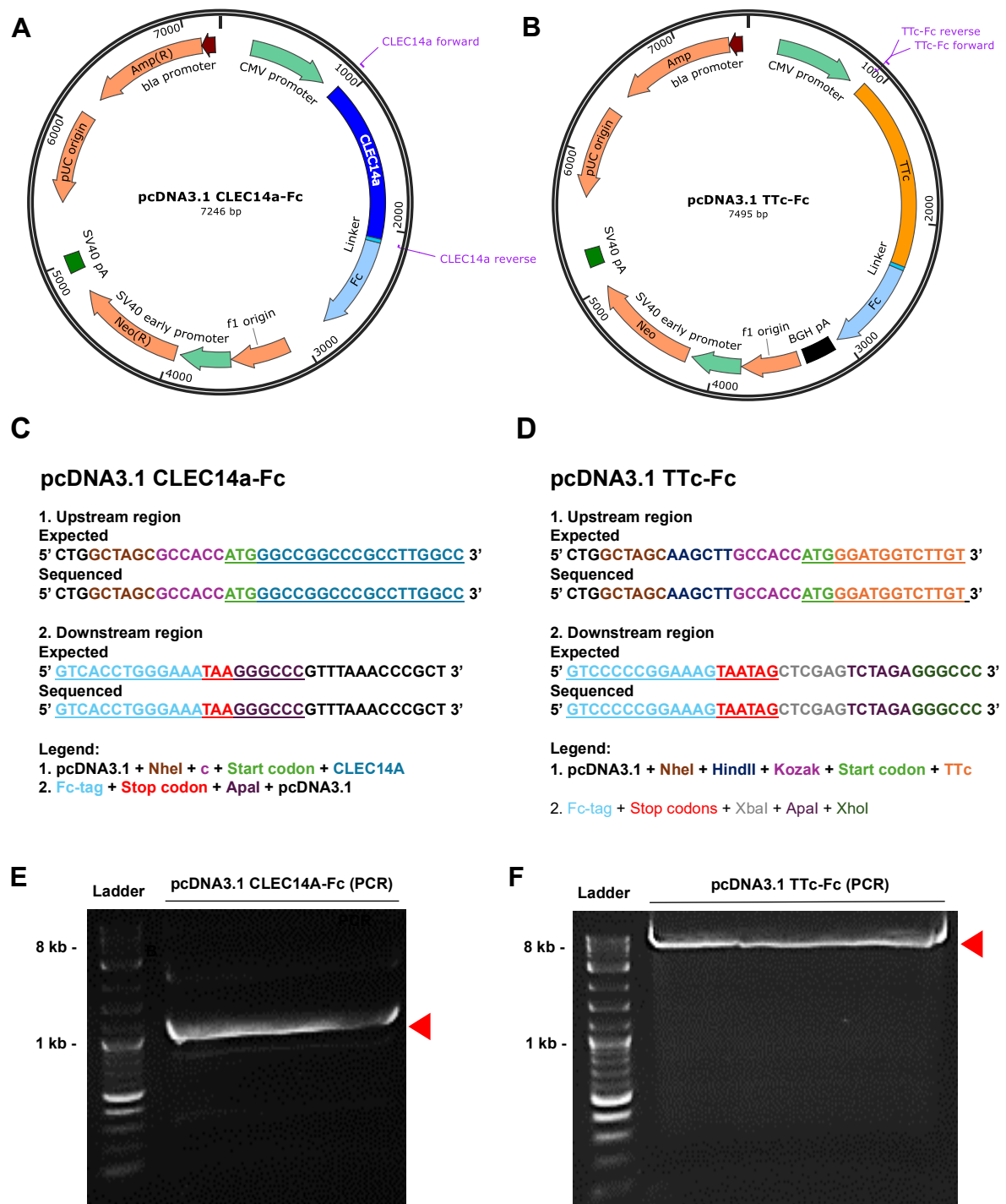
### **3.3.2.2 DNA Assembly for Constructing pcDNA3.1 CLEC14A-TTc-Fc**

The CLEC14A-TTc protein is genetically engineered for a conjugate vaccine by linking the extracellular domain of the CLEC14A sequence to the TTc sequence. To achieve this, a previously generated pcDNA3.1 TTc-Fc plasmid obtained from Dr Fernanda Nicole Escobar Riquelme (University of Birmingham) was used.

To genetically link CLEC14A and TTc, the CLEC14A-Linker sequence and pcDNA3.1 TTc-Fc sequence were amplified by PCR, using primers designed to generate overlapping ends. Specifically, for CLEC14A-Linker (1185 bp), the primers CLEC14A forward (CLEC14A.F5) and CLEC14A reverse (CLEC14A.R5) were used to amplify the CLEC14A sequence with the addition of a GSS linker (excluding the Fc tag) from pcDNA3.1 CLEC14A-Fc (Figure 3.12A). For pcDNA3.1 TTc-Fc (7495 bp), the primers TTc-Fc forward (pcTTcFc.F4) and TTc-Fc reverse (pcTTcFc.R3) were used to amplify and linearise the pcDNA3.1 TTc-Fc vector (Figure 3.12B) (The primer sequences are shown in Table 2-4).

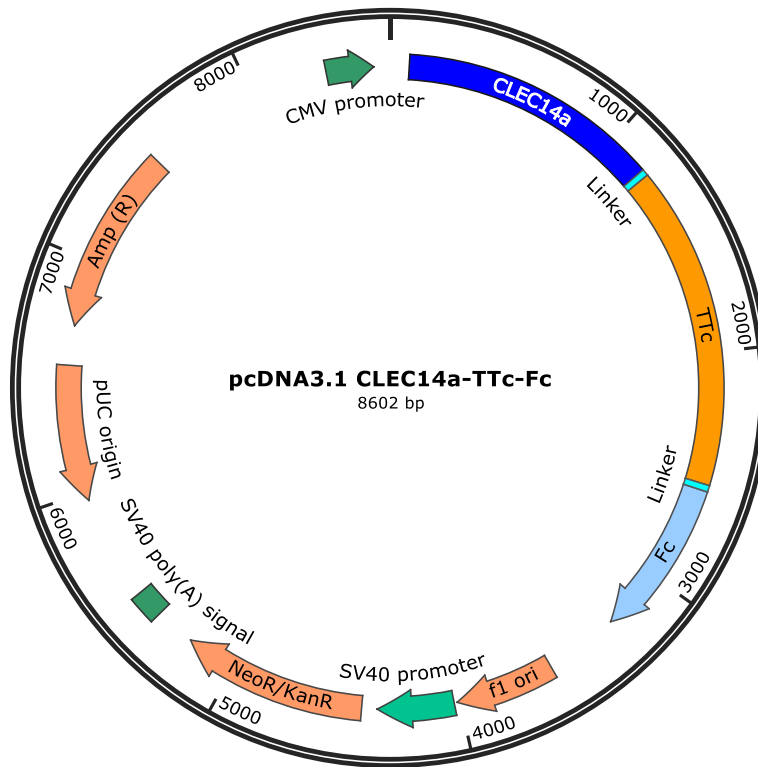
PCR conditions were optimised, including amplification time, temperature, and primer lengths (data not shown). The optimal annealing times were 1 minute for CLEC14A-Linker and 5 minutes for pcDNA3.1 TTc-Fc. The expected and sequenced sequences of the upstream and downstream regions for pcDNA3.1 CLEC14A-Fc and pcDNA3.1 TTc-Fc vectors are shown (Figure 3.12C and Figure 3.12D). Figure 3.12F and Figure 3.12D presents the PCR results of CLEC14A-Linker and linearised pcDNA3.1 TTc-Fc. Subsequently, the PCR products were purified and quantified using gel, and the concentration was adjusted for cloning.

The CLEC14A-Linker and pcDNA3.1 TTc-Fc PCR products were assembled using the NEBuilder HiFi DNA Assembly Master Mix. The assembled constructs were then transformed into 5-alpha chemically competent *E. coli*. Single bacterial colonies were selected, and after plasmid isolation, the constructs were analysed by Sanger sequencing to confirm successful assembly. Figure 3.13A depicts the complete pcDNA3.1 CLEC14A-TTc-Fc plasmid map, designed using SnapGene, along with the sequencing results of the upstream, downstream, and linker regions. The sequencing results confirmed the expected input sequences, aligning with the in silico subcloning design in SnapGene (Figure 3.13B).



**Figure 3.12 PCR cloning for constructing the pcDNA3.1 CLEC14A-TTc-Fc vector.** SnapGene-generated plasmid maps of (A) pcDNA3.1 CLEC14A-Fc and (B) pcDNA3.1-TTc-Fc vectors. (C and D) The expected and sequenced sequences of the upstream and downstream regions for A) pcDNA3.1 CLEC14A-Fc and B) pcDNA3.1 TTc-Fc vectors. (E and F) DNA gel electrophoresis images depicting PCR amplification of the CLEC14A-Linker (1185 bp) and pcDNA3.1-TTc-Fc (7495 bp) sequences, which were subsequently cloned to construct the pcDNA3.1 CLEC14A-TTc-Fc vector.

A



B

### pcDNA3.1 CLEC14a-TTc-Fc

#### 1. Upstream region

Expected

5' CTGGCTAGCGCCACCATGGGCCGGCCCGCCTTGGCC 3'

Sequenced

5' CTGGCTAGCGCCACCATGGGCCGGCCCGCCTTGGCC 3'

#### 2. Linker 1

Expected

5' TCCACCGGAGGCAGCGCGGGGCTCTGGCGGA AAA 3'

Sequenced

5' TCCACCGGAGGCAGCGCGGGGCTCTGGCGGA AAA 3'

#### 3. Linker 2

Expected

5' CAAATGACGGAGGCAGCGGAGGAGGCTCCGGAGGAGAGCCC 3'

Sequenced

5' CAAATGACGGAGGCAGCGGAGGAGGCTCCGGAGGAGAGCCC 3'

#### 4. Downstream region

Expected

5' CCCCCGAAAGTAATAGCTCGAGTCTAGAGGGCCC 3'

Sequenced

5' CCCCCGAAAGTAATAGCTCGAGTCTAGAGGGCCC 3'

Legend:

1. pcDNA3.1 + NheI + Kozak + Start codon + CLEC14A

2. CLEC14a + Linker + TTC

Legend:

1. TTC + Linker + Fc-tag

2. Fc-tag + Stop codons + XhoI + XbaI + ApaI

**Figure 3.13 pcDNA3.1 CLEC14A-TTc-Fc assembly analysed by sequencing.** A) Vector map of pcDNA3.1 CLEC14A-TTc-Fc designed on SnapGene. B) Representative Sanger sequencing results of the upstream, downstream and linker regions of pcDNA3.1 CLEC14A-TTc-Fc, aligned with their expected sequences for comparison.

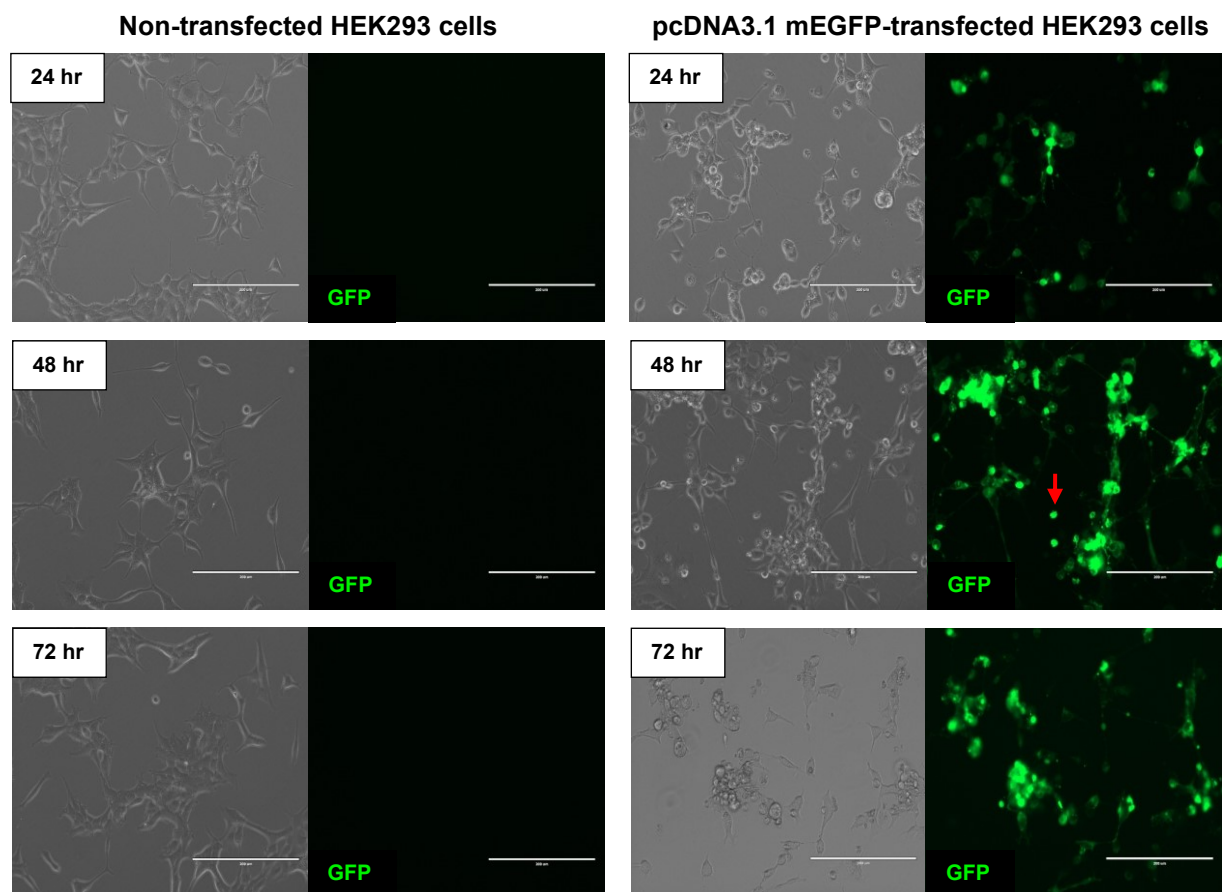
### **3.3.2.3 Transfection and protein production in HEK293 cells**

HEK293 cells were used as a mammalian protein expression system due to their high transfection efficiency, robust growth, human-like post-translational modifications, and scalability for large-scale protein production (Tan et al., 2021). Firstly, the transfection conditions using the transfection reagent (Lipofectamine 3000) were optimised. Cells were transfected with a control plasmid, pcDNA3.1 mGFP, an expression vector for GFP, following the manufacturer's guidelines. At the same time, a transfection-only Lipofectamine 3000 as a non-transfected control was run in parallel. Cells were harvested at 24-, 48-, and 72-hours post-transfection, where cell viability and GFP expression were assessed using the FITC channels; no GFP signal was observed in the non-transfected control cells (Figure 3.14, left). In transfected cells, GFP signal was observed as early as 24 hours post-transfection, with signal intensity proportional to protein expression peaking at 48 hours and remaining at high levels 72 hours (Figure 3.14, right). These results indicate that GFP expression is highest 48 hours after transfection.

To transiently produce CLEC14A-Fc and CLEC14A-TTc-Fc proteins,  $1 \times 10^6$  HEK293 cells at passage 8-10 were transfected with pcDNA3.1 CLEC14A-Fc or CLEC14A-TTc-Fc plasmids using optimised Lipofectamine 3000. Culture supernatants were collected at 24-, 48-, and 72-hours post-transfection (with an additional 96-hour time point for CLEC14A-TTc-Fc). Total protein from the supernatant was precipitated using the TCA-deoxycholate method and quantified using the BCA assay or Nanodrop. Equal amounts of total protein from each sample were separated by SDS-PAGE and stained with Coomassie blue to visualise CLEC14A-Fc (Figure 3.15A) and CLEC14A-TTc-Fc

(Figure 3.16A). Replicate gels were transferred to membranes for Western blot analysis.

Membranes were probed with anti-CLEC14A (green) and anti-human Fc (red) antibodies (Figure 3.15B and Figure 3.16B). CLEC14A-Fc and CLEC14A-TTc-Fc proteins were detected as ~90 kDa and ~140 kDa bands under reducing conditions, respectively (Figure 3.15B and Figure 3.16B, indicated by red arrows).

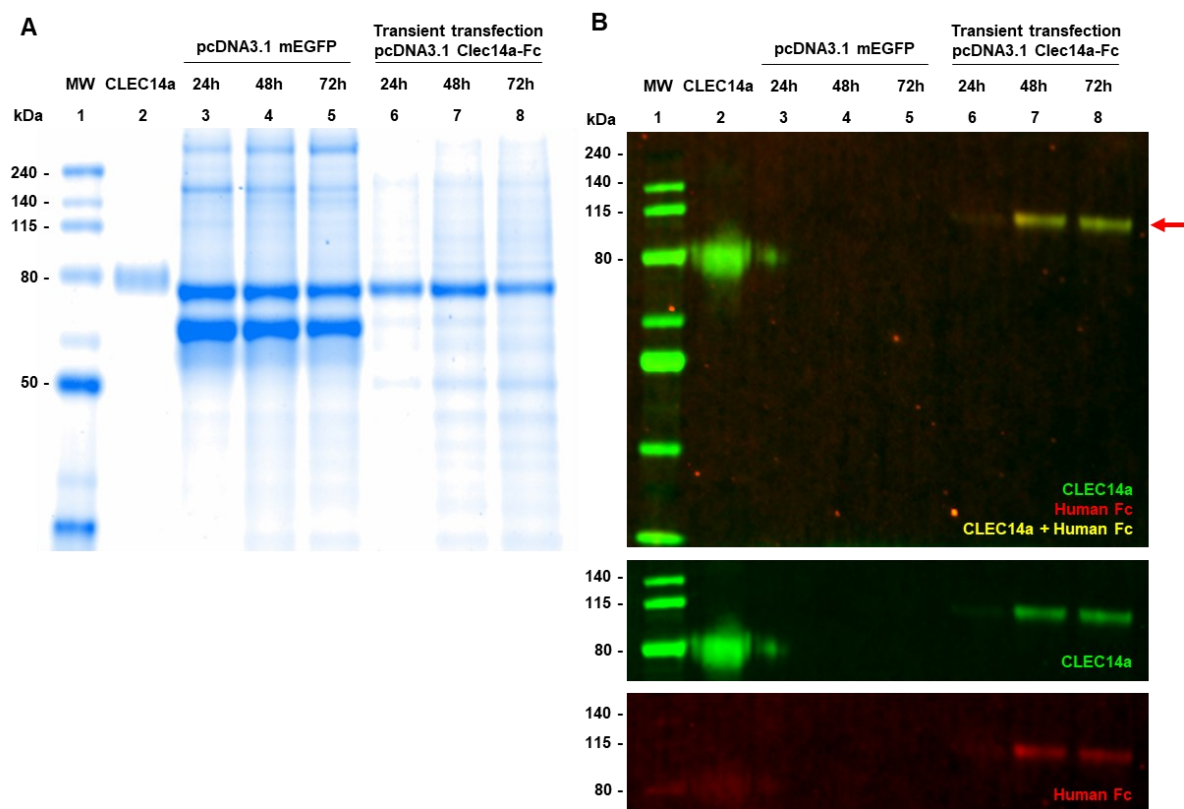


**Figure 3.14 Optimisation of Transfection Timing in HEK293 Cells Using Lipofectamine 3000.** HEK293 cells were transfected with the pcDNA3.1-mGFP vector, while non-transfected HEK293 cells were negative control. Fluorescence microscopy assessed the protein expression level at 24-, 48-, and 72-hours post-transfection. Scale bar: 200  $\mu$ m.

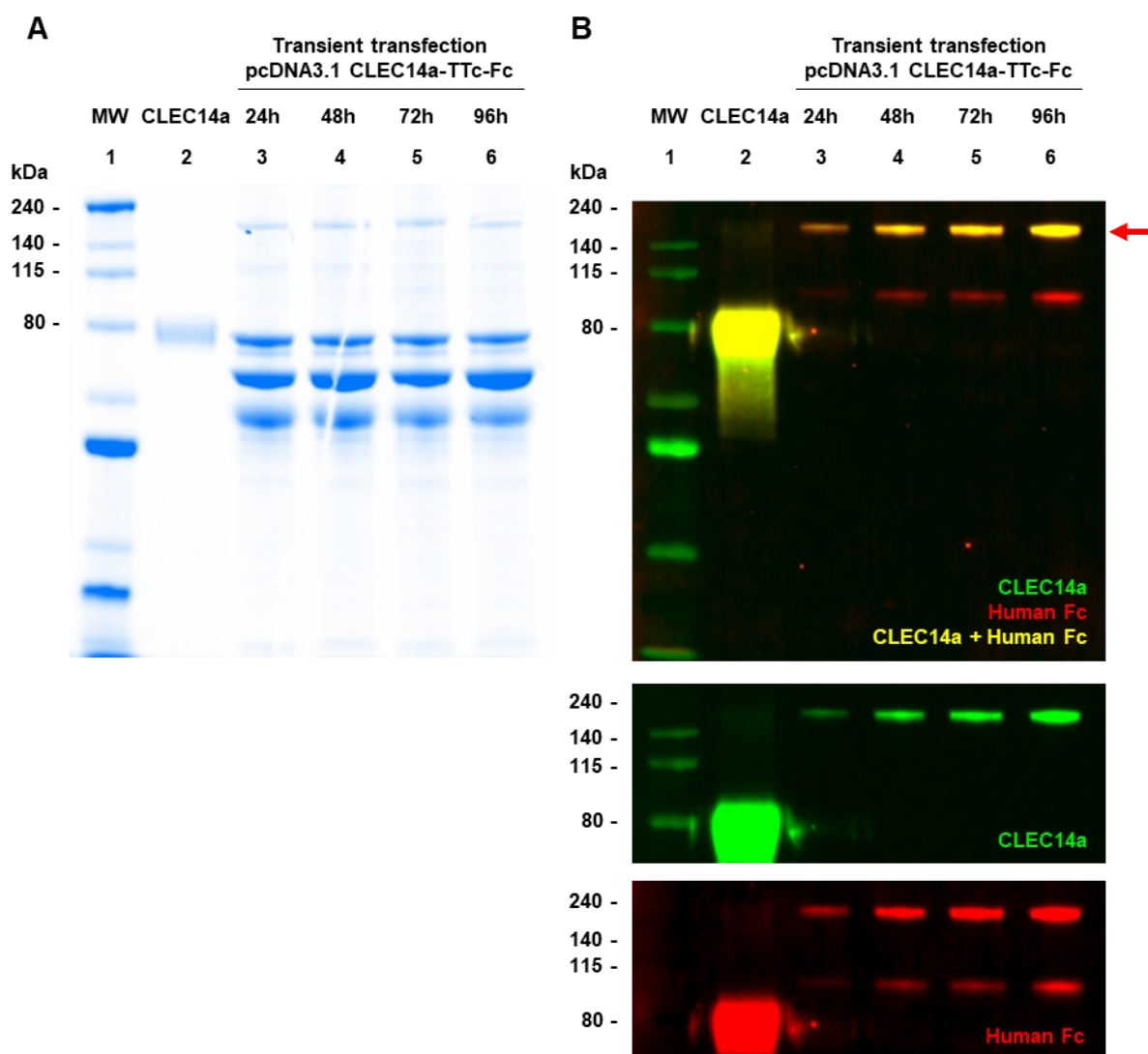
We generated stable HEK293 cell lines expressing CLEC14A-Fc or CLEC14A-TTc-Fc for large-scale, consistent, reproducible protein production. pcDNA3.1 CLEC14A-Fc or CLEC14A-TTc-Fc plasmids were linearized with *ScaI* and transfected into HEK293 cells using Lipofectamine 3000. Transfected cells were selected using G418 antibiotic resistance. The amount of antibiotic G418 was optimised to generate a stable cell line. A high dose of G418 (500 µg/ml) was initially used for the first three days to select stably transfected cells, followed by a lower maintenance dose of 300 µg/ml to sustain selection. Successfully transfected stable cell lines were cultured for approximately one month, and frozen stocks were banked for future use.

Culture supernatants were collected on day 4, and total proteins in the supernatant were analysed by gel electrophoresis, the same method for transient transfection. Equal amounts of proteins were loaded per well for SDS-PAGE. One gel was stained with Coomassie blue to visualise the total protein content (Figure 3.17A). In contrast, a replicate gel was transferred to a membrane and analysed by western blot using anti-CLEC14A (green) and anti-human Fc (red) antibodies (Figure 3.17B). CLEC14A-Fc and CLEC14A-TTc-Fc proteins were detected at ~90 kDa and ~145 kDa, respectively (Figure 3.17B, indicated by blue and red arrows).

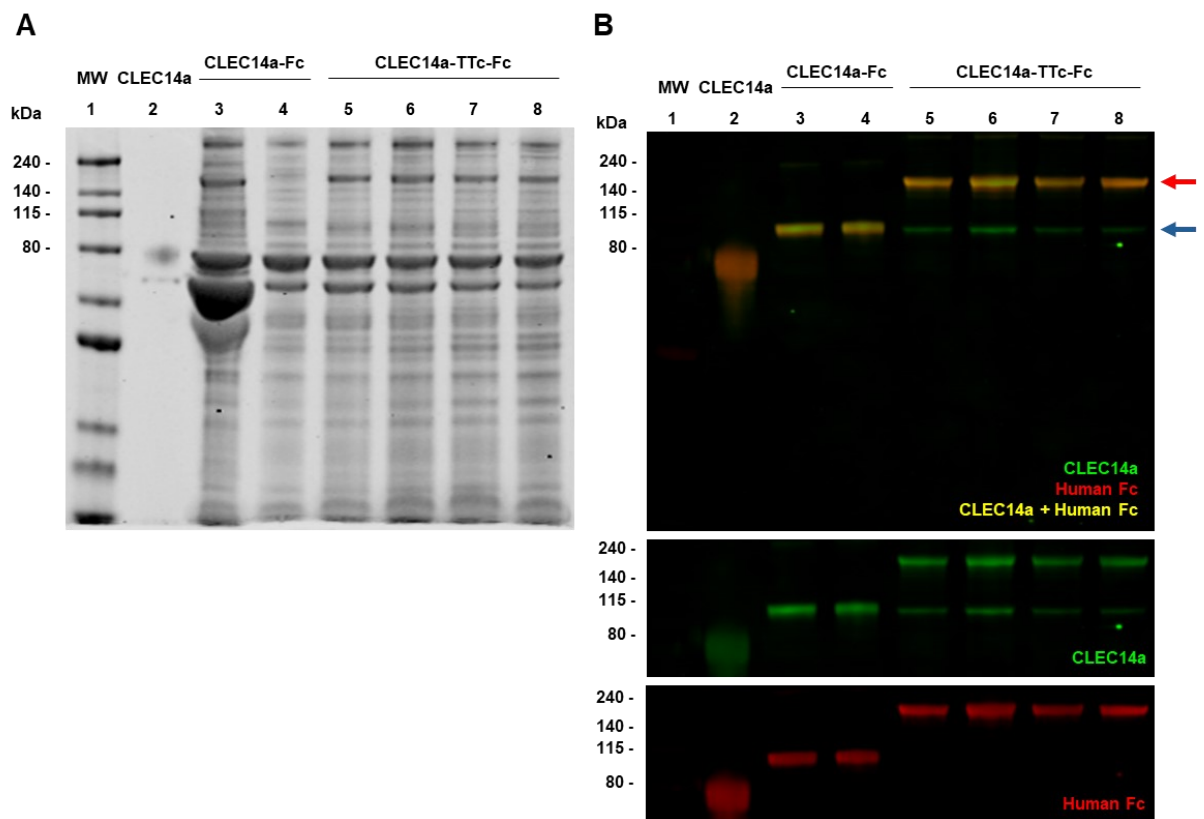
These results confirm successful transient and stable expression of CLEC14A-Fc and CLEC14A-TTc-Fc in HEK293 cells.



**Figure 3.15 CLEC14A-Fc expression in the supernatant HEK293 cells transfected with pcDNA3.1 CLEC14A-Fc vector.** HEK293 cells were transiently transfected with the pcDNA3.1 CLEC14A-Fc vector. Cells were transfected with either pcDNA3.1 CLEC14A-Fc or pcDNA3.1-mEGFP as a control. SDS-PAGE separated protein samples and (A) stained with Coomassie blue for protein content and (B) transferred to a PVDF membrane to detect CLEC14A protein (green) and the Fc tag (red) by western blot. Supernatants were collected at 24-, 48-, and 72-hours post-transfection. CLEC14A-Fc protein was detected as a ~90 kDa band under reducing conditions. MW: Molecular weight in kDa.



**Figure 3.16 CLEC14A-TTc-Fc expression in the supernatant of transiently transfected HEK293 cells after transfected with pcDNA3.1 CLEC14A-TTc-Fc vector.** HEK293 cells were transiently transfected with pcDNA3.1 CLEC14A-TTc-Fc vector, and supernatants were collected on Days 1, 2, 3 and 4. Protein samples were precipitated using trichloroacetic acid (TCA) and analysed by SDS-PAGE. One gel was stained with Coomassie blue to assess total protein content. A second mirror gel was transferred to a PVDF membrane for western blot analysis, detecting CLEC14A protein (green) and the Fc tag (red). A His-tagged commercial CLEC14A protein was used as a positive control. CLEC14A-TTc-Fc protein was identified as a ~145 kDa band under non-reducing conditions. Molecular weights (MW) are shown in kDa.



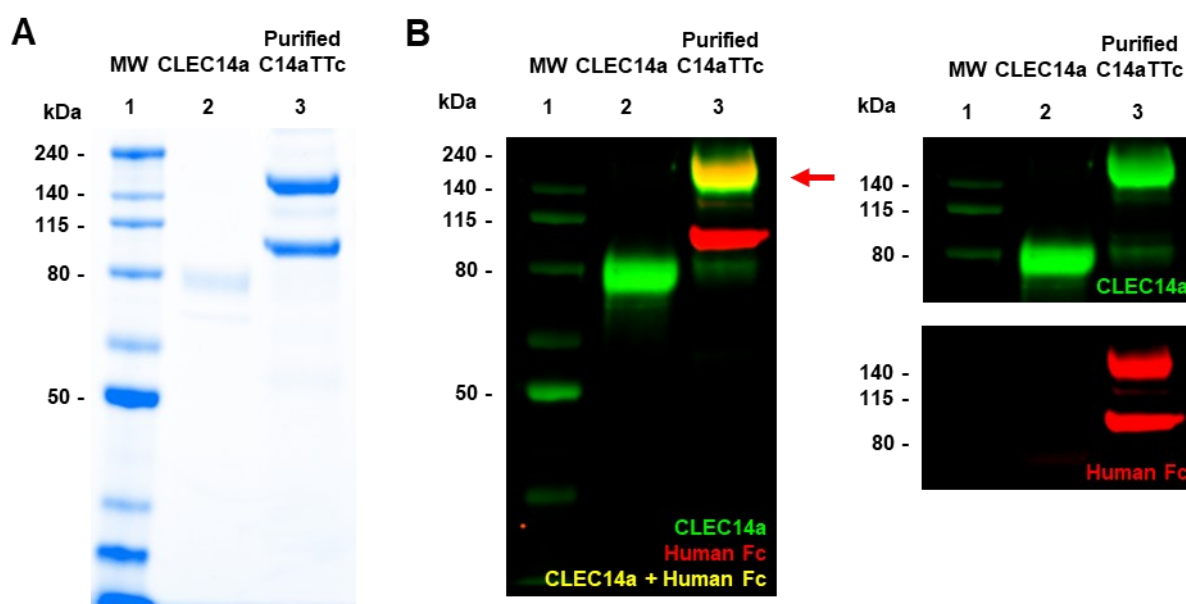
**Figure 3.17 Large-scale production of CLEC14A-Fc and CLEC14A-TTc-Fc in the supernatant of stably transfected HEK293 cells.** HEK293 cells were stably transfected with pcDNA3.1 CLEC14A-Fc or CLEC14A-TTc-Fc vector, and supernatants were collected on Day 4. Protein samples were precipitated using TCA and analysed by SDS-PAGE. (A) A gel stained with Coomassie blue was used to assess total protein content. (B) A second gel was transferred to a PVDF membrane for western blot analysis, detecting CLEC14A protein (green) and the Fc tag (red). A His-tagged commercial CLEC14A protein served as a positive control. CLEC14A-Fc and CLEC14A-TTc-Fc proteins were identified as bands at ~90 kDa and ~145 kDa, respectively. Molecular weights are indicated in kDa.

#### **3.3.2.4 Protein Purification**

CLEC14A-Fc and CLEC14A-TTc-Fc proteins were purified through affinity chromatography. The CLEC14A-Fc protein was specifically purified to serve as a control in Western blot analyses. First, the HiTrap Protein A column was equilibrated with PBS, and then culture supernatant from CLEC14A-Fc and CLEC14A-TTc-Fc stably producing cells passed through to the column at a flow rate of 1 ml/min. The column was washed, and the Fc-tagged proteins were eluted in 0.1 M citric acid (pH 2.7–2.8). The purified proteins were then dialysed in PBS overnight.

Purified proteins were quantified using Nanodrop, and the yield of purified CLEC14A-TTc-Fc protein was approximately 1.5–2 mg/L. Following quantification, the purified protein was analysed by SDS-PAGE and Western blot (Figure 3.18). The SDS-PAGE for CLEC14A-TTc-Fc (Figure 3.18A) demonstrated a relatively high purity using a single-step purification method, eliminating the need for an additional polishing step. The Western blot for CLEC14A-TTc-Fc (Figure 3.18B), detected with anti-CLEC14A (green) and anti-human Fc (red), confirmed the expected ~145 kDa band. The overlapping signals (yellow) verified the successful production and identity of the CLEC14A-TTc-Fc protein. Additionally, two smaller bands were observed on the membrane, indicating that CLEC14A probably undergoes cleavage during production. (Khan, 2016).

These results together confirm the successful construction of the pcDNA3.1 CLEC14A-Fc and CLEC14A-TTc-Fc plasmids, as well as the production and purification of the respective proteins. CLEC14A-TTc-Fc will be used in the following immunisation experiment.



**Figure 3.18 Purification of CLEC14A-TTc-Fc (C14aTTc) protein.** The CLEC14A-TTc-Fc was purified from the supernatant of pcDNA3.1 CLEC14A-TTc-Fc stably transfected HEK293 cells and run on duplicate gels. (A) One gel was stained with Coomassie Blue, (B) while the other was transferred to a PVDF membrane for a Western blot assay to detect CLEC14A protein (green) and the Fc-tag (red). A His-tagged commercial CLEC14A protein was used as a positive control. CLEC14A-TTc-Fc protein was identified as a ~145 kDa band under non-reducing conditions. Molecular weights are shown in kDa.

### **3.3.3 CLEC14A-TTc vaccination breaks immune tolerance to CLEC14A and induces a CLEC14A-specific IgG1 response**

Since CLEC14A is a self-antigen and cannot trigger an immune response, a carrier-priming protocol was optimised to overcome tolerance and induce antibody production (Figure 3.19A) (Escobar-Riquelme, 2024). Briefly, mice (n=5) were primed intraperitoneally (i.p.) with 50 µg of TTc protein in alum. After three to four weeks, they were immunised with 50 µg of CLEC14A-TTc in alum via i.p. Two weeks later, mice were boosted with CLEC14A-TTc. Anti-CLEC14A antibodies were detected using ELISA at different time points.

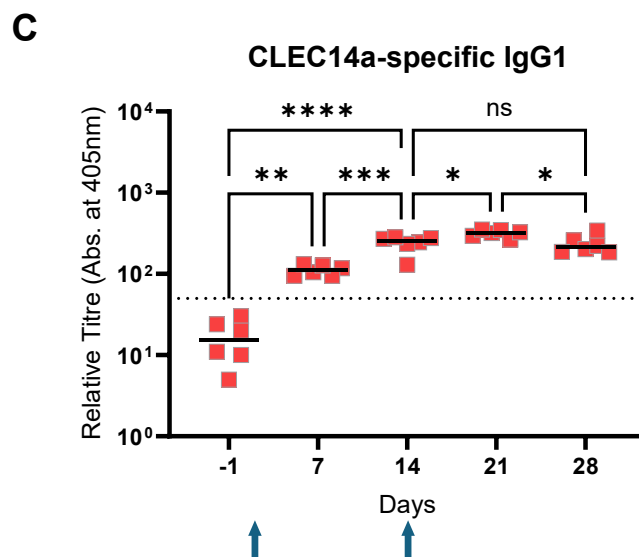
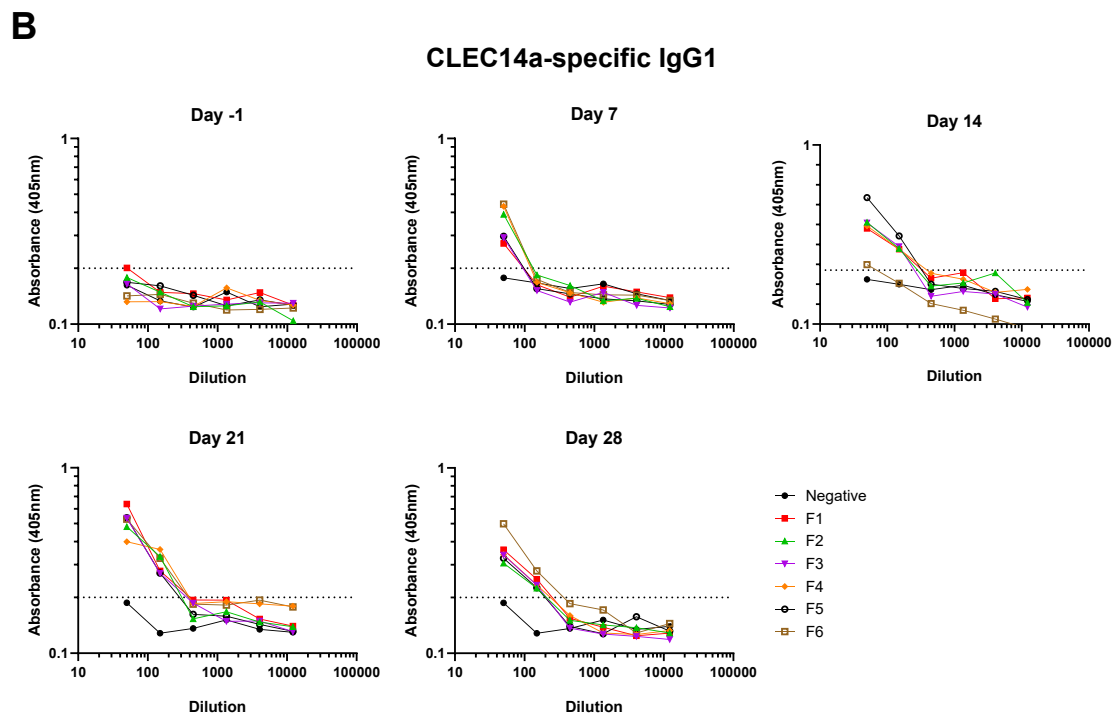
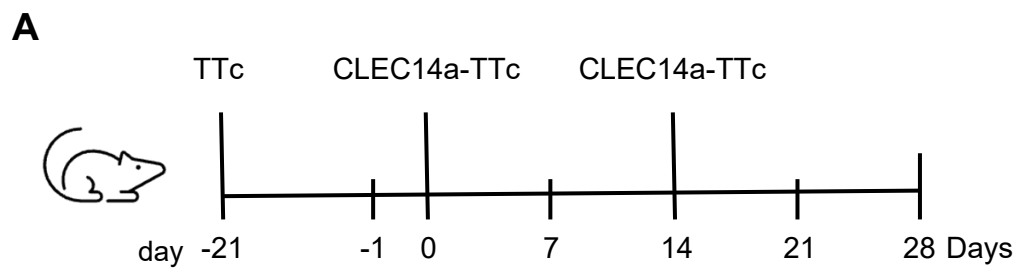
Our primary interest was in measuring IgG1 due to its significant role in the immune response, particularly its involvement in antibody-dependent cellular cytotoxicity (ADCC), opsonisation, and neutralisation, which are crucial for combating extracellular pathogens and toxins, and are often indicative of a Th2-type response induced by alum adjuvant (Schulte et al., 2008). This is particularly relevant for vaccine-induced anti-tumour responses. Furthermore, previous work from our lab and collaborators on ROBO4 vaccination has shown that the IgG1 isotype is the most important and prominently induced isotype in this type of vaccination strategy (Escobar-Riquelme, 2024; Zhuang et al., 2015).

However, assessing the full spectrum of antibody classes (e.g., IgM, other IgG subclasses) at every time point was constrained by the limited blood sample volumes that could be collected from mice at early experimental stages (typically only 10-20 µL of venous blood). This small volume of sera was often only sufficient for a single isotype check per ELISA plate. Therefore, while class-switching to various IgG subclasses and the initial IgM response are critical aspects of adaptive immunity, our analysis primarily

focused on IgG1, which represents the mature, high-affinity antibody response generated through T-cell-dependent mechanisms and class-switch recombination (Andrade et al., 2000).

Throughout the immunisation period, no adverse effects were observed. At the end time point, tissues, including the spleen, lymph nodes, kidneys, heart, liver, and pancreas, were harvested. Future work should involve a comprehensive analysis of these harvested tissues to assess potential off-target effects and confirm the safety profile in the long term.

The commercial His-tagged CLEC14A protein was used to detect CLEC14A-specific antibodies in sera. Figure 3.19B depicts the ELISA binding curves of CLEC14A-specific IgG1 in sera from individual mice at days -1, 7, 14, 21, and 28. As anticipated, no CLEC14A-specific IgG1 antibodies were identified on day -1 before immunisation (Figure 3.19C). The antibody became detectable on day 7 compared to non-injected CLEC14A-TTc mice. Following the second boost, antibody titres significantly increased, peaking on day 21. By day 28, the titres showed a slight decline compared to day 21. These data suggest that carrier priming alongside CLEC14A-TTc vaccination could break self-tolerance to CLEC14A and induce a CLEC14A-specific IgG1 antibody response.



**Figure 3.19 CLEC14A-TTc immunisation induces a CLEC14A-specific IgG1 response.** (A) Protocol of immunisation. Mice were intraperitoneally primed with 50 µg of TTc formulated in a 1:1 (v/v) ratio with alum (n=6). Three weeks later, the mice were boosted with 50 µg of CLEC14ATTc in a 1:1 (v/V) ratio with alum. A second boost was administered 15 days after the first. Blood samples were collected on Days -1, 7, 14, 21 and 28. (B) Anti-CLEC14A IgG1 dilution curves. The sera were analysed for anti-CLEC14A antibodies using ELISA. Each graph corresponds to a specific time point. Normal mouse sera served as a negative control. Each line represents the sera from an individual mouse. Detection lines indicate the threshold used to determine CLEC14A relative titres. (C) CLEC14A-specific IgG1 titres following immunisation. Each symbol represents an individual mouse (n = 6). Bars indicate the median and the threshold set at 50 (detection line). Statistical analysis was performed using ordinary one-way ANOVA for multiple comparison (\*\*\*\* p < 0.0001; \*\*\* p < 0.001; \*\* p < 0.01; \* p < 0.05; ns, not significant).

### 3.4 Discussion

In this study, we confirmed the expression of CLEC14A in tumour endothelial cells of mouse models (4T1, E0771, and LLC1). Immunofluorescence staining and qRT-PCR analyses demonstrated that CLEC14A is highly expressed in tumour vasculature but absent in normal tissues, further suggesting its specificity as a therapeutic target (Figure 3.1 and Figure 3.2). These findings align with previous studies that CLEC14A is identified as a tumour endothelial marker with no significant role in physiological angiogenesis (Mura et al., 2012; Noy et al., 2016).

This study's successful construction and expression of CLEC14A-TTc and CLEC14A-Fc fusion proteins demonstrate a robust strategy for developing conjugate cancer vaccines targeting tumour angiogenesis. This work involves genetically linking the extracellular domain of CLEC14A, a tumour endothelial marker, to the potent immunogenic fragment of tetanus toxin (TTc) to enhance immune recognition and antibody-mediated anti-tumour effects.

Initial cloning and subcloning strategies were optimised to overcome common molecular biology challenges. Incorporating the *ScaI* enzyme during subcloning significantly improved fragment separation, effectively reducing unwanted ligation products and enhancing overall cloning efficiency.

The presence of expected protein bands at ~90 kDa for CLEC14A-Fc and ~145 kDa for CLEC14A-TTc-Fc on Western blot analyses confirmed successful expression and secretion of the target proteins (Figure 3.17). The production of stable cell lines provided a reliable and reproducible platform for large-scale protein production.

Affinity chromatography purification using Protein A columns yielded CLEC14A-TTc-Fc protein in quantities sufficient for subsequent experimental applications, although minor degradation bands indicated proteolytic cleavage of the CLEC14A component during protein expression. This observation confirms previous findings that CLEC14A proteins can undergo partial proteolytic processing (Khan, 2016). Future studies should investigate strategies to minimise such cleavage events, possibly through modifications in culture conditions, protease inhibitors, or protein engineering approaches.

We developed a conjugate vaccine using TTc as a carrier protein genetically linked to the extracellular domain of mouse CLEC14A to enhance the immune response against CLEC14A. The successful cloning and expression of CLEC14A-TTc in HEK293 cells provided the recombinant protein for immunisation studies. Protein purification and validation via SDS-PAGE and Western blotting confirmed the integrity of the CLEC14A-TTc fusion protein, ensuring its suitability for further immunogenicity assessments (Figure 3.18).

Previous studies have demonstrated that TTc as a carrier can enhance immune responses against weak antigens by eliciting strong CD4<sup>+</sup> T cell activation (Chotprakaikiat et al., 2016; Xu et al., 2021). Our approach leveraged this strategy to increase CLEC14A-specific immunogenicity, aiming to generate an immune response targeting CLEC14A-expressing tumour vasculature. The protein expression and purification results set the stage for in vivo immunisation and tumour inhibition studies, which will be crucial in determining the vaccine's efficacy in cancer models.

The CLEC14A-TTc vaccine effectively overcame self-tolerance to CLEC14A and induced a robust IgG1 response in mice (Figure 3.19C). Furthermore, incorporating CpG oligodeoxynucleotides (ODNs) as an adjuvant may further enhance this immune response. CpG ODNs act as immune stimulants by mimicking bacterial DNA, activating Toll-like receptor 9 (TLR9) and improving innate and adaptive immune responses (Krieg, 2007). Specifically, types B (ODN1826) and C (ODN2395) CpG ODNs have been shown to significantly improve the immune response against NP (4-hydroxy-3-nitrophenyl acetyl) as reported in (Escobar-Riquelme, 2024). Furthermore, in my experiments, I observed that combining CpG B with the ROBO4TTc vaccine in alum resulted in higher ROBO4-specific IgG1 titres than alum alone (see Appendix C). This suggests that CpG ODNs could similarly boost the antibody response against CLEC14A.

### **3.5 Conclusion**

The CLEC14A-TTc conjugate vaccine was successfully generated by genetically linking the extracellular domain of mouse CLEC14A to TTc through successful cloning, expression, and purification. Subsequent analysis demonstrated that CLEC14A-TTc vaccination effectively induced a CLEC14A-specific IgG1 antibody response. Future investigations may explore the impact of CLEC14A vaccination on tumour growth.

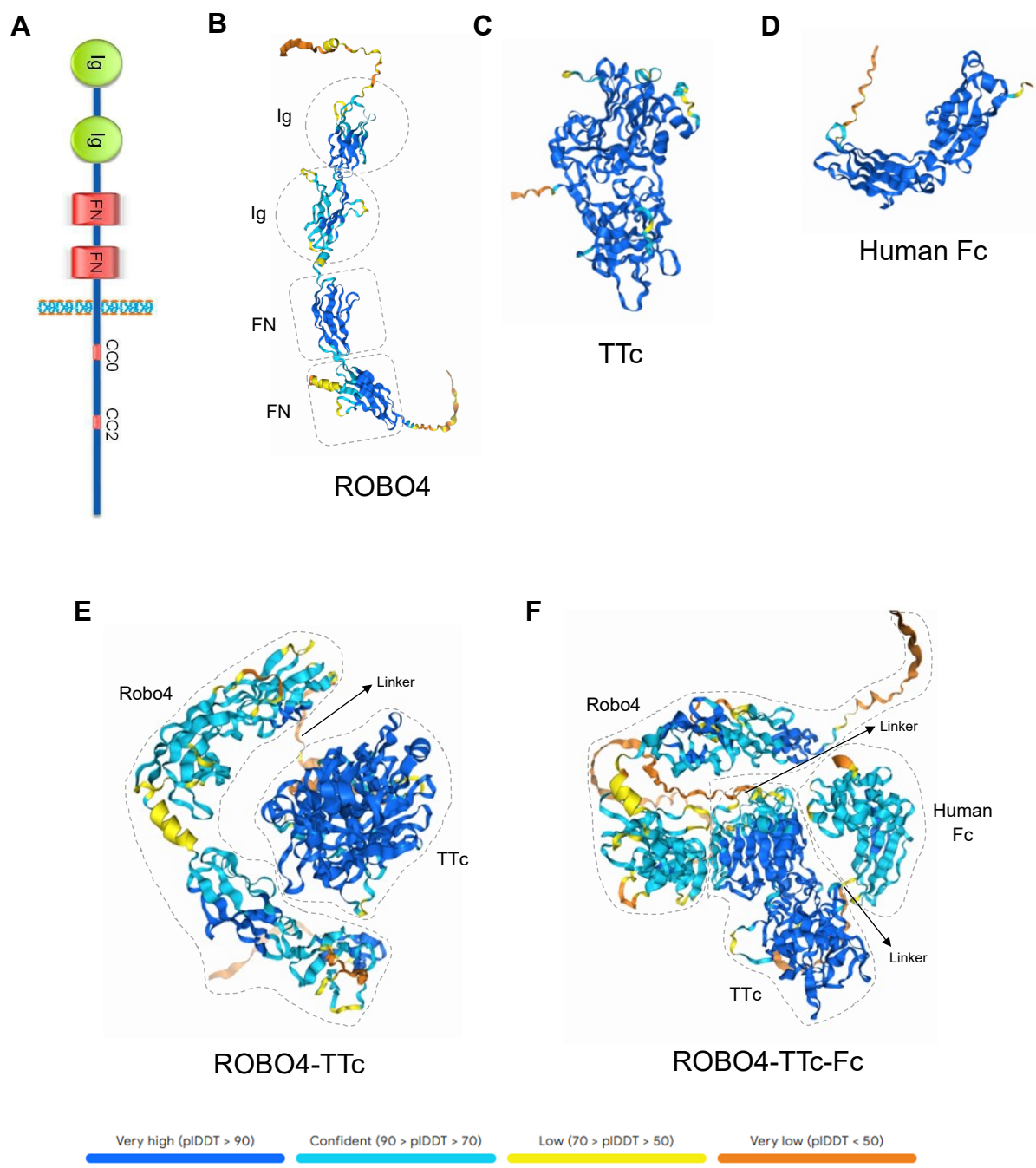
## **Chapter 4: ROBO4-TTc conjugate vaccine enhances adaptive immunity and alleviates innate immune suppression in TNBC (4T1) tumours compared to TTc-vaccinated controls.**

### **4.1 Introduction**

Angiogenesis, the formation of new blood vessels, is a fundamental hallmark of cancer, enabling tumour growth and metastasis by supplying oxygen and nutrients (Hanahan, 2022; Hanahan & Weinberg, 2011). Therefore, anti-angiogenic therapies have become a type of cancer treatment.

Within the tumour microenvironment, conditions such as hypoxia and low shear stress drive molecular changes in endothelial cells, leading to the expression of tumour endothelial markers (TEMs), such as Roundabout guidance receptor 4 (ROBO4), a transmembrane receptor primarily expressed on endothelial cells (Figure 4.1A). ROBO4 plays a crucial role in angiogenesis by regulating endothelial cell migration, proliferation, and tube formation in cancer (Dai et al., 2019; Park et al., 2003).

The predicted 3D structure of ROBO4 (Figure 4.1), as estimated by AlphaFold, reveals a multi-domain extracellular region comprising two immunoglobulin (Ig)-like domains and two fibronectin type-III repeats (Xiao et al., 2022). Notably, the structure also suggests regions that are not fully folded, particularly the linker regions and certain tails. This less-structured or partially exposed nature of the protein may enhance its susceptibility to proteolytic cleavage (Figure 4.1B).



**Figure 4.1 Structure of the ROBO4 proteins.** (A) ROBO4 receptor structure, adapted from Alan Zhuang's thesis. ROBO4 comprises two Ig and two fibronectin domains in the extracellular region. (B, C, D, E, and F) Predicted 3D structures of ROBO4, TTc, Human Fc, ROBO4-TTc, and ROBO4-TTc-Fc generated using AlphaFold. The images illustrate the predicted folding and linker positioning by displaying the detailed spatial arrangements of each domain and the fusion constructs.

Preclinical studies have highlighted the therapeutic potential of targeting ROBO4 (Hirobe et al., 2022). Studies have shown that the ROBO4 vaccine and anti-ROBO4 CAR T cells can significantly reduce tumour growth in cancer models (Hirobe et al., 2022; Zhuang et al., 2015). Both monoclonal antibody and CAR T cell therapies face challenges, including high production costs that limit accessibility, particularly in developing countries.

Active vaccination, such as recombinant protein vaccine, can elicit polyclonal antibody responses targeting multiple epitopes, which may enhance immune efficacy.

However, due to insufficient T-cell activation, ROBO4 is a self-antigen, limiting its ability to provoke a robust immune response (Zhuang et al., 2015). By linking a weak antigen to an immunogenic carrier protein, such vaccines induce carrier-specific CD4<sup>+</sup> T cells to support B-cell activation, and antibody production targets the weak antigen (Rappuoli et al., 2019).

Our lab developed a conjugate vaccine that can induce high titres of antibodies specific to ROBO4 (Zhuang et al., 2015). By chemically linking the extracellular domain of ROBO4 to a common foreign vaccine carrier protein, chicken gamma globulin (CGG), autoreactive B cells can recruit T cell help from primed carrier-specific memory T helper cells. This protocol could efficiently induce specific autoreactive anti-tumour vessel antibodies that suppress tumour growth.

Our lab plans to translate this vaccine protocol to carrier proteins used in human vaccination. Tetanus toxin, a non-toxic fragment (TTc), has been selected as the carrier protein. TTc is one of the most widely used carrier proteins in developing conjugate vaccines (Bayart et al., 2022). ROBO4, genetically linked to TTc, has been generated.

The Lewis Lung Cancer (LLC1) model, a non-inflamed, poorly immunogenic tumour resistant to immune-checkpoint inhibitor therapies (ICT), was used in further studies (Sun et al., 2021). These studies showed that ROBO4-TTc in an alum vaccine can induce the production of a high titre of ROBO4-specific antibodies and retard tumour growth in this model (Escobar-Riquelme, 2024). Our vaccine strategy may therefore improve cancer therapy outcomes for ICT-resistant tumours.

Triple-negative breast cancer (TNBC) is an aggressive and heterogeneous subtype of breast cancer. TNBC poses a significant clinical challenge due to the absence of the oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Aysola et al., 2013) and the limited immune response of immune checkpoint inhibitor treatment (ICT) (Guo et al., 2024). TNBC treatment relies primarily on chemotherapy, radiotherapy and surgery, which is associated with high recurrence rates and poor prognosis (Obidiro et al., 2023), which underscores the need for novel therapeutic approaches.

The 4T1 murine model is widely used in TNBC research due to its aggressive growth, immune evasion, and spontaneous metastasis to the lungs, liver, and bones. This model closely mimics human TNBC, making it valuable for studying tumour biology and evaluating novel treatments (Arroyo-Crespo et al., 2019). Tumour angiogenesis is a key driver of TNBC progression, facilitating growth and metastasis. Indeed, TNBC vasculature is often abnormal and dysfunctional, leading to hypoxia, increased proliferation, and immune evasion (Ribatti et al., 2016).

Targeting angiogenesis presents a potential therapy for TNBC therapy. While vascular endothelial growth factor (VEGF) inhibitors have been explored in clinical trials, their

effectiveness is often limited by adaptive resistance mechanisms (Haibe et al., 2020). Alternative approaches targeting non-VEGF angiogenic regulators, such as ROBO4, offer new therapeutic opportunities (Dai et al., 2019). Our ROBO4-TTc vaccine may provide another potential method to enhance TNBC treatment.

## 4.2 Chapter aims

Our lab has developed a conjugated vaccine that induces an antibody response against the tumour vessel-expressed antigen ROBO4. This vaccine can inhibit tumour growth in immune checkpoint therapy (ICT)-resistant tumours, such as the Lewis Lung cancer model (LLC1). This chapter aims to evaluate the effect of this ROBO4 conjugate vaccine on another ICT-resistant tumour, the triple-negative breast cancer model (4T1).

The specific objectives of this chapter are as follows:

- i. Confirm ROBO4 expression on the 4T1 mouse model.
- ii. Produce and purify the ROBO4TTc protein.
- iii. Assess ROBO4-specific antibody generation and its impact on tumour growth.
- iv. Analyse vascular integrity, immune infiltration, and endothelial interactions using multiplex imaging.

This chapter addresses these objectives by providing insights into tumour-immune interactions and exploring the mechanism of ROBO4TTc vaccination as cancer immunotherapy.

## **4.3 Results**

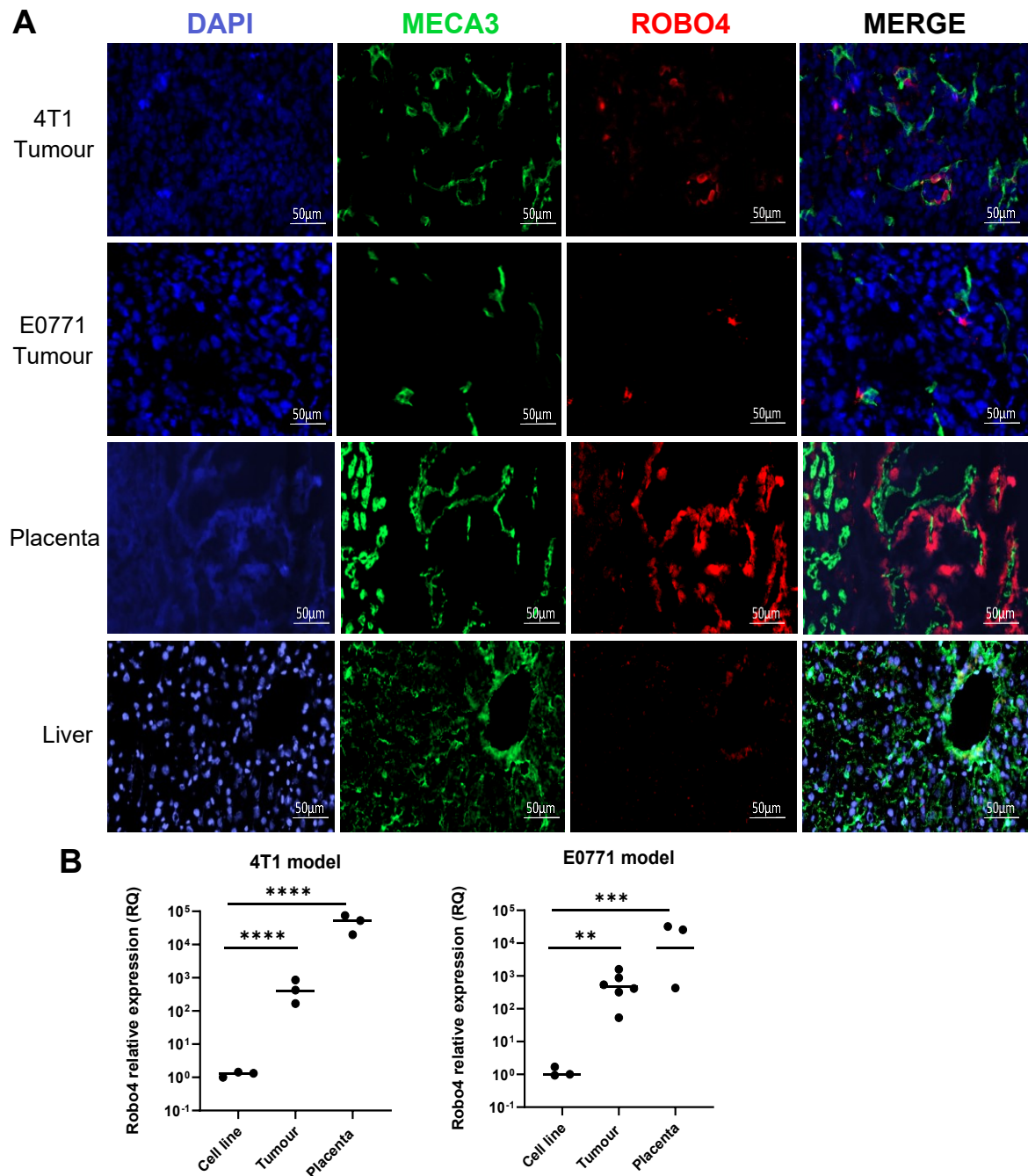
### **4.3.1 ROBO4 is highly expressed in the triple-negative breast cancer (TNBC) mouse tumour**

To assess ROBO4 expression in tumours and control tissues, various mouse models were developed using different tumour cell lines and transplantation techniques. These experiments utilized syngeneic tumour models, where tumour cells derived from a mouse of a particular inbred strain are implanted into a genetically identical (syngeneic) mouse of the same strain. This approach ensures immunological compatibility between the host and the tumour, allowing for the study of tumour-immune interactions in an immunocompetent setting. Specifically, the E0771 cell line was used for C57BL/6 mouse models, whilst the 4T1 cell line was used for BALB/c mouse models. Breast tumour cells, 4T1 and E0771, were transplanted subcutaneously into the fourth mammary fat pad of the recipient mice. Mice were culled three to four weeks post-transplantation, and tumours were harvested. Placenta tissues were used as a positive control for ROBO4 antibody staining.

ROBO4 protein expression in the tissues was assessed using immunofluorescence staining. Tumour and control tissues were stained with anti-ROBO4 (polyclonal rabbit IgG) or a rabbit IgG isotype control and anti-MECA32 (a pan-endothelial cell antigen expressed in most mouse endothelial cells) (Bayart et al., 2022). ROBO4 was visualised in red and MECA32 in green, with most ROBO4 signals (red) colocalising (yellow) or closely localising with MECA32 signals (green) in 4T1 and E0771 tumours (Figure 4.2A). Immunofluorescence analysis revealed strong ROBO4 expression on mouse placental blood vessels, while no expression was detected in normal adult

mouse tissue (liver). Adult healthy tissue typically exhibits little to no ROBO4 expression (Huminiecki et al., 2002; Zhuang et al., 2015). ROBO4 expression was observed on some blood vessels in 4T1 and E0771 tumours (Figure 4.2A). These findings demonstrate that ROBO4 is highly expressed in tumour-associated blood vessels, particularly in the 4T1 tumour, but is absent in blood vessels of healthy tissues (e.g., liver). Furthermore, ROBO4 mRNA expression was assessed in the tumours using qRT-PCR. This analysis showed high levels of ROBO4 expression in 4T1 and E0771 tumours compared to the cell line, suggesting that transplantation of the tumour cells led to an upregulation of this endothelial-specific marker ROBO4 (Figure 4.2B, data from Escobar-Riquelme (2024)).

This suggests that ROBO4 may be a potential target, and all these findings indicate that the 4T1 and E0771 tumour models are suitable for this study.



**Figure 4.2 ROBO4 Expression in 4T1 and E0771 tumours.** (A) Immunofluorescence staining presents ROBO4 protein expression in mouse breast tumours (4T1 and E0771 models) and healthy mouse placenta (positive control) and liver (negative control) tissues. Four weeks post-transplantation into the mammary fat pad, tumours were harvested for analysis. Staining involved labelling ROBO4 in red, endothelial cells with MECA32 in green, and nuclei with DAPI in blue. Scale bars: 50  $\mu$ m. (B) mRNA expression of *Robo4*. *Robo4* expression in 4T1 and E0771 tumours was normalised to *B2m* and compared to their corresponding cell lines. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons on log-transformed data. Significance levels: \*\*\*\*  $p < 0.0001$ ; \*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ . mRNA data from Escobar-Riquelme (2024).

#### **4.3.2 ROBO4-TTc production and purification**

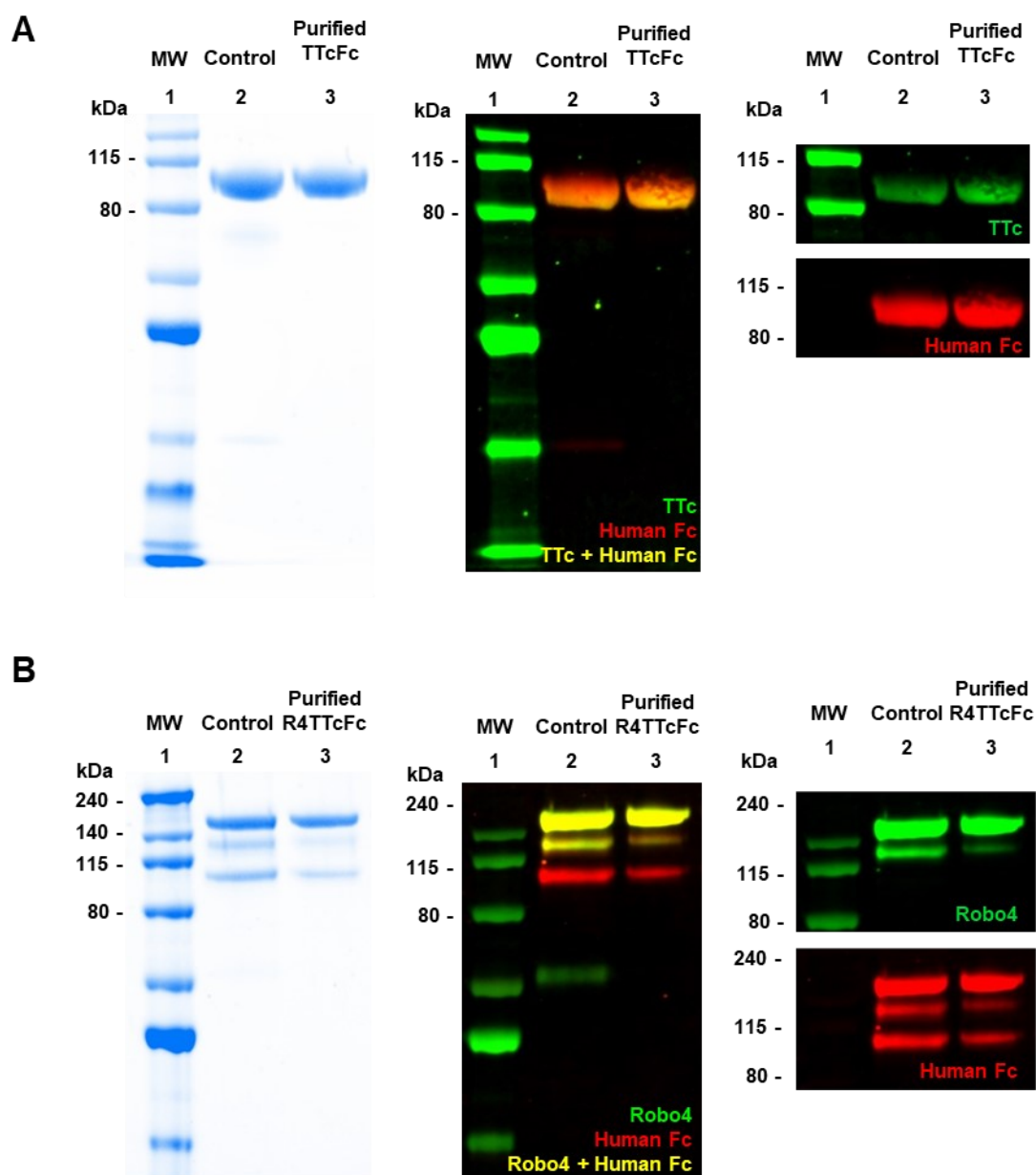
To produce TTc and ROBO4TTc proteins for vaccination, HEK293 cell lines stably producing TTc or ROBO4TTc were obtained from Dr. Fernanda Nicole Escobar-Riquelme (University of Birmingham). As described in Escobar-Riquelme's PhD thesis (Escobar-Riquelme, 2024), the extracellular domain of mouse ROBO4 was coupled to tetanus toxin fragment C (TTc) using a GSS DNA linker to enable the formation of ROBO4TTc for protein expression. The GSS linker—a flexible glycine-serine-serine sequence—connects the ROBO4, TTc, and Fc domains to maintain the structural integrity and flexibility of the fusion proteins. An Fc tag was incorporated into the sequence for detecting and downstream purification of ROBO4-TTc. HEK293 cells were transfected with pcDNA3.1-ROBO4TTc to express the constructs stably and were subsequently selected, following the protocol detailed in Chapter 1 (CLEC14ATTc protein production).

The predicted 3D structures of ROBO4, TTc, Human Fc, ROBO4-TTc, and ROBO4-TTc-Fc were generated using AlphaFold (Figure 4.1). The amino acid sequences of each protein were submitted to the AlphaFold structure prediction tool to model their 3D conformations (for the complete DNA and amino acid sequences, see Appendix A). The images illustrate the predicted folding patterns and linker positioning, showing the spatial organisation of each domain and fusion construct.

HEK293 cells stably expressing TTc/ROBO4-TTc were cultured, and the cell supernatant was harvested on day 4 by centrifugation and filtered and stored at -20°C. Affinity chromatography (as described in Chapter 1) was used to purify Fc-tagged TTc (Figure 4.3A) and ROBO4TTc (Figure 4.3B) proteins, which were then stored at 4°C. Proteins were then analysed using SDS-PAGE for protein purity assessment and

Western blot to confirm protein identity. The first gel was stained with Coomassie Blue to assess total protein content, while the second gel was transferred to a PVDF membrane and stained with anti-TTc/ROBO4 (green) and anti-Human Fc (red) antibodies. The expected sizes of TTc-Fc and ROBO4TTc-Fc are 80.34 kDa and 131.66 kDa, respectively.

In summary, TTc and ROBO4TTc proteins were successfully produced, purified and stored for immunisation experiments.



**Figure 4.3 Analysis of purified TTc-Fc and ROBO4TTc-Fc proteins from stable HEK293 cell lines.**

(A) TTc-Fc (TTcFc) and (B) ROBO4TTc-Fc (ROBO4TTcFc or R4TTcFc) protein analysis. Supernatants were processed and run on duplicate SDS-PAGE gels. One gel was stained with Coomassie Blue to visualise total protein (Left), and the other was used for Western blotting (Right), detecting TTc or ROBO4 protein in green and Fc-tag in red. Molecular weights are approximate: ROBO4TTcFc, 131.66 kDa; TTcFc, 80.34 kDa; TTc, 53.69 kDa; and Fc, 26 kDa. The anti-ROBO4 antibody is shown in green, and the anti-human Fc antibody is shown in red. Lane 1: protein ladder, Lane 2: TTcFc or ROBO4TTcFc conjugate protein control, Lane 3: purified TTcFc or ROBO4TTcFc protein used for priming mice.

#### **4.3.3 ROBO4-TTc vaccination induces a ROBO4-specific IgG1 response and restricts the tumour growth in the TNBC mouse model**

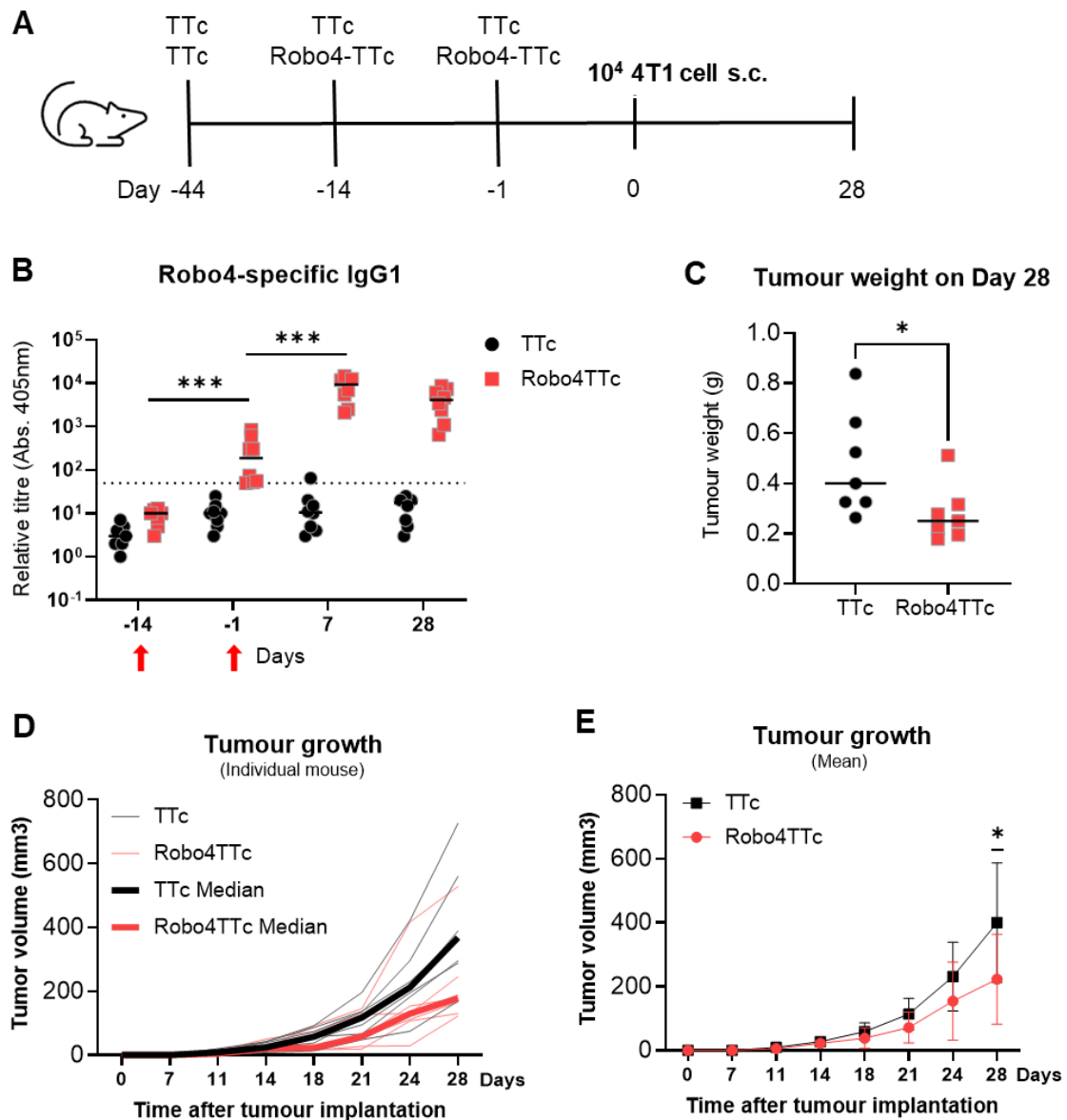
To evaluate the effect of the ROBO4TTc vaccine, an optimised carrier-priming and boost strategy (Escobar-Riquelme, 2024) was employed. Briefly, BALB/c mice (n=16) were primed intraperitoneally (i.p.) with alum-precipitated TTc (Figure 4.4A). Three to four weeks later, mice were i.p. immunised with either TTc (n=8) or ROBO4TTc (n=8) in alum, and a second boost was administered two weeks later. One day after the second boost, all mice were subcutaneously (s.c.) injected with  $2 \times 10^4$  4T1 cells into the fourth mammary fat pad. Blood samples were collected on -21 d (before immunisation with ROBO4-TTc), -14 d (7 days after the first immunisation with ROBO4-TTc), -1 day (before the second boost), 8 d (8 days after the second immunisation with ROBO4-TTc), and 28 d after the transplantation of tumour cells (tumour cell transplantation as day 0). Tumour size was measured weekly, and all mice were culled on 28 d. No side effects or adverse events were observed in mice during the experiment.

ROBO4-specific IgG1 antibodies were quantified by ELISA. As expected, no ROBO4-specific IgG1 antibodies were detected on -21 d (before immunisation; Figure 4.4B). By day -14 d (one-week post-first immunisation with ROBO4-TTc), ROBO4-specific antibodies were detectable in the ROBO4TTc group. However, by 8 d (one-week post-second immunisation with ROBO4-TTc), ROBO4-specific antibody titres in the ROBO4TTc group increased significantly compared to -14 and -21 d, reaching their peak. From 8 d to 28 d, antibody titres remained stable without significant fluctuations. In contrast, as expected, no ROBO4-specific IgG1 was detected at any time in the TTc group.

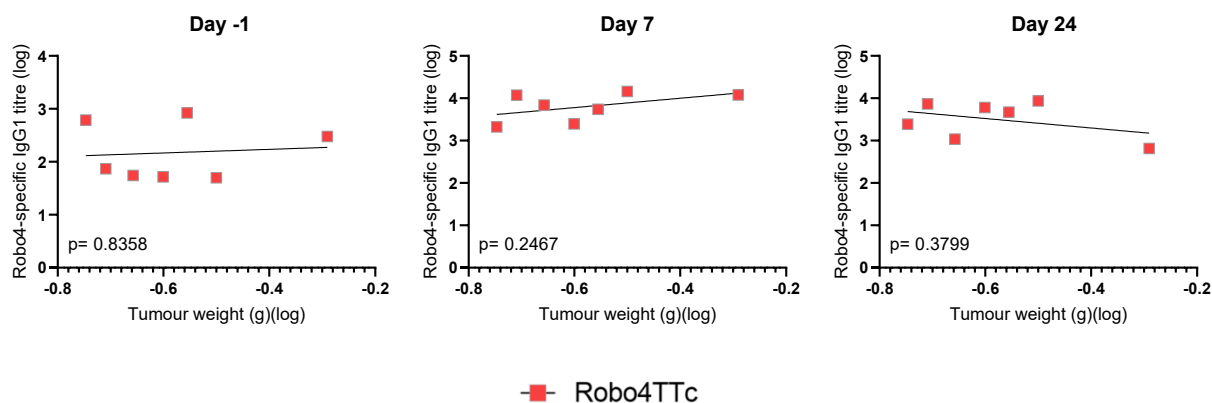
Tumour growth data were plotted for individual mice, with median and mean values and error bars representing the standard deviation (SD) (Figure 4.4D and E). Tumours were detectable approximately 10 days post-transplantation. Tumour growth in the ROBO4TTc group was significantly slower than in the TTc group.

Correlation analyses were performed between the final tumour weights (Day 24) and ROBO4-specific IgG1 antibody titres on -1, 7, and 24 d (Figure 4.5). Statistical analysis was conducted using data from the ROBO4-TTc groups in the 4T1 tumour experiments (Figure 4.4). A negative correlation trend was observed between ROBO4-specific antibody titres on Day 24 and the final tumour weights (Day 24) (Figure 4.5).

These findings demonstrate that the ROBO4TTc vaccine successfully overcomes self-tolerance, inducing a robust ROBO4-specific IgG1 antibody response and significantly inhibiting tumour growth in 4T1 models.



**Figure 4.4 Restricts tumour growth after injection of ROBO4-TTc.** (A) Immunisation and tumour challenge protocol. Mice were i.p. primed with TTc in alum. Three weeks later, two groups were immunised with TTc or ROBO4TTc with alum. A second boost was administered 15 days after the first. One day after the second booster, all mice were s.c. transplanted with 4T1 cells. Blood samples were collected on -14, -1, 7, and 28 d. (B) ROBO4-specific IgG1 titres post-immunization. The arrow shows the ROBO4-TTc boost (n=8). Each symbol represents an individual mouse. Bars indicate the median, and a threshold was set at 50. (C) Tumour weight. (D) Tumour growth curves. The data was presented as the median of each group with individual data lines for each mouse. (E) Mean tumour growth curves, where error bars represent the standard deviation (n=7). Statistical significance was assessed using two-way ANOVA with Tukey's multiple comparisons test for matched data, \*\*\*\*p=0.0002. Tumour weights were compared using the Mann-Whitney test (\*p < 0.05), with bars representing the median weight. Statistical significance was assessed using the Mann-Whitney test, \*\*\*\*p=0.0002.

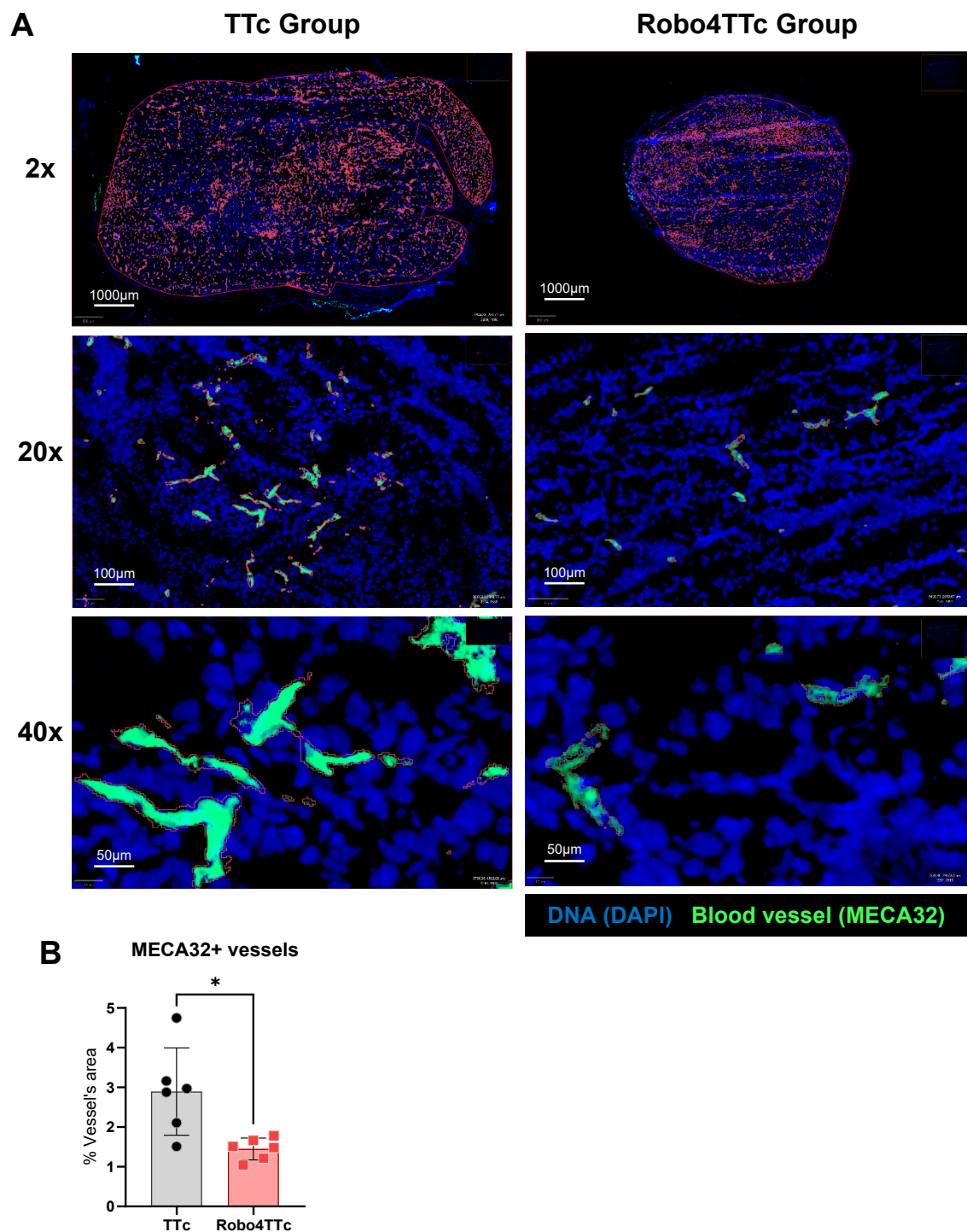


**Figure 4.5 Correlation analysis between ROBO4-specific IgG1 antibody titres and tumour weights at Day 24.** ROBO4TTc were analysed on -1, 7 and 24 d before and after the 4T1 tumour cell transplantation (n=7). Nonlinear regression (curve fit) was used for statistical analysis. Each symbol represents an individual mouse.

#### **4.3.4 ROBO4TTc vaccination reduces vascularity in the tumour**

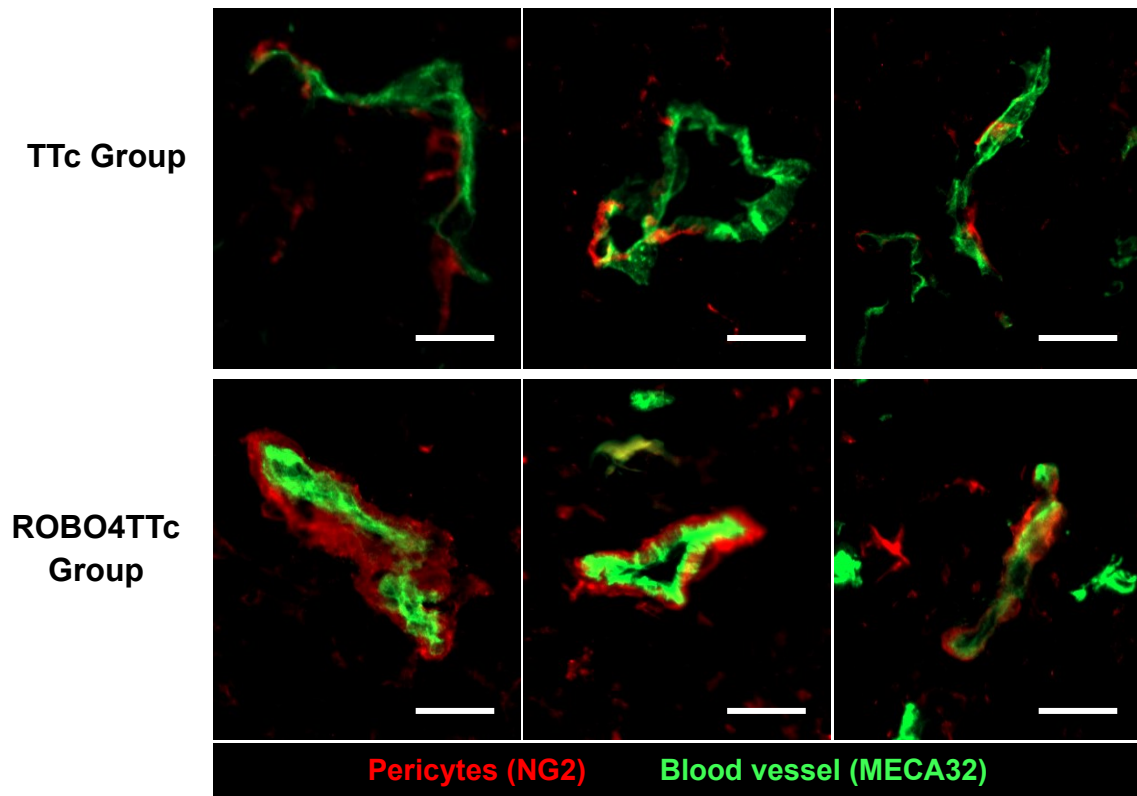
Following confirmation of the induction of ROBO4-specific antibody response after the ROBO4-TTc vaccination, we next assessed the influence of ROBO4 vaccination on angiogenesis in 4T1 tumours. Tumour vasculature was evaluated using immunofluorescence staining, with MECA32 (green) for vessel endothelial cells and DAPI (blue) for nuclei staining (Figure 4.6A). The percentage of vessel area relative to the total tumour section was quantified using QuPath software, with vessel regions automatically delineated in red (Bankhead et al., 2017; Mazor et al., 2022). MECA32+ vessel area was significantly reduced in tumours from the ROBO4TTc group compared to controls (TTc: n=6; ROBO4TTc: n=6) (Figure 4.6B). These findings suggest that the ROBO4TTc vaccination inhibits tumour growth and is associated with a reduction in tumour vascularisation.

To investigate whether ROBO4 targeting induces alterations in tumour vessels, the same tumour sections were stained with two different markers, neuron-glial antigen 2 (NG2) and platelet-derived growth factor receptor alpha (PDGFR- $\beta$ ), to assess pericyte coverage (Figure 4.7A and Figure 4.8A). Quantification of NG2<sup>+</sup> pericyte coverage relative to CD31<sup>+</sup> endothelial cells was analysed using ImageJ (Figure 4.7B). The data indicated that NG2<sup>+</sup> pericyte coverage significantly increased in the tumours from ROBO4TTc-treated mice compared to controls ( $p < 0.05$ ) (Figure 4.7B). In contrast, PDGFR- $\beta$ <sup>+</sup> pericyte coverage remained unchanged between groups (Figure 4.8B) suggesting selective pericyte alterations. Increased pericyte coverage is a recognised mechanism of vascular normalisation (Li et al., 2025). These findings indicate that ROBO4TTc treatment reduces tumour vessels and normalises them, further indicating increased tumour-infiltrating lymphocytes (TILs).

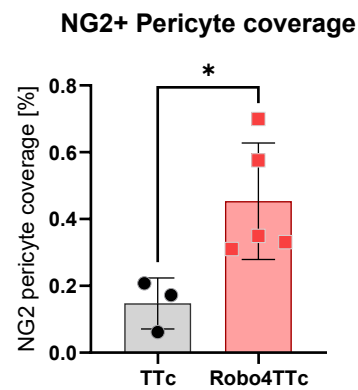


**Figure 4.6 Reduced vascular density in tumour ROBO4TTc vaccination.** Representative images of tumours from TTc (n = 6) and ROBO4TTc (n = 6) groups stained with MECA32 (green) to identify endothelial cells and DAPI (blue) to visualise nuclei. Blood vessel area was quantified using QuPath software, with vessel regions automatically delineated in red. Statistical analysis was performed using the Mann–Whitney test (\*p < 0.05). Bars represent the mean ± SD. Scale bars: 1000 µm, 100 µm, and 50 µm.

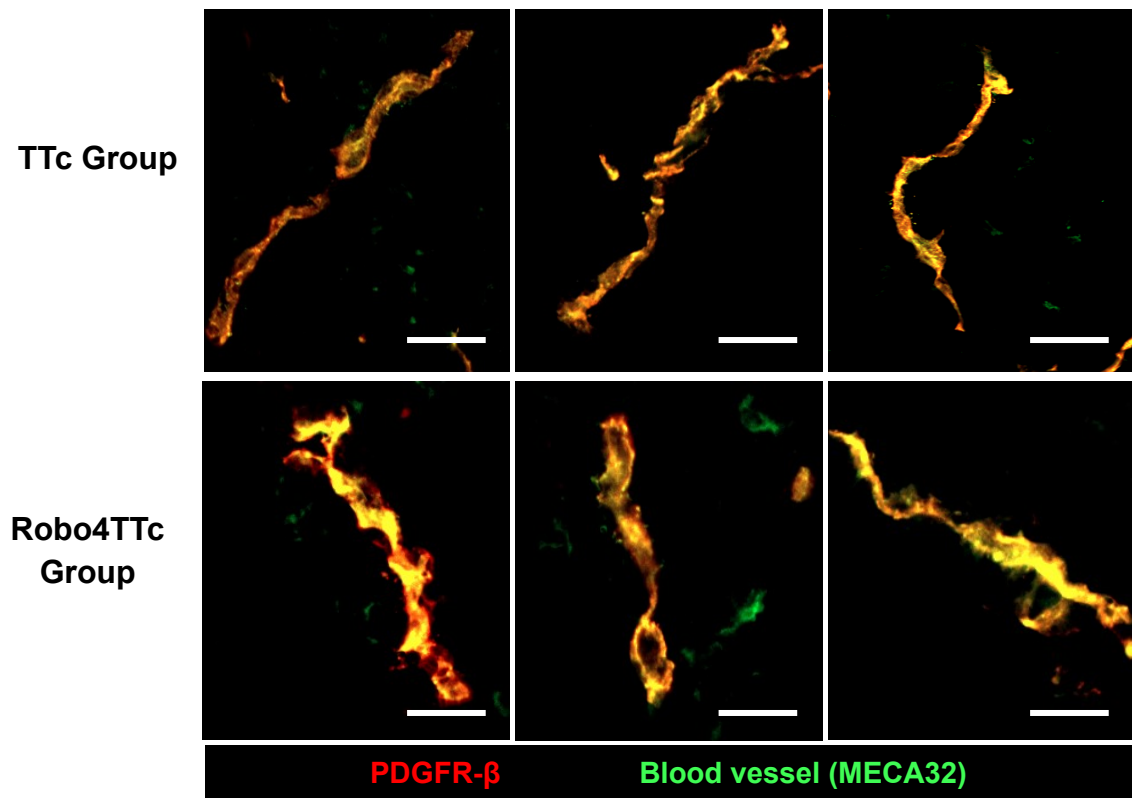
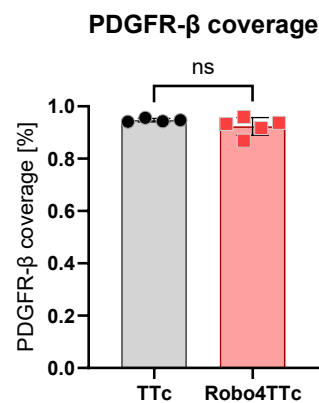
**A**



**B**



**Figure 4.7 Altered vessel coverage by neuron-glia antigen 2 (NG2)+ pericytes in tumours following ROBO4TTc vaccination.** Immunostaining of tumour sections from mice 4 weeks post-treatment with the ROBO4TTc (TTc: n=3, ROBO4TTc: n=5). Endothelial cells were labelled with MECA32 (green) and pericytes with NG2 (red). The ratio of NG2+ cells to MECA32+ cells was quantified using ImageJ. Statistical significance was assessed using the Mann-Whitney test (\*p < 0.05). Bars represent the mean  $\pm$  SD. Scale bar: 50  $\mu$ m.

**A****B**

**Figure 4.8 Vessel coverage by platelet-derived growth factor receptor alpha (PDGFR-β)+ pericytes remains unchanged following ROBO4TTc vaccination.** Immunostaining of tumour sections from mice 4 weeks post-treatment with the ROBO4TTc vaccine (TTc: n=4, ROBO4TTc: n=5). Endothelial cells were labelled with MECA32 (green), and pericytes were stained with PDGFR-β (red). The ratio of PDGFR-β+ cells to MECA32+ cells was quantified using ImageJ. Statistical significance was assessed using the Mann-Whitney test (\*p < 0.05). Bars represent the mean ± SD. Scale bar: 50 μm.

#### 4.3.5 The ROBO4TTc vaccine enhances lymphocyte infiltration.

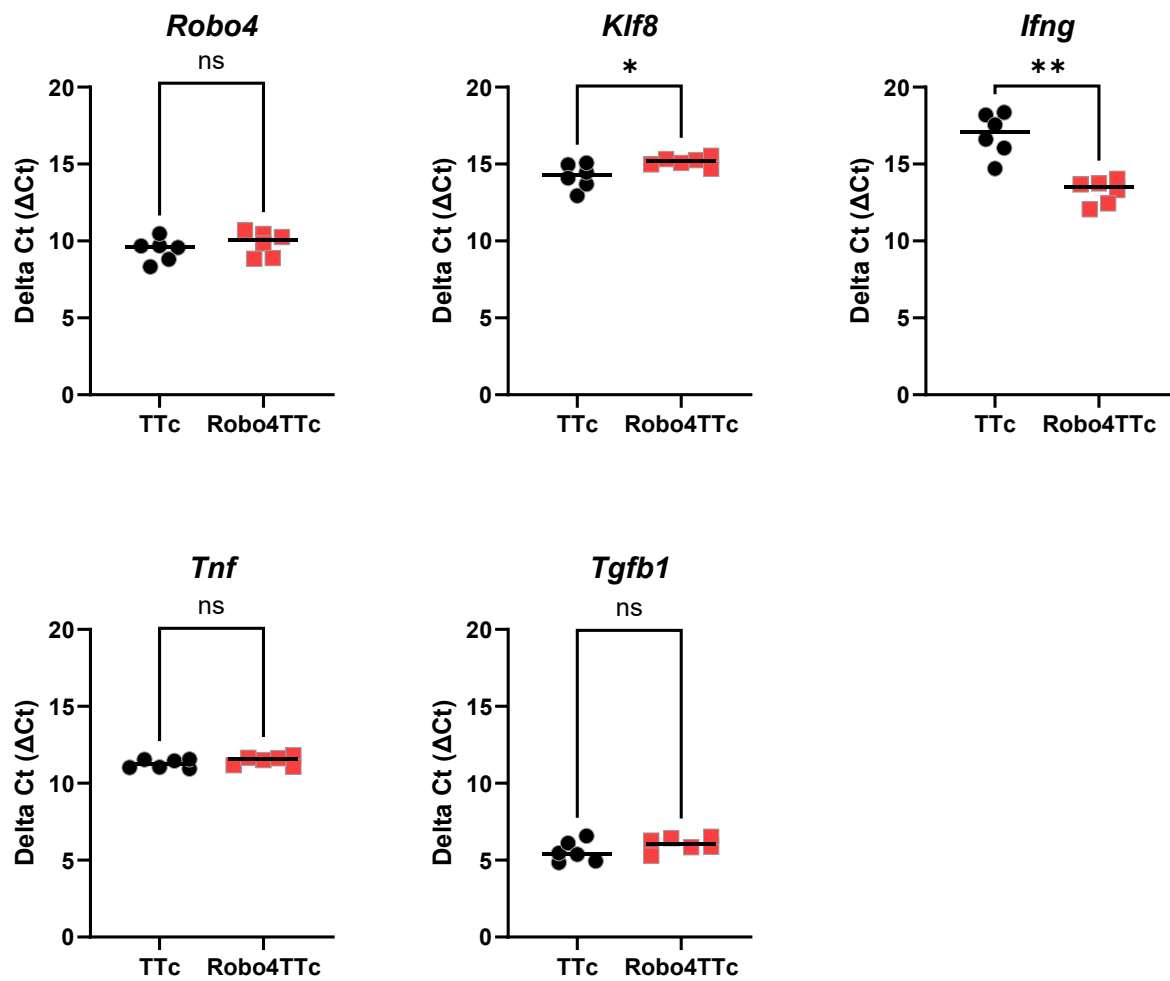
The same tumour tissue from the previous experiment was used to explore further the immune response induced by vaccination. Vessel marker *Robo4*, transcription factor *Kruppel-like factor (Klf8)*, and cytokine expression levels for *Ifng*, *Tnf*, and *Tgfb1* were quantified using qRT-PCR (Figure 4.9). Gene expression was analysed using the  $\Delta C_t$  method, with normalisation to a housekeeping gene ( $\beta 2m$ ).

*Robo4* mRNA levels were slightly reduced in tumours from the ROBO4TTc treatment group. Still, there was no significant difference compared to the control group, suggesting that *Robo4* mRNA levels may not be a sensitive indicator for assessing the immediate effects of vaccination on the tumour vasculature. This is likely due to the complex post-transcriptional and post-translational regulation of *Robo4* expression, which is known to be influenced by dynamic microenvironmental cues such as shear stress and inflammatory cytokines within the tumour (Dai et al., 2019). Furthermore, while mRNA levels reflect gene transcription, they do not always directly correlate with protein abundance or functional activity, as protein expression and stability are subject to additional regulatory mechanisms. Therefore, protein-level analysis or functional assays of ROBO4's role in angiogenesis might provide a more accurate assessment of treatment efficacy than mRNA expression alone.

*Klf8* has multiple functions supporting breast cancer cell metastasis and invasion and is associated with drug-resistant metastasis and poor survival in triple-negative breast cancer patients (Le Minh et al., 2023). It is known to regulate various cellular processes, including epithelial-to-mesenchymal transition (EMT), which can contribute to immune evasion by cancer cells and alter the tumour microenvironment (Wang et al., 2007). As expected, *Klf8* is highly expressed in 4T1 cells. Interestingly, *Klf8*

expression is significantly reduced upon ROBO4TTc treatment, suggesting that the ROBO4TTc vaccine inhibits tumour growth, probably by affecting tumour progression.

Cytokine expression data further demonstrate that *Ifng* levels were significantly increased in tumours from the ROBO4TTc treatment group compared to the control group. This upregulation of *Ifng* likely stems from the enhanced infiltration and activation of CD4+ and CD8+ T cells. *Ifng* is a key cytokine produced primarily by these T cell subsets, and its elevated presence suggests a robust anti-tumour immune response, promoting macrophage activation, antigen presentation, and potent cytotoxic CD8 T-cell effector functions, all of which contribute to tumour suppression (Jorgovanovic et al., 2020). However, the expression levels of *Tnf* and *Tgfb1* (IL-4 and other cytokines not shown here) were similar between the two groups. The ROBO4TTc in alum injection primarily induced ROBO4-specific IgG1 production (Escobar-Riquelme, 2024). As an adjuvant, alum typically induces a Th2 response, promoting IL-4 production and IgG1 class switching. These data suggest that ROBO4TTc in an alum-based vaccine may enhance cytokine production to support an anti-tumour response.

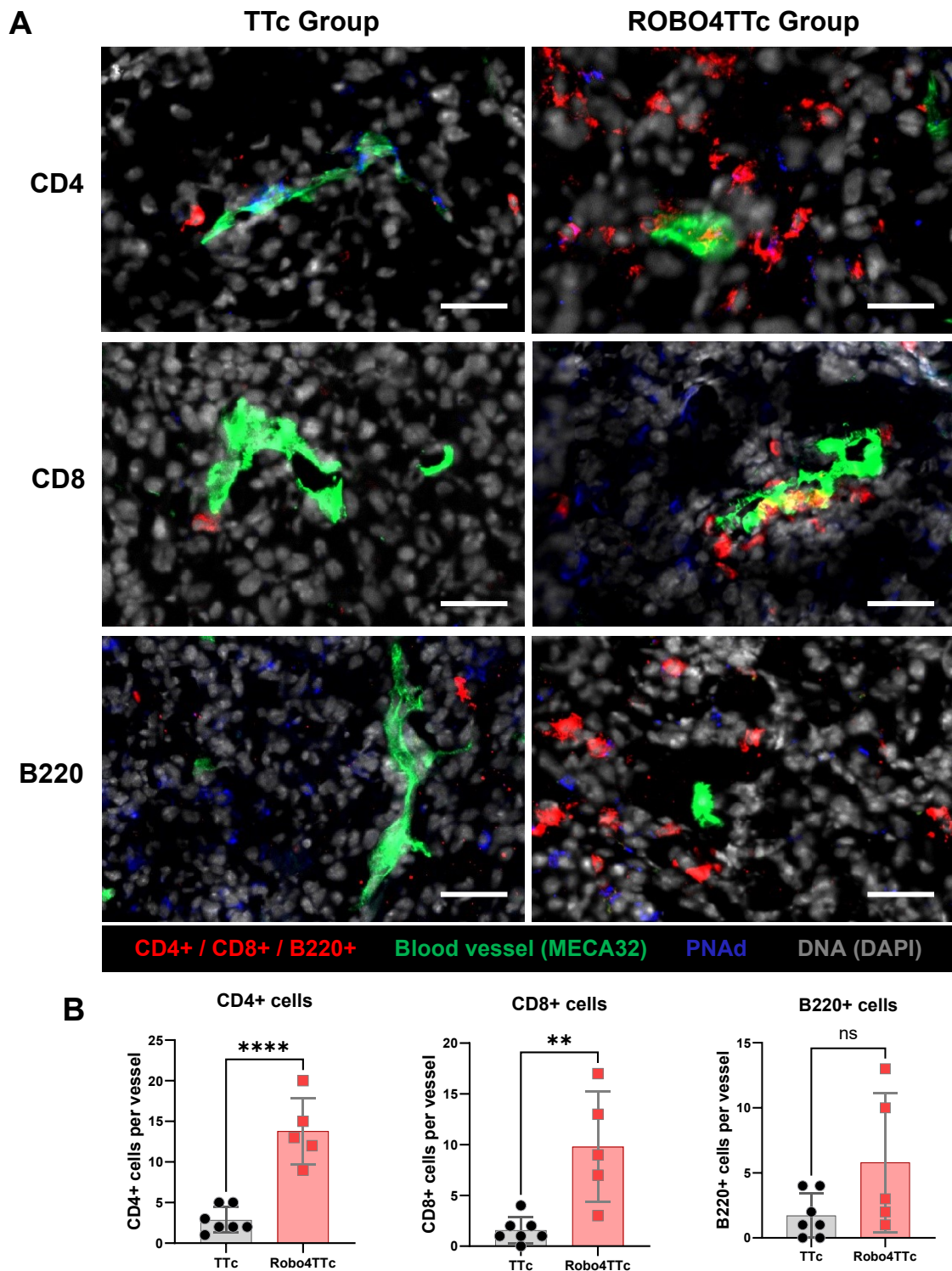


**Figure 4.9 Analysis of gene expression levels in 4T1 tumours from TTc- and ROBO4-TTc-vaccinated mice.** mRNA expression levels of *Robo4*, *Klf8*, *Ifng*, *Tnf*, and *Tgfb1* were measured in tumours from mice vaccinated with ROBO4TTc (n = 6) or TTc (n = 6). Each symbol represents an individual mouse. Gene expression was analysed using the  $\Delta$ Ct method, with expression normalised to a housekeeping gene. Statistical comparisons between groups were performed using a two-tailed Mann-Whitney test. Data are presented as median values. Statistical significance: ns, not significant ( $p \geq 0.05$ ); \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

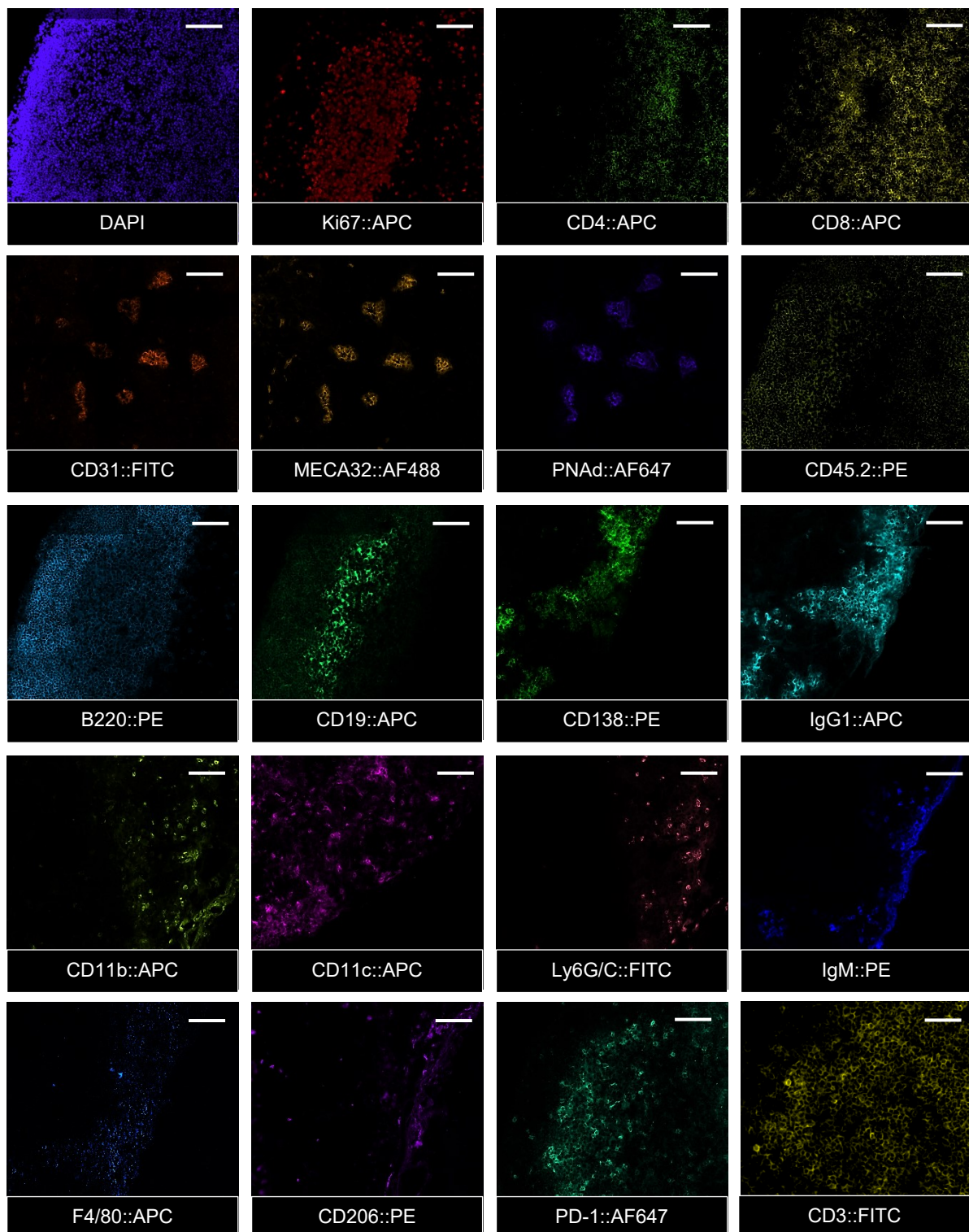
To further investigate the immune response in tumour tissue, the spatial distribution of immune cells in tumour sections was assessed by staining for CD4, CD8, and B220, along with MECA32 co-staining to identify endothelial cells (Figure 4.10). PNA<sup>+</sup> (blue) was used as a marker for high endothelial venules (HEVs). Immune cell infiltration was quantified within a 100  $\mu$ m radius surrounding tumour-associated blood vessels (MECA32<sup>+</sup>) (Figure 4.10A), with three randomly selected vessels analysed per tumour (TTc: n=7; ROBO4TTc: n=5). Significantly more CD4<sup>+</sup> and CD8<sup>+</sup> T cells were detected closely localized to tumour vasculature in the ROBO4TTc group compared to the TTc group (Figure 4.10B). These findings suggest the ROBO4TTc vaccine may enhance T-cell infiltration into tumours via the vasculature.

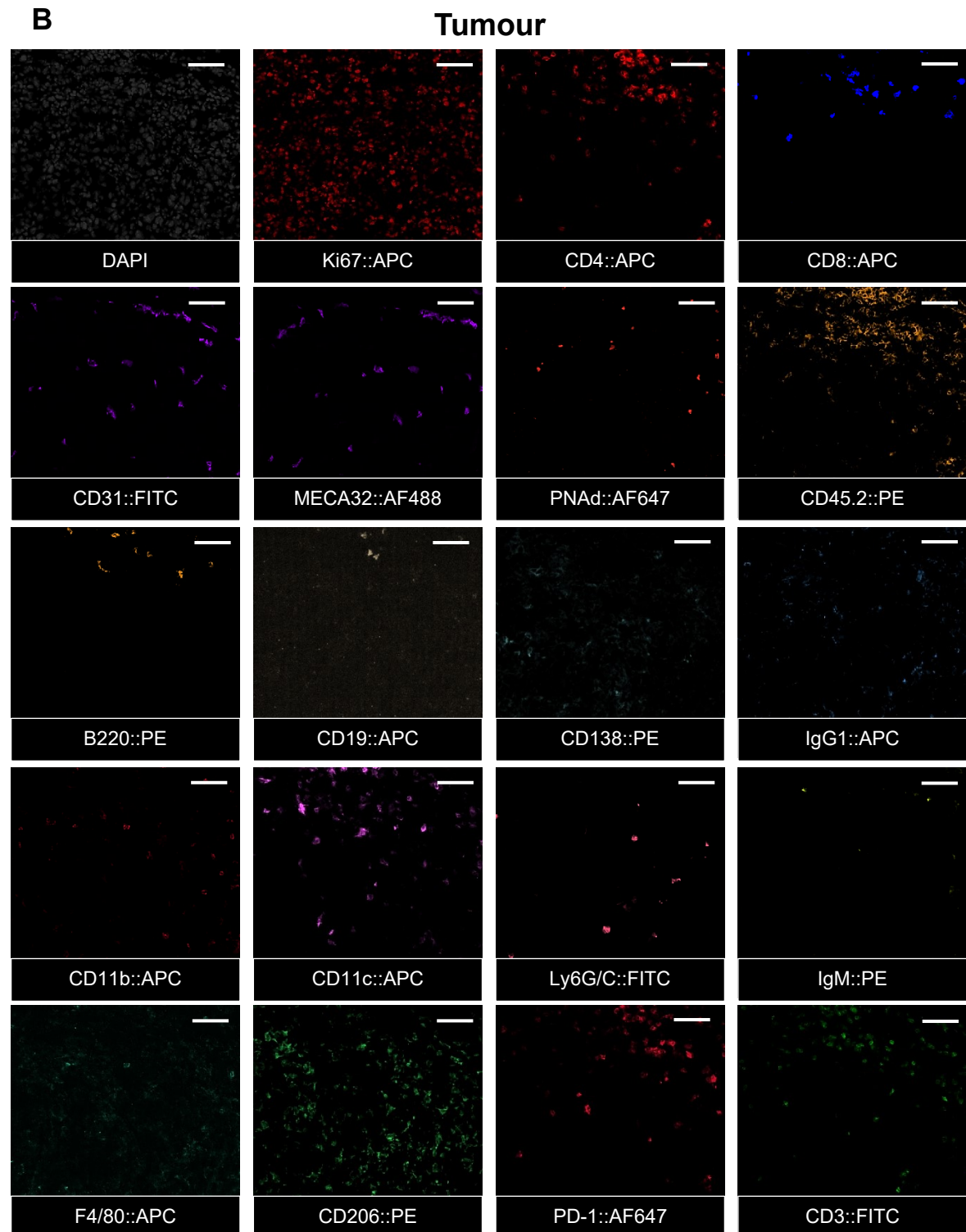
Multiplex imaging was performed to investigate whether immune cells were localised within specific tumour regions or diffusely distributed and whether tumour vasculature altered following the ROBO4TTc vaccination. I employed the MACSima system (Miltenyi Biotech) for multiplex imaging and designed and optimised a panel of 20 markers to identify significant immune cell populations and key vascular structures (Table 2-12). Multiplex immunofluorescence staining of individual markers in the lymph node and 4T1 tumour is shown in Figure 4.11.

To validate the staining panel, I first tested it on a lymph node sample before applying it to tumour tissues from the vaccine experiment. Figure 4.12A and B present seventeen-colour composite images highlighting key immune and structural markers in the tumour-draining lymph node (inguinal lymph node) and 4T1 tumour. Figure 4.13A and B focuses on B cell and T cell areas. B cells are identified by B220 (yellow) and CD19 (turquoise), while plasma cells are labelled with CD138 (green). Antibody-producing cells are further distinguished by IgG1 (aqua) and IgM (blue) staining.



**Figure 4.10 Increased T cell infiltration into tumours after ROBO4TTc vaccination.** Representative images of tumours from TTc and ROBO4TTc group, showing CD4+, CD8+ and B220+ (red) and MECA32 staining (green) to identify endothelial cells and DAPI staining (blue) for nuclei (TTc: n=7 and ROBO4TTc: n=5 mice). Statistical analysis was done using the Mann-Whitney test: ns, not significant ( $p \geq 0.05$ ); \*\*,  $p < 0.01$ ; \*\*\*\*,  $p < 0.0001$ . The bars represent the mean with SD. Scale bar: 50  $\mu$ m.

**A****Lymph Node**



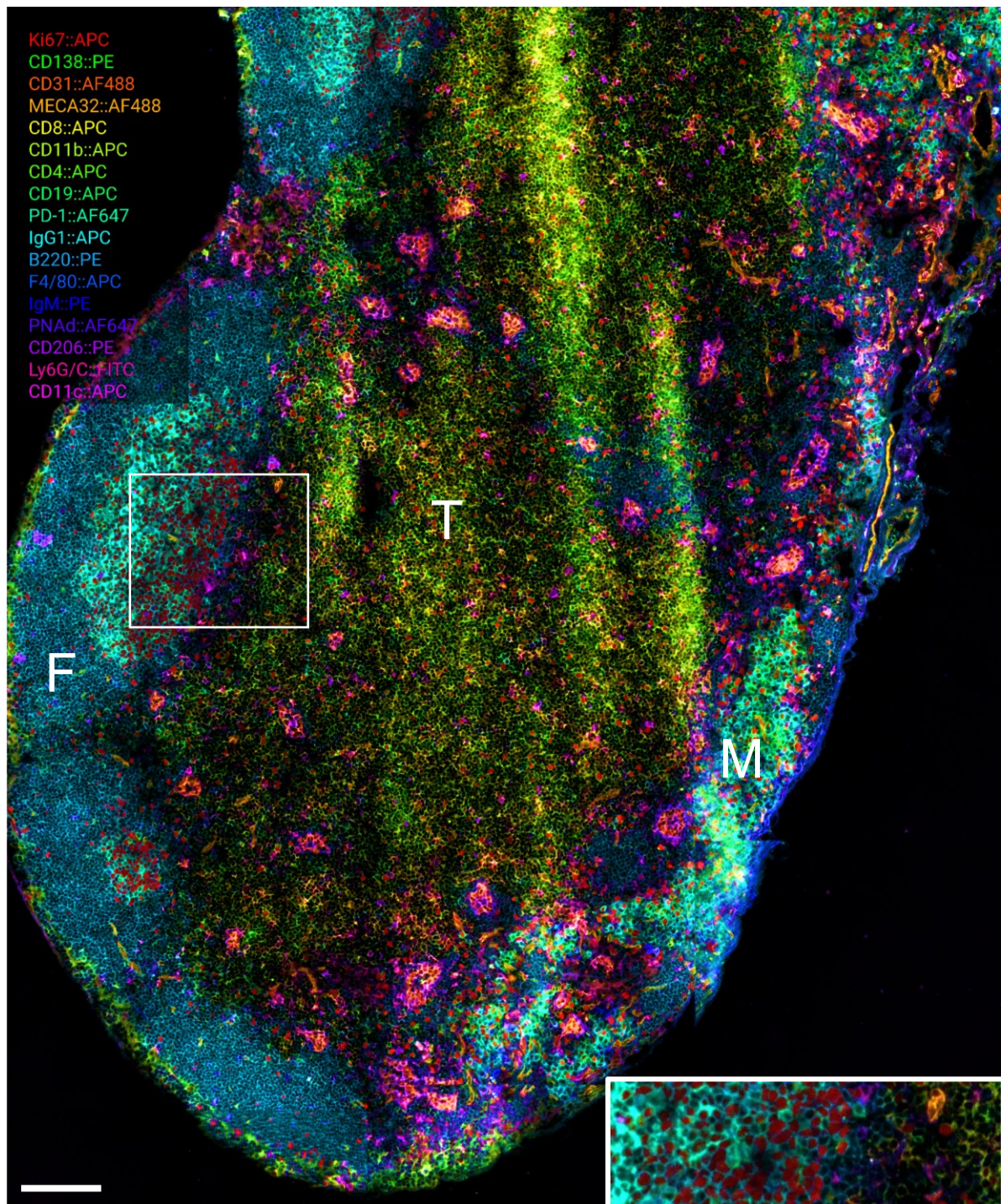
**Figure 4.11 Multiplex immunofluorescence staining of individual markers in the lymph node (A) and 4T1 tumour (B).** Stains include DAPI (nuclei), Ki67 (proliferation), CD4 (helper T cells), CD8 (cytotoxic T cells), CD3 (T cells), CD31 (endothelial cells), MECA32 (blood vessels), PNA (high endothelial venules), CD45.2 (leukocytes), B220 (B cells), CD19 (B cells), CD138 (plasma cells), IgG1 and IgM (antibodies), CD11b (myeloid cells), CD11c (dendritic cells), Ly6G/C (neutrophils), F4/80 (macrophages), CD206 (M2 macrophages), and PD-1 (exhausted T cells). Scale bars: 100  $\mu$ m.

CD4<sup>+</sup> helper T cells appear in red, and CD8<sup>+</sup> cytotoxic T cells are displayed in dark blue. A clear germinal centre is visible in the tumour-draining lymph node, containing IgM<sup>+</sup> immune complexes presented on follicular dendritic cells. IgM<sup>+</sup> plasma cells and class-switched IgG1<sup>+</sup> plasma cells are found in the medullary region. Figure 4.14A and B highlight myeloid cell populations. CD11b<sup>+</sup> monocytes (green) are mainly in subcapsular and interfollicular areas. As expected, CD11c<sup>+</sup> cells (turquoise) are found in the T zone and co-localize with CD11b<sup>+</sup> cells in the interfollicular region. Ly6G/C<sup>+</sup> granulocytes (e.g., neutrophils) appear in dark blue, while F4/80<sup>+</sup> macrophages (purple) are mainly present in the medullary area. CD206 marks M2-polarized macrophages or a secreted receptor from macrophages, showing a diffuse pattern in the T zone. Figure 4.15A and Figure 4.15B focuses on endothelial markers. CD31 (blue) labels endothelial cells of both blood and lymphatic vessels and some macrophages. Blood vessels expressing the pan-endothelial cell antigen (MECA32) are shown in green, while high endothelial venules (HEVs), labelled by peripheral lymph node addressin (PNAd, also called MECA79), appear in red. As expected, HEVs are primarily located in the T zone, with some also found in the medullary area. Other types of vessels are mainly concentrated in the medullary region.

All markers exhibit the expected staining patterns in the lymph node, confirming their specificity. The panel was performed on tumour sections from the ROBO4TTc vaccine group and the control group (three sections from each group). Tumour tissue contains extensive necrotic areas, resulting in a higher background than the lymph node. Sections with proper and precise morphology, particularly those from the tumour edge region, were selected for further analysis.

**A**

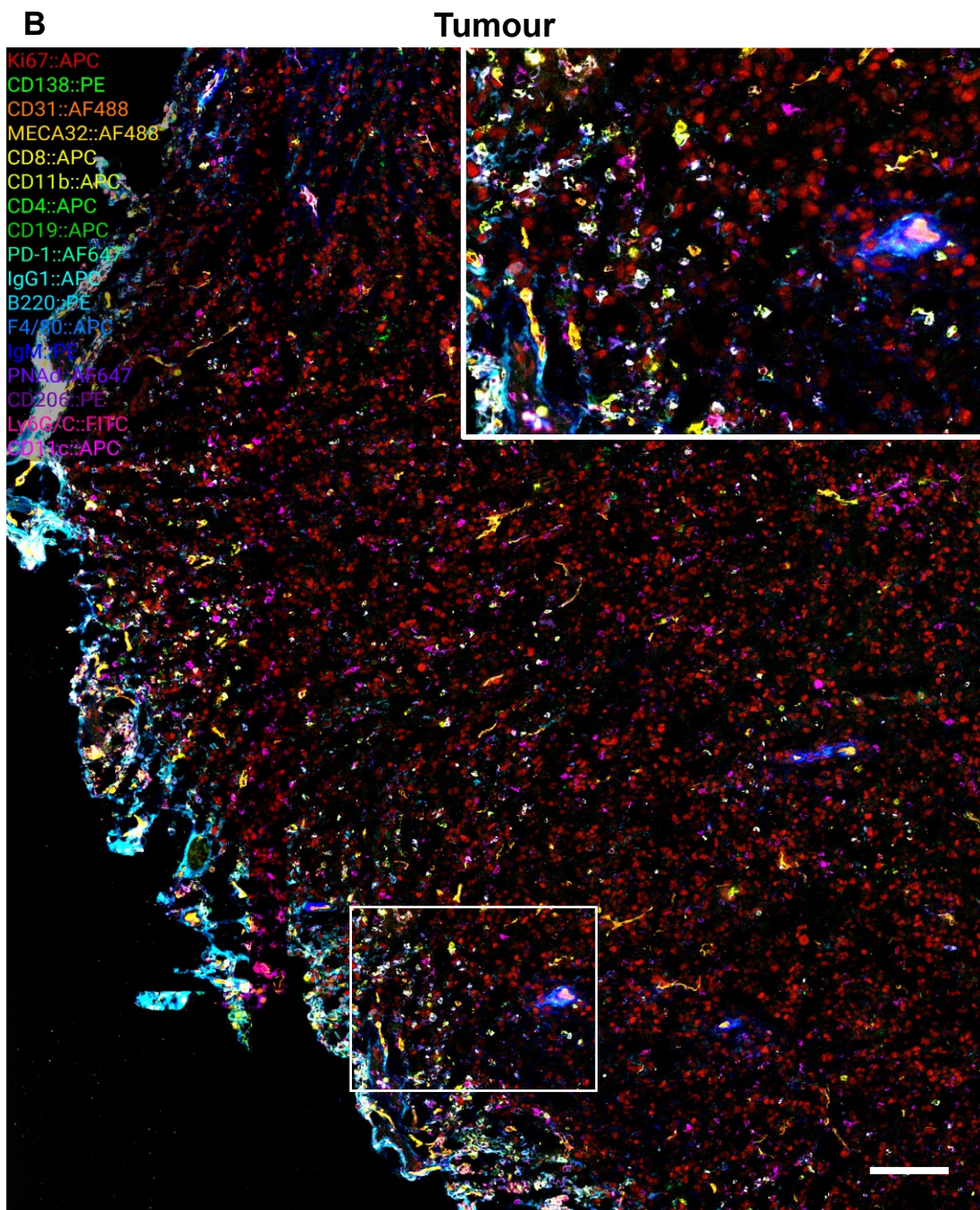
## Lymph Node



**F: Follicle**

**T: T-cell zone**

**M: Medulla**

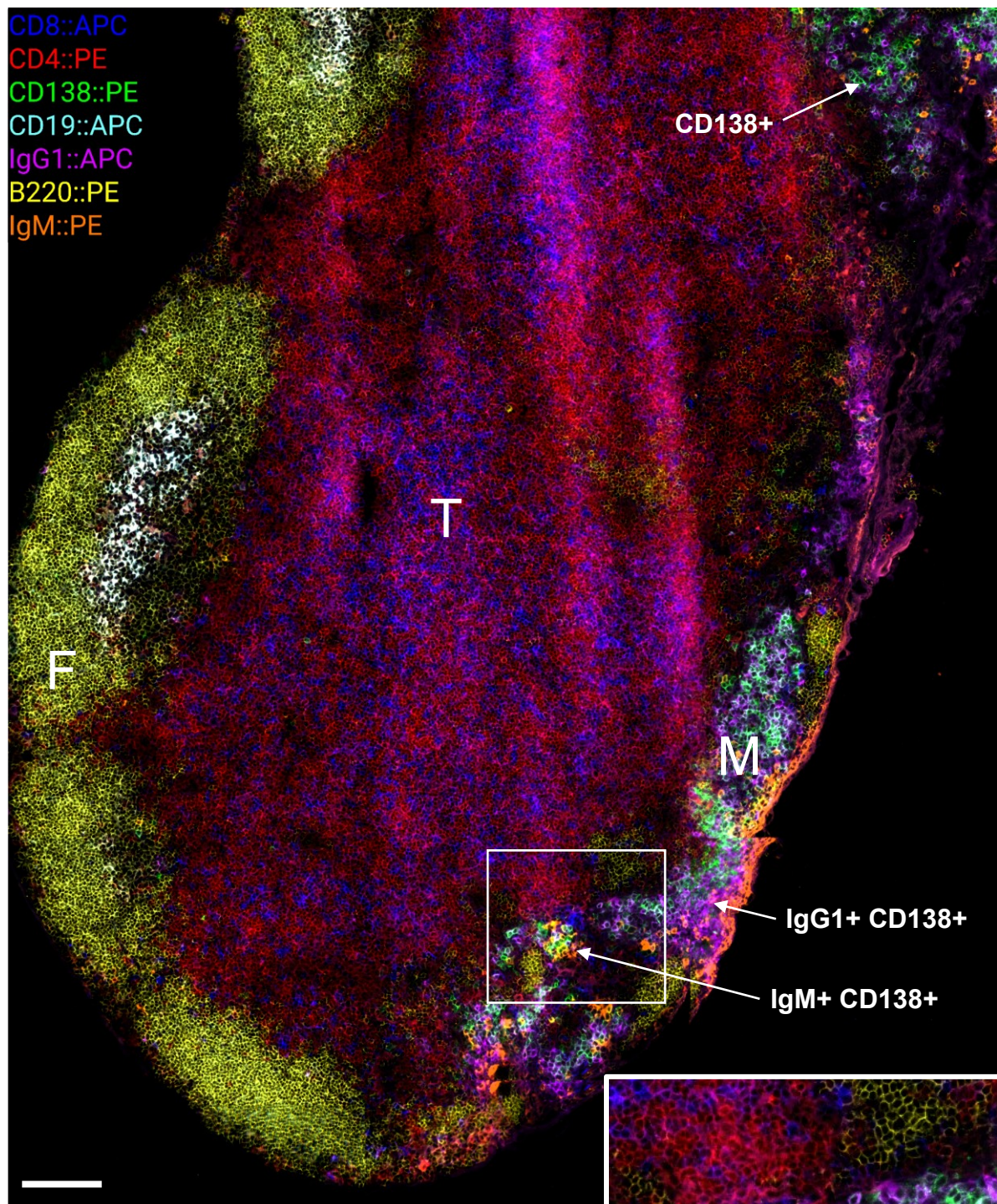


**Figure 4.12** Seventeen-colour composite images highlighting key immune and structural markers in a lymph node (A) and 4T1 tumour (B) using the MACSima multiplex imaging system.

Ki67 (proliferation; red), CD4 (helper T cells; pale green), CD8 (cytotoxic T cells; yellow), CD31 (endothelial cells; orange), MECA32 (blood vessels; gold), PNAd (high endothelial venules; dark blue), B220 (B cells; turquoise), CD19 (B cells; dark green), CD138 (plasma cells; dark green), IgG1 and IgM (antibodies; aqua and blue, respectively), CD11b (myeloid cells; lime), CD11c (dendritic cells; purple), Ly6G/C (neutrophils; pink), F4/80 (macrophages; dark blue), CD206 (M2 macrophages; dark purple), and PD-1 (exhausted T cells; pale green). Scale bars: 3 mm.

**A**

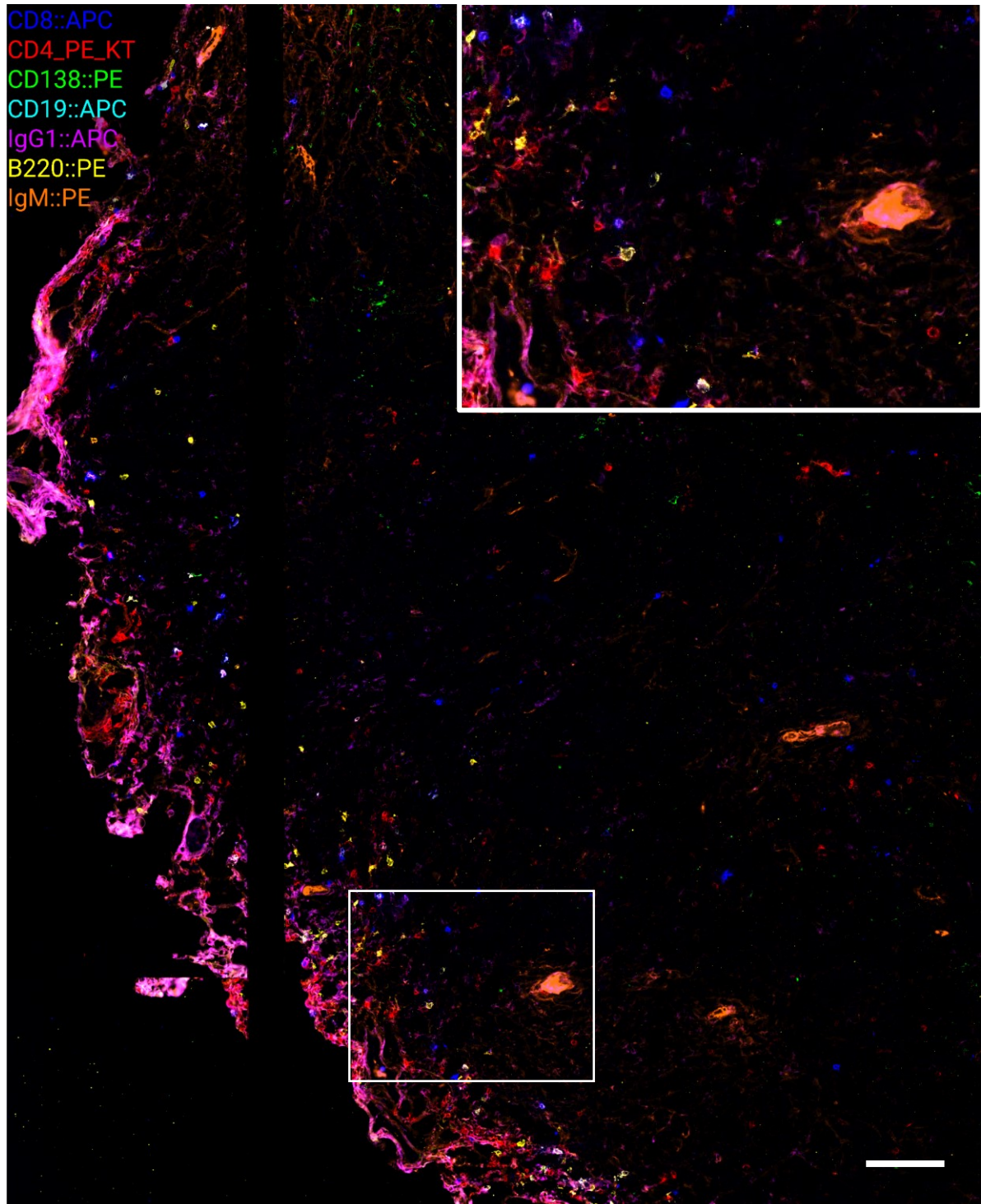
**Lymph Node – T and B cells**



**F: Follicle**

**T: T-cell zone**

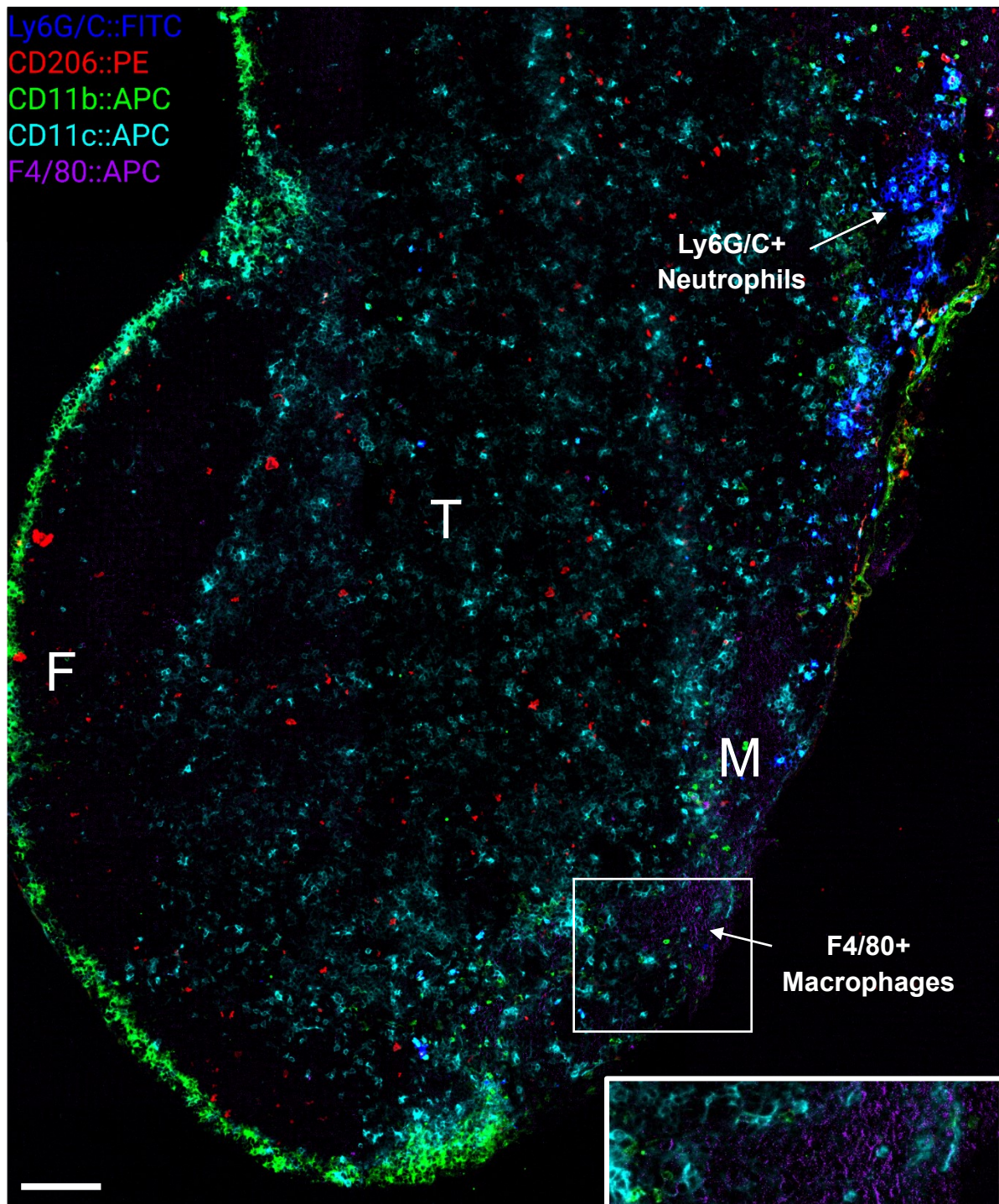
**M: Medulla**

**B****Tumour – T and B cells**

**Figure 4.13** Images show the germinal centre structures with T and B cell markers in a lymph node (A) and 4T1 tumour (B). CD4 (helper T cells; red), CD8 (cytotoxic T cells; blue), B220 (B cells; yellow), CD19 (B cells; turquoise), CD138 (plasma cells; green), and IgG1/IgM (antibodies; purple and orange, respectively). Scale bars: 3 mm.

**A**

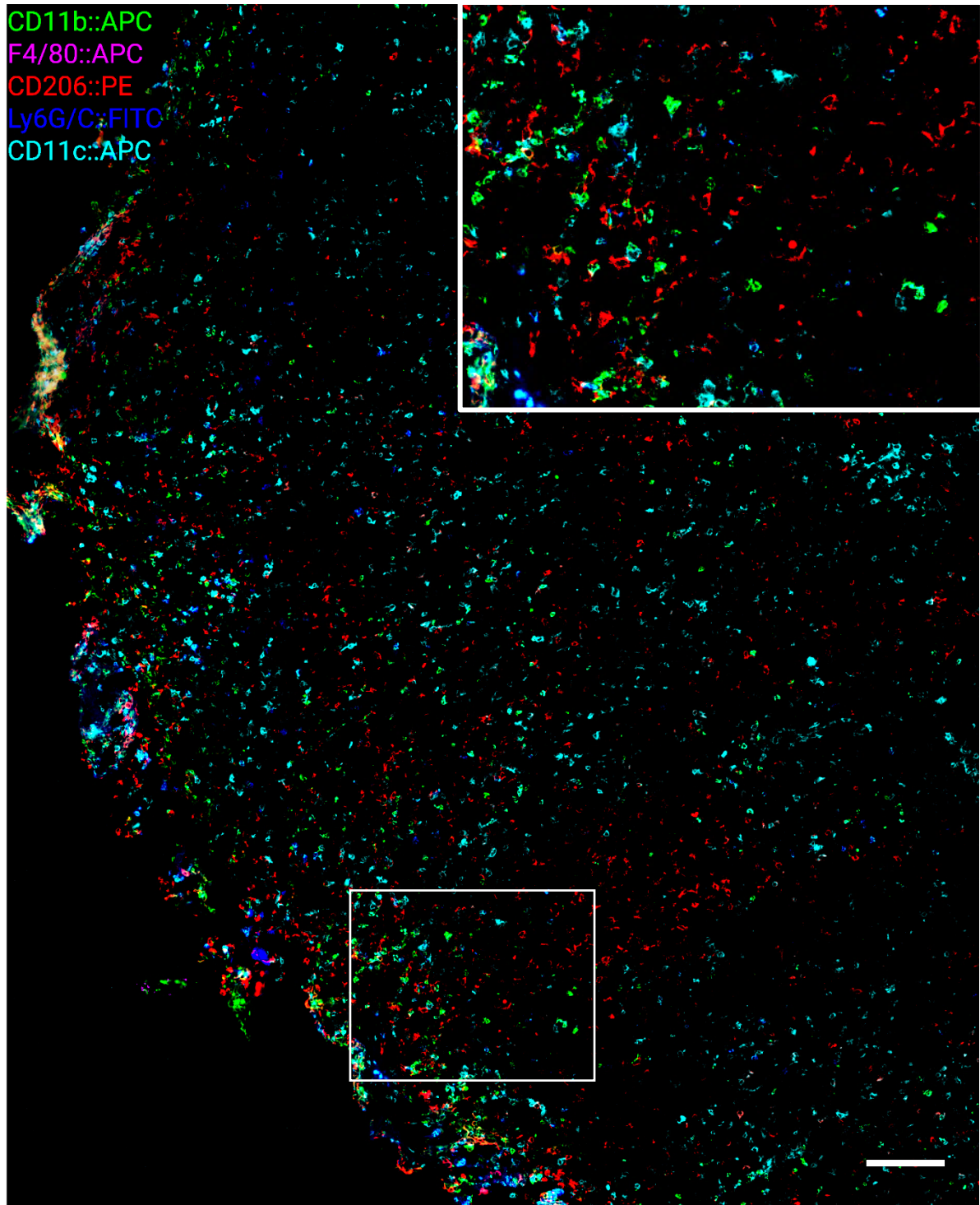
**Lymph Node – Myeloid cells**



**F: Follicle**

**T: T-cell zone**

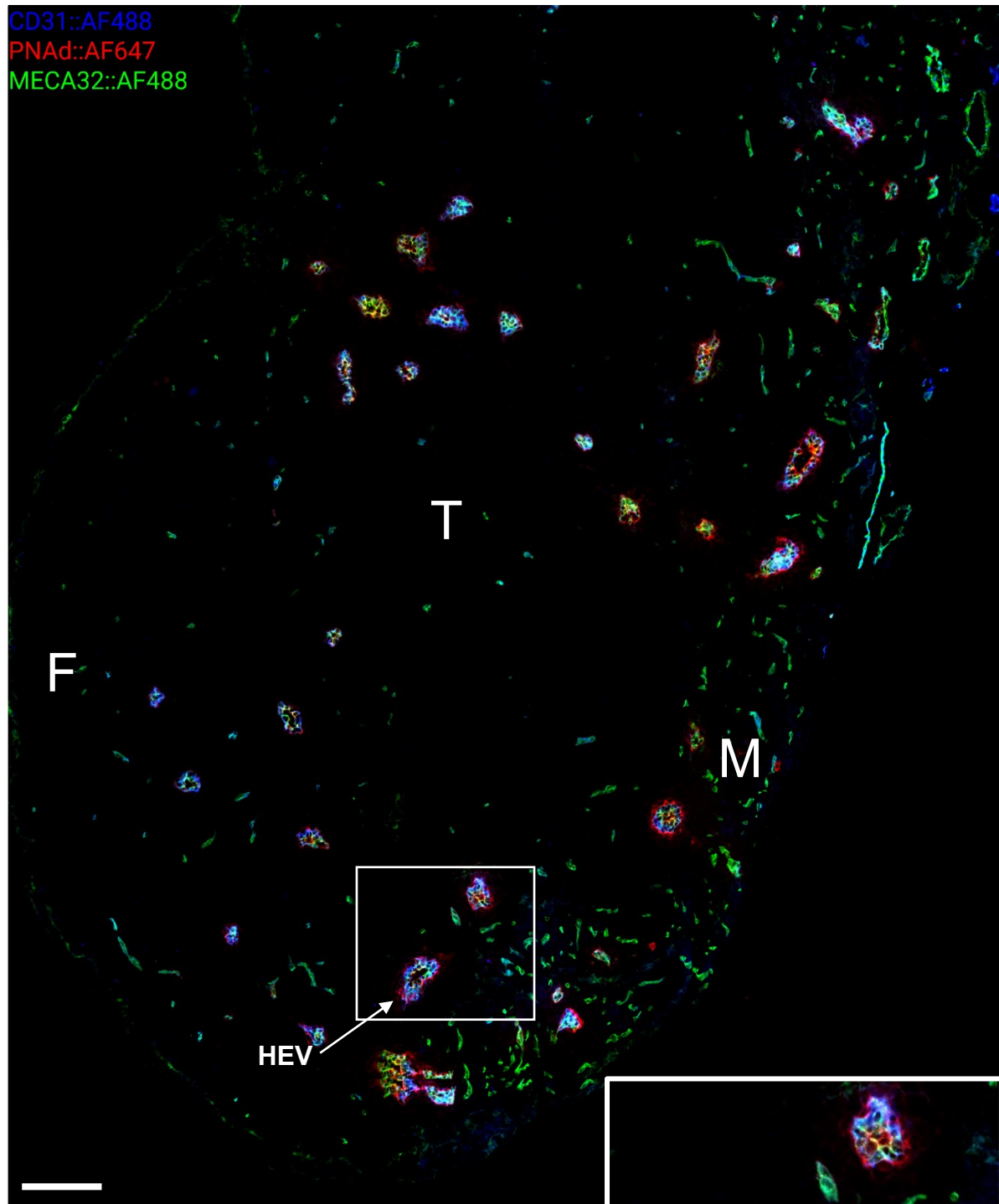
**M: Medulla**

**B****Tumour – Myeloid cells**

**Figure 4.14** Images display myeloid cell markers in a lymph node (A) and 4T1 tumour (B). CD11b (myeloid cells; turquoise), CD11c (dendritic cells; purple), Ly6G/C (neutrophils; dark blue), F4/80 (macrophages; purple), and CD206 (M2 macrophages; red). Scale bars: 3 mm.

**A**

## **Lymph Node – Vessels**

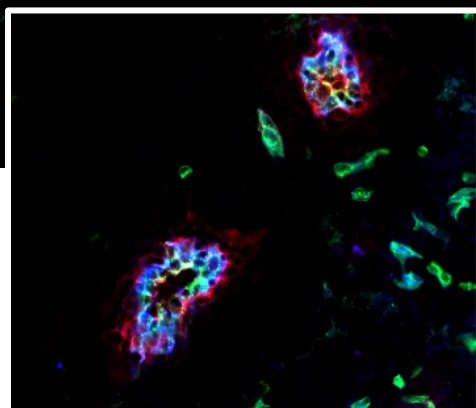


**F: Follicle**

**T: T-cell zone**

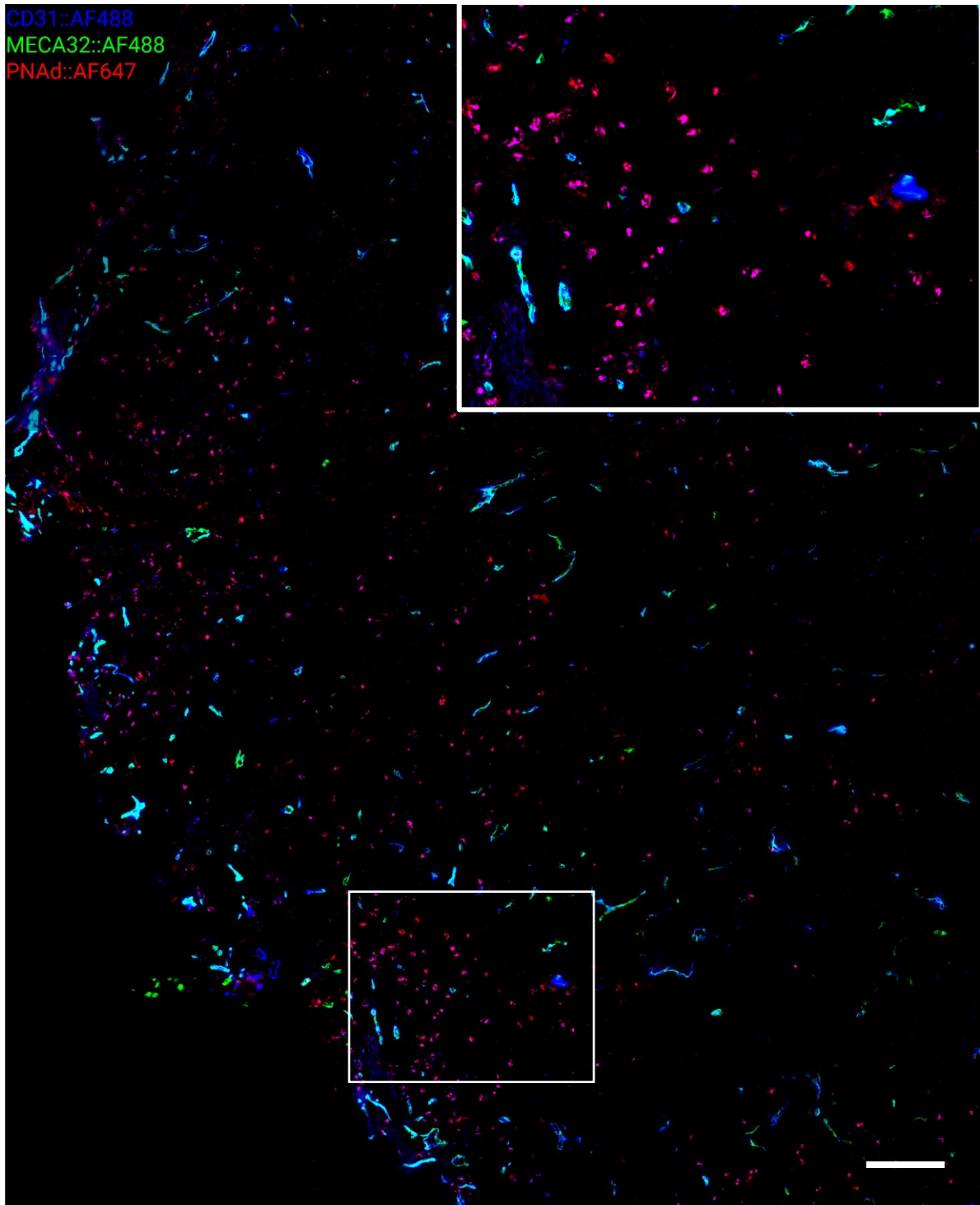
**M: Medulla**

**HEV: High endothelial venules**



**B**

**Tumour – Vessels**



**Figure 4.15** Images highlight vascular markers in a lymph node (A) and 4T1 tumour (B). CD31 (endothelial cells; blue), MECA32 (blood vessels; green), and PNAd (high endothelial venules; red). Scale bars: 3 mm.

Representative images of non-necrotic areas indicated by clear cellular morphology from tumours from TTc and ROBO4TTc vaccinated tumours from both groups (n=3) show CD4<sup>+</sup> T cells (blue), CD8<sup>+</sup> T cells (red), B cells (B220<sup>+</sup>, green), dendritic cells (CD11c<sup>+</sup>, purple), blood vessels (MECA32<sup>+</sup>, white), and nuclei (DAPI, grey) (Figure 4.16A and B).

To annotate and calculate the cell populations on the multiplex imaging using MACS<sup>®</sup> iQ View Analysis Software, the Previously explained workflow was used for the spatial analysis of multiplex imaging on MACS iQ View (see Appendix B for the detailed workflow graph). Cell type annotation began with segmenting the selected regions of interest using specific settings outlined in the Table 2-14. Highly auto-fluorescent cells were excluded by gating out FITC-positive cells in the initial images. CD45<sup>+</sup> cells were gated and subsequently sub-gated into B220<sup>+</sup>, CD11b<sup>+</sup>, Ly6G/C<sup>+</sup>, CD3<sup>+</sup>, CD11c<sup>+</sup>, Ki67<sup>+</sup>, and PD-1<sup>+</sup> populations. CD3<sup>+</sup> T cells were further subdivided into CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which were then analysed for Ki67 and PD-1 expression. Statistical comparisons between groups were performed using the Mann–Whitney test.

Spatial cell profiling of 4T1 tumours from TTc- and ROBO4TTc-vaccinated groups revealed distinct immune landscape alterations (Figure 4.17). Despite a lower overall infiltration of CD45<sup>+</sup> immune cells in the ROBO4TTc group compared to the TTc group (mean 25% vs 55% of total cells), there was a relative enrichment of B220<sup>+</sup> B cells (mean 5%) and CD3<sup>+</sup> T cells (mean 15%), accompanied by a notable reduction in Ly6G/C<sup>+</sup> neutrophils (mean 25%) in the total CD45<sup>+</sup> cells. Strikingly, the ROBO4TTc group showed a significant increase in CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets, constituting an average of 4% and 8% of the CD45<sup>+</sup> population, respectively, compared to <1% in the TTc group. Furthermore, these T cell subsets exhibited elevated Ki67 expression,

indicative of active proliferation. However, higher levels of PD-1 expression were also detected on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, suggesting a potentially activated or exhausted phenotype. The direct contribution of vaccine-induced ROBO4-specific CD8<sup>+</sup> T cells to tumour inhibition in this specific model would require further investigation (e.g., through depletion studies). It is important to note that a previous the ROBO4 vaccine in LLC1 tumours showed that its anti-tumour effects persisted even in CD8-deficient mice (Zhuang et al., 2015). This suggests that while CD8<sup>+</sup> T cells may be activated, the primary mechanism of tumour suppression in this vaccination strategy is likely antibody-dependent cellular cytotoxicity (ADCC), as supported by the dependence on B cells and IgG1 (Zhuang et al., 2015). Nevertheless, the increased infiltration and activation markers on CD8<sup>+</sup> T cells observed here still indicate an enhanced adaptive immune response within the tumour microenvironment that could contribute to overall tumour control or provide a foundation for synergistic therapies.

Notably, these immune cells were closely associated and appeared to interact near blood vessels in ROBO4TTc tumours (Figure 4.16B), which may contribute to enhanced antitumor immunity. In contrast, tumours from the TTc group exhibited significantly higher vessel density (Figure 4.6) but lower immune cell infiltration, with immune cells appearing more dispersed across the section rather than in close contact (Figure 4.16B). This apparent paradox is a hallmark of the abnormal tumour vasculature that develops in untreated malignancies. These vessels, though numerous, are often highly chaotic, leaky, and structurally dysfunctional (Yang et al., 2025). This disorganization and poor integrity create an inefficient system for immune cell extravasation and trafficking from the bloodstream into the tumour parenchyma (Yang et al., 2025). Furthermore, the chaotic nature of these vessels often leads to

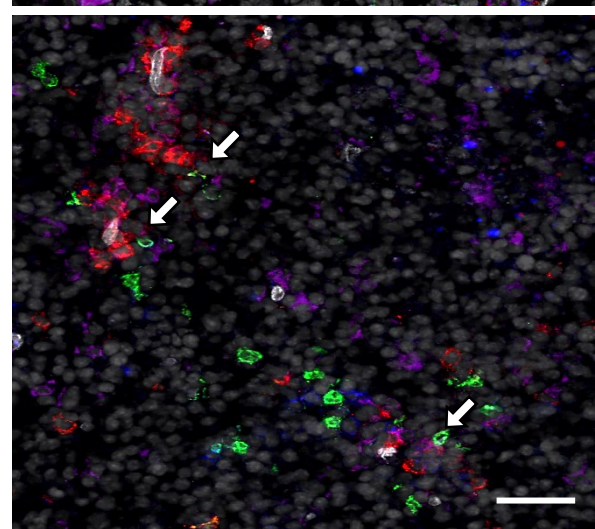
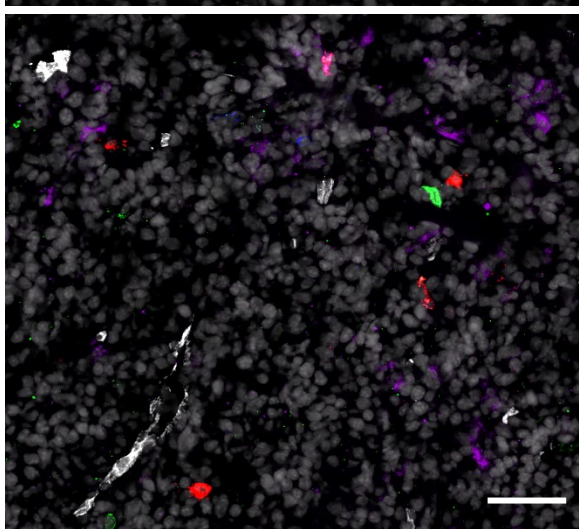
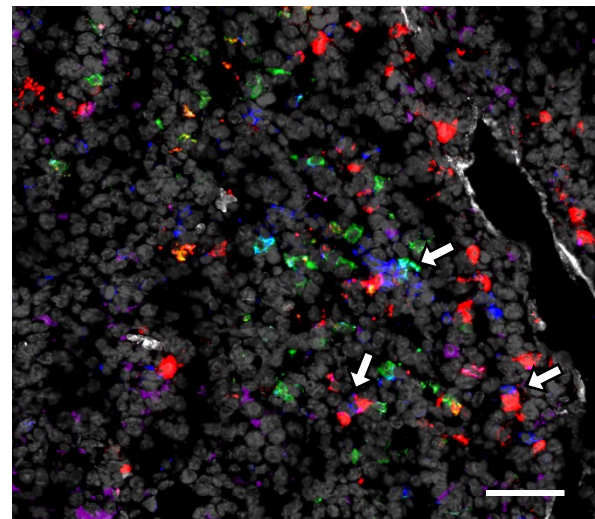
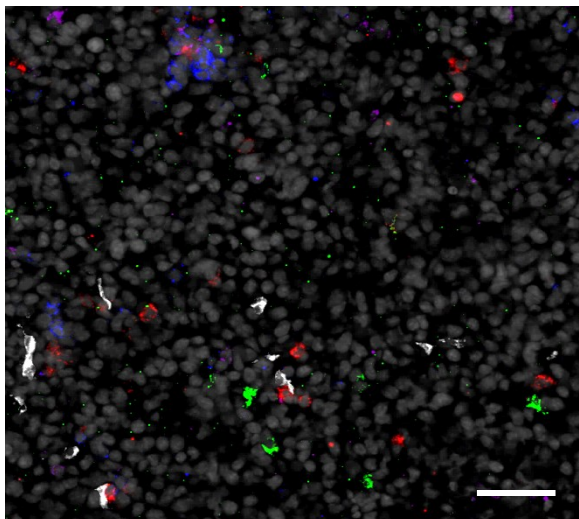
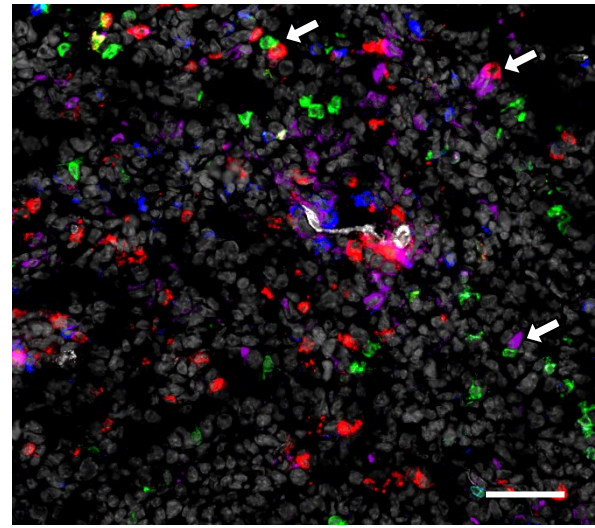
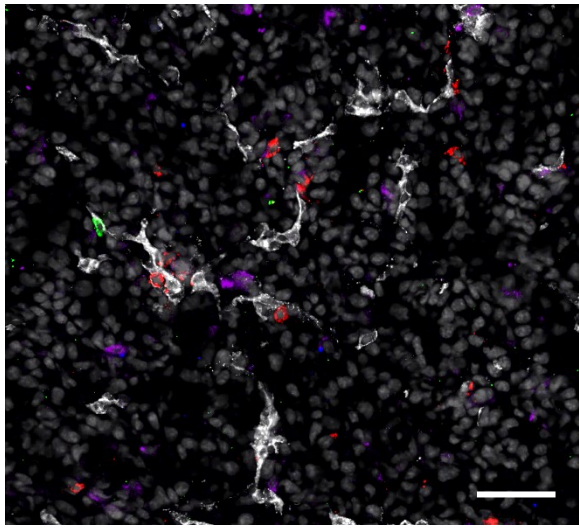
regions of severe hypoxia within the tumour, which itself promotes an immunosuppressive microenvironment that actively repels or deactivates infiltrating immune cells (Ribatti et al., 2016).

While these findings are promising, a more detailed and systematic analysis of tissue sections stained with this technique is warranted. Due to time constraints, such analysis could not be performed within this project's scope. Future studies should aim to validate antibody specificity, incorporate additional markers (e.g., CXCL13), include larger sample sizes, and develop stereological approaches to allow robust quantification and statistical assessment of the spatial relationships observed.

**A**

**TTc Group**

**Robo4TTc Group**



CD4 T cells

CD8 T cells

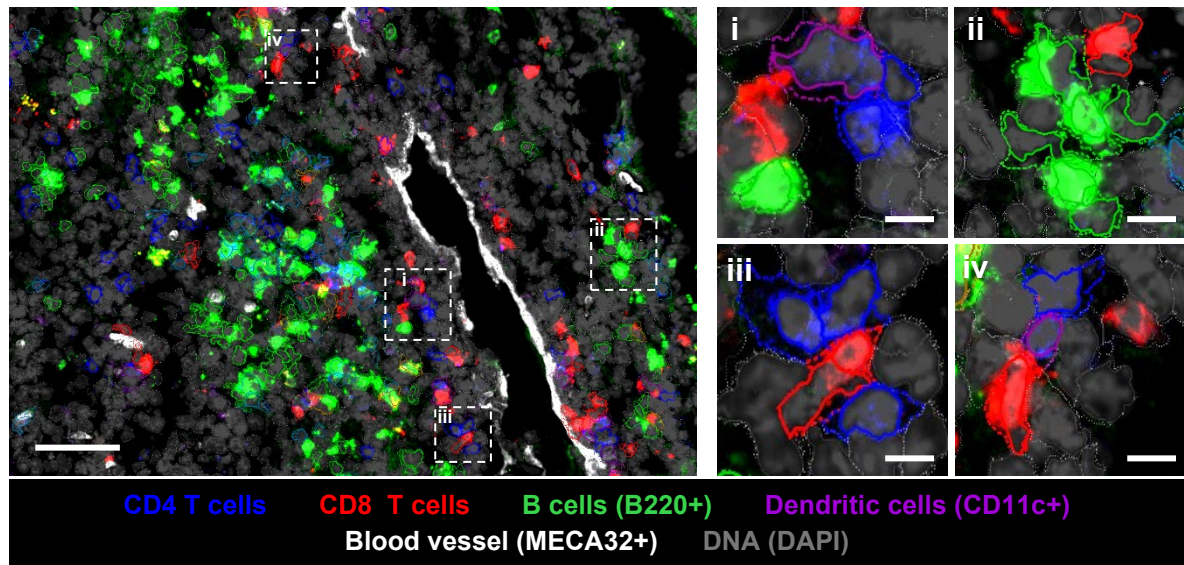
B cells (B220+)

Dendritic cells (CD11c+)

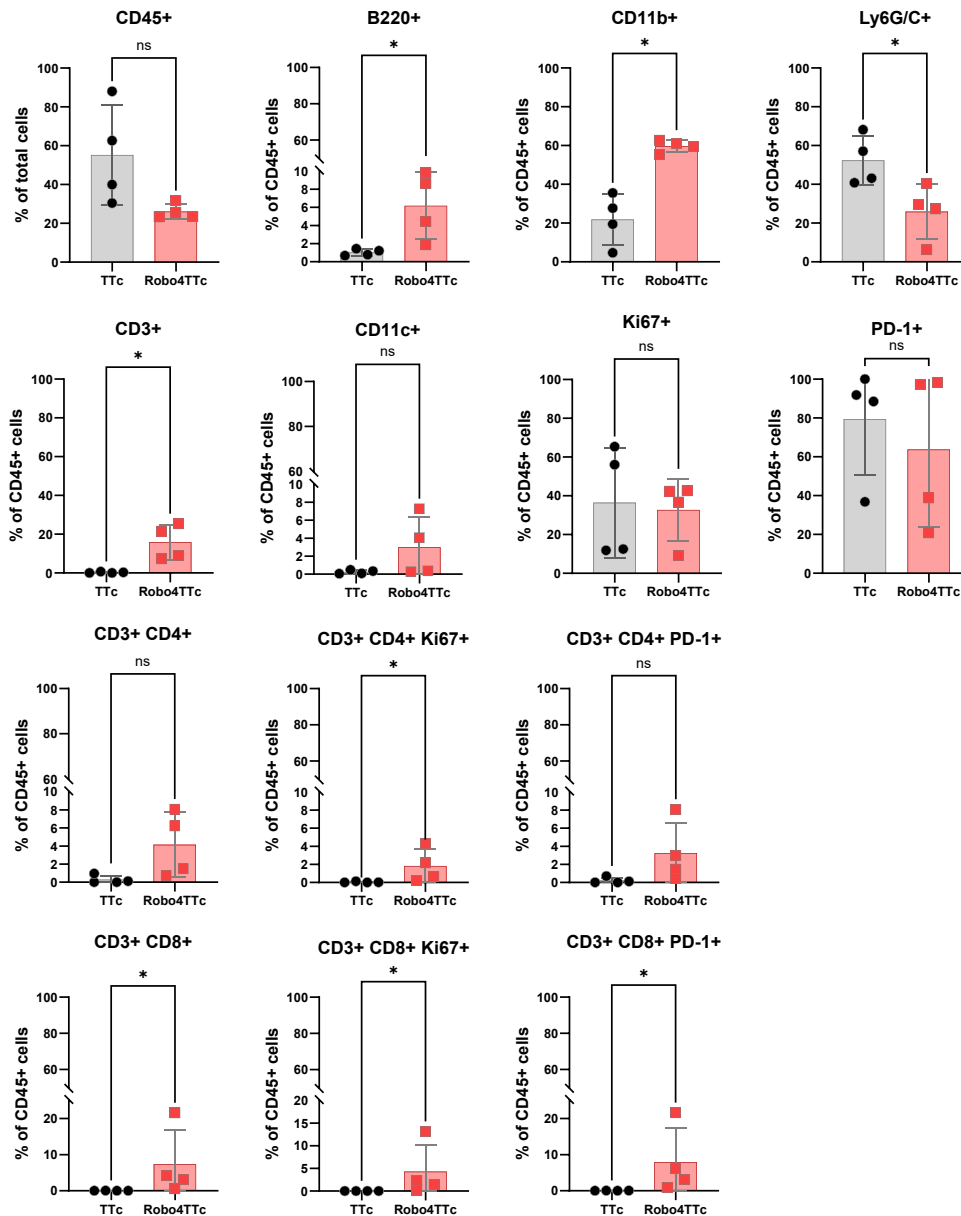
Blood vessel (MECA32+)

DNA (DAPI)

**B**



**Figure 4.16 ROBO4TTc promotes immune cell infiltration and spatial interactions within 4T1 tumours.** (A) Representative multiplex images of 4T1 tumours from TTc and ROBO4TTc-vaccinated groups (n = 3). (B) Annotating of immune cell populations in 4T1 tumours following ROBO4TTc vaccination, analysed using MACS iQ View. Images show CD4<sup>+</sup> T cells (blue), CD8<sup>+</sup> T cells (red), B cells (B220<sup>+</sup>, green), dendritic cells (CD11c<sup>+</sup>, purple), blood vessels (MECA32<sup>+</sup>, white), and nuclei (DAPI, grey). Scale bars: 10  $\mu$ m and 200  $\mu$ m.



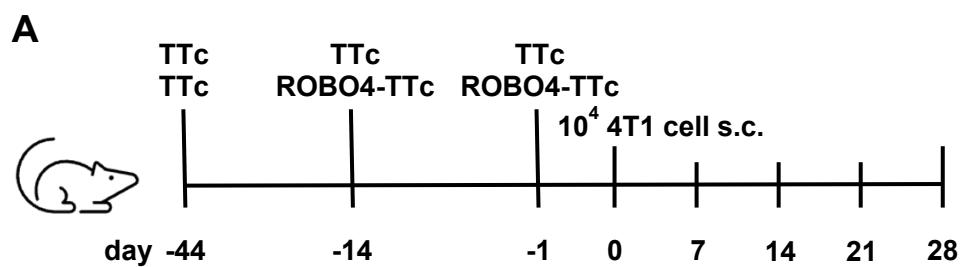
**Figure 4.17 Spatial immune profiling of 4T1 tumours from TTc- and Robo4TTc-vaccinated mice.**

Quantification of immune cell populations was performed using MACS iQ View software based on annotated cell types. Cell type annotation began with segmenting the selected regions of interest using specific settings outlined in the accompanying table. Highly auto-fluorescent cells were excluded from the analysis. CD45<sup>+</sup> cells were gated and subsequently sub-gated into B220<sup>+</sup>, CD11b<sup>+</sup>, Ly6G/C<sup>+</sup>, CD3<sup>+</sup>, CD11c<sup>+</sup>, Ki67<sup>+</sup>, and PD-1<sup>+</sup> populations. CD3<sup>+</sup> T cells were further subdivided into CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which were then analysed for Ki67 and PD-1 expression. Data are presented as the percentage of CD45<sup>+</sup> lymphocytes. Statistical comparisons between groups were performed using the Mann–Whitney test. Results are shown as mean ± SD. Significance is indicated as follows: ns (not significant,  $p \geq 0.05$ ), \* $p < 0.05$ .

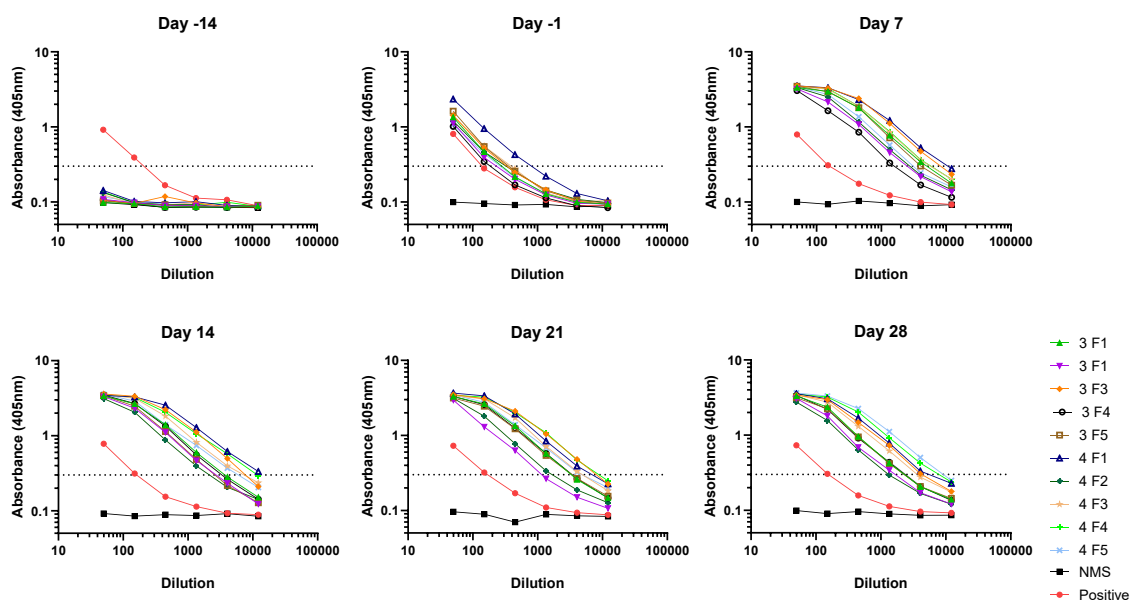
#### **4.3.6 ROBO4TTc vaccination modulates the tumour microenvironment by enhancing adaptive immunity and reducing myeloid suppression in TNBC (4T1) tumours.**

To investigate any further details about changes in the immune cell populations in the 4T1 tumour after ROBO4TTc vaccination, spectral cytometry was selected. To do this, the same experimental protocol was performed as shown before (Figure 4.18A). Briefly, BALB/c mice (n=16) were primed i.p. with alum-precipitated TTc (Figure 4.18A). Four weeks later, mice were i.p. immunised with either TTc (n=8) or ROBO4TTc (n=8), and a second boost was administered two weeks later. One day after the second boost, all mice were subcutaneously injected with  $2 \times 10^4$  4T1 cells into the fourth mammary fat pad.

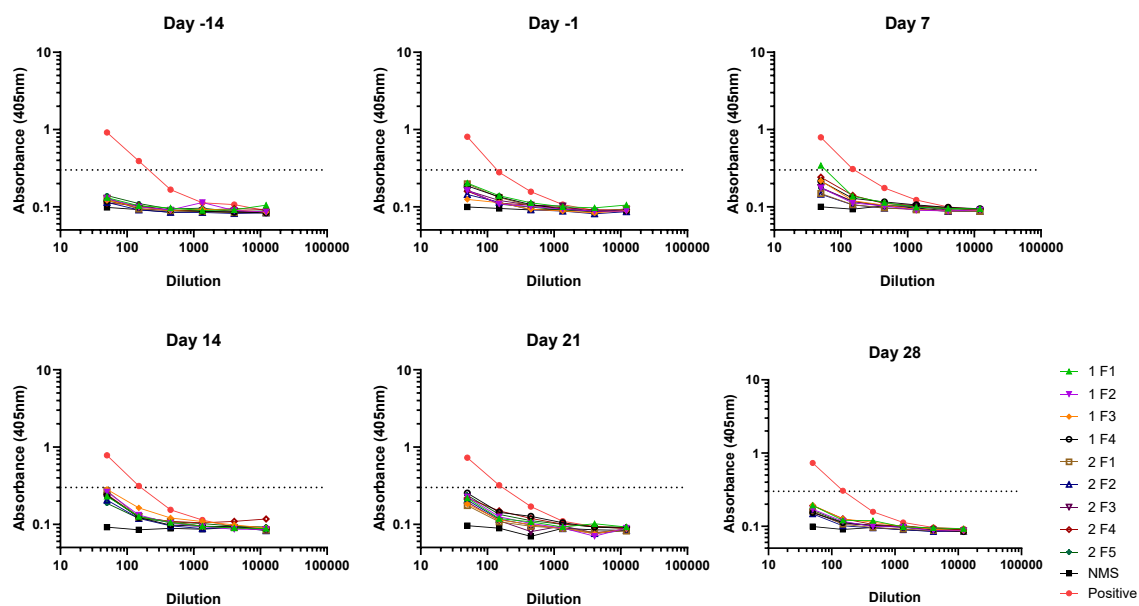
ROBO4-specific IgG1 antibodies in sera were quantified using ELISA. As expected, no ROBO4-specific IgG1 antibodies were detected on day -14 (before immunised with ROBO4-TTc; Figure 4.18B). One day before the tumour cell implantation (two weeks post-first immunisation with ROBO4-TTc), ROBO4-specific antibodies were detected in the ROBO4TTc group. By day 7 (one week post-second immunisation with ROBO4-TTc), ROBO4-specific antibody titres in the ROBO4TTc group increased significantly compared to both day -1 and the TTc group, reaching their peak. From day 7 to day 28, antibody titres remained stable without significant fluctuations. In contrast, no ROBO4-specific IgG1 was detected at any time in the TTc group (Figure 4.18B and C).

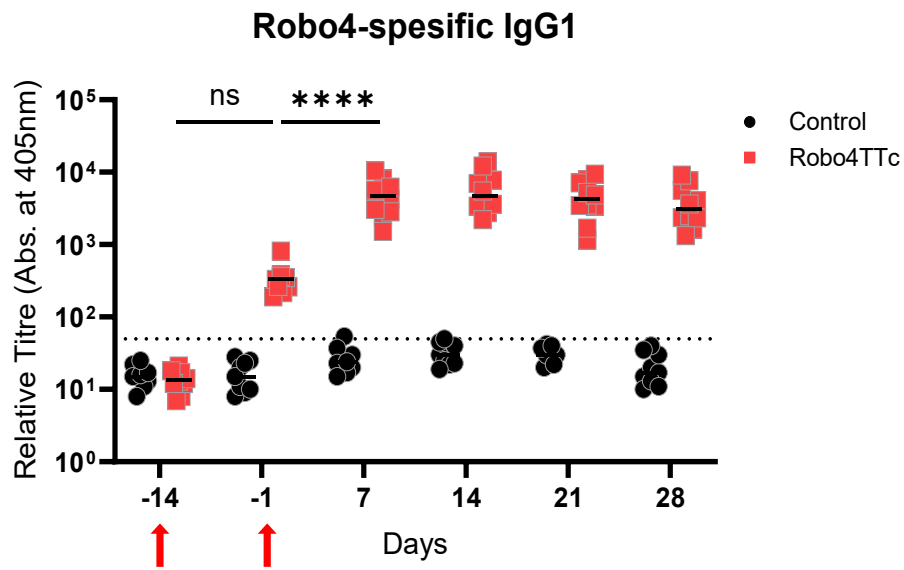
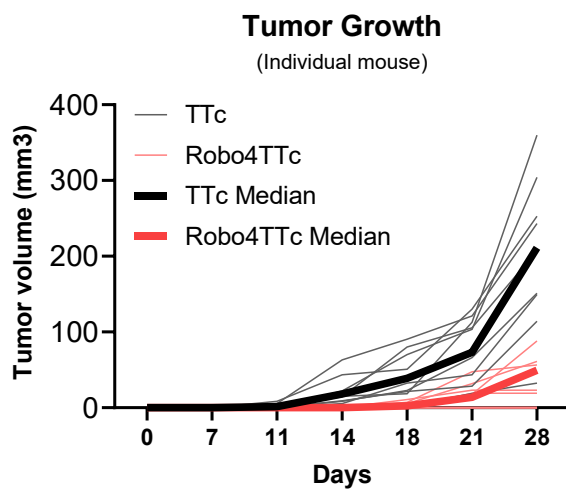
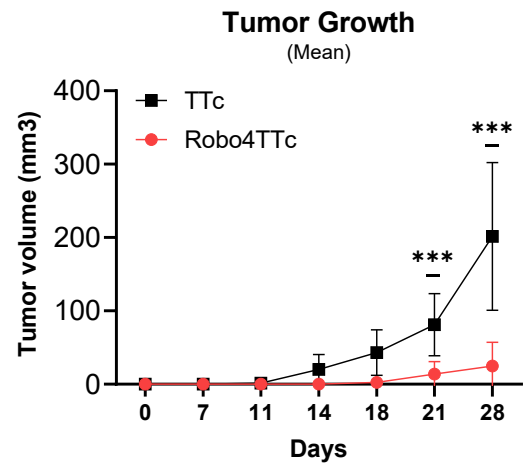
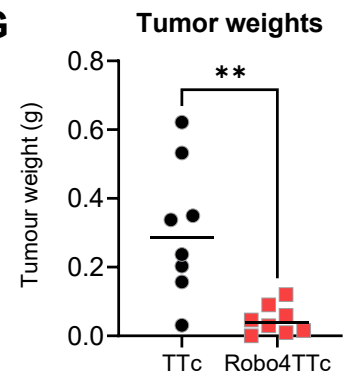


**B**      **ROBO4TTc Group**



**TTc Group**



**C****D****E****F****G**

**Figure 4.18 ROBO4TTc vaccine restricts tumour growth in the TNBC mouse model (4T1).** (A) Experimental strategy for ROBO4TTc vaccine immunisation in vivo. Mice were i.p. primed with TTc or ROBO4TTc in alum. 50 µg of the respective antigen was given on Days 30 and 45 post-initial priming. One day post the second booster, all mice were subcutaneously implanted with 4T1 cells. All mice were culled on Day 28. (B) ROBO4-specific IgG1 dilution curves from the ROBO4TTc and TTc groups. Serum samples were analysed to check ROBO4-specific antibodies by ELISA on Days -14, -1, 7, 14, 21, and 28. Data points for TTc (n=9) and ROBO4TTc (n=10) are shown for each time point. A sample from a previous experiment served as a positive control, and normal mouse serum as a negative control. Each line represents individual mouse sera. Dashed black lines indicate the threshold for determining ROBO4-specific titres. (C) ROBO4-specific IgG1 response in ROBO4TTc and TTc group. Each symbol represents an individual mouse, with bars showing the median. The dashed line shows detection at 50. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test (ns > 0.5; \*\*\*\*p < 0.0001). (D) Individual tumour growth curves. Data for TTc (n=8) and ROBO4TTc (n=8) mice are shown. (E) Mean tumour growth curves. Error bars represent the standard deviation. Data were analysed using a two-tailed Mann-Whitney test (\*\*\*p < 0.001). (F) Representative tumour images: Images from the TTc and ROBO4TTc groups on Day 28. (G) Tumour weights. Data were analysed using a two-tailed Mann-Whitney test (\*\*p < 0.01).

Tumour growth data were plotted as individuals with median and mean values with error bars representing the standard deviation (SD; Figure 4.18D). Tumours were detectable approximately 10 days post-transplantation. Tumour growth in the ROBO4TTc group was significantly slower than in the TTc group, mainly from day 18 onwards, with significant differences in tumour volume observed on days 21 and 24. On day 28, all mice were culled, and tumours were collected for weighing and flow cytometry analysis. Tumours in the ROBO4TTc group were significantly smaller than those in the TTc group (Figure 4.18D). Notably, two mice in the ROBO4TTc group remained tumour-free.

As tumour growth significantly reduced following ROBO4TTc vaccination, we sought to identify the mechanism driving this by characterising broad immune cell populations in tumours. Figure 4.19 outlines the gating strategy for identifying significant immune cell populations in 4T1 tumours and draining lymph nodes. The strategy involved excluding artefacts by removing events from the first and last few seconds of acquisition. Singlet cells were selected based on side scatter (SSC) and forward scatter (FSC), followed by the exclusion of dead cells. Live CD45<sup>+</sup> immune cells were gated, after which CD19<sup>+</sup>B220<sup>+</sup> and CD19<sup>+</sup>B220<sup>-</sup> cells were identified as B and B1 cells. CD19<sup>-</sup> cells were further classified based on CD11b and CD11c expression. CD11b<sup>low</sup> cells were subdivided into CD4<sup>+</sup> and CD8<sup>+</sup> T cells, while CD11b<sup>high</sup> cells were analysed for MHC-II<sup>+</sup> CD11c<sup>+</sup> dendritic cells (DCs). Additionally, Ly6C<sup>-</sup>F4/80<sup>+</sup> macrophages and Ly6C<sup>+</sup> neutrophils were identified. As expected, mainly myeloid cells were detected in 4T1 tumours.

The data revealed notable differences across multiple immune cell populations following vaccination with ROBO4TTc (Figure 4.20). First, the proportion of live cells

was slightly higher in ROBO4TTc-vaccinated mice (30–45%) than in controls (10–25%). CD45<sup>+</sup> immune cells also showed an increasing trend, with 25–35% in ROBO4TTc-vaccinated mice versus 20–25% in controls (Figure 4.20A).

Further analysis showed an enrichment of CD11b<sup>low</sup> cells in the ROBO4TTc group, while CD11b<sup>high</sup> cells were significantly lower than in controls. Both B (B220<sup>+</sup>CD19<sup>+</sup>) and B1 (B220<sup>-</sup>CD19<sup>+</sup>) cells accounted for 0.5–1.5% of total CD45<sup>+</sup> cells in the ROBO4TTc group, nearly double the control (~0.5% of total CD45<sup>+</sup> cells). CD4<sup>+</sup> T cells were 10–30% in ROBO4TTc compared to 5–10% in controls, while CD8<sup>+</sup> T cells increased from <5% in controls to 5–10% in the ROBO4TTc group. Dendritic cells also showed a trend toward elevation, rising from a mean of 2% in controls to 3% in the ROBO4TTc group. In ROBO4TTc tumours, neutrophils were significantly reduced (20–50% of CD45<sup>+</sup> cells) compared to the TTc group (40–80% of CD45<sup>+</sup> cells). Macrophages showed no significant differences. The immune profiling data aligns well with the observed reduction in tumour growth following ROBO4TTc vaccination, suggesting a meaningful immunological shift within the tumour microenvironment. Specifically, vaccination was associated with increased infiltration of adaptive immune populations, CD4<sup>+</sup> and CD8<sup>+</sup> T cells and B cells, while overall myeloid cell presence, particularly CD11b<sup>+</sup> populations, was diminished.

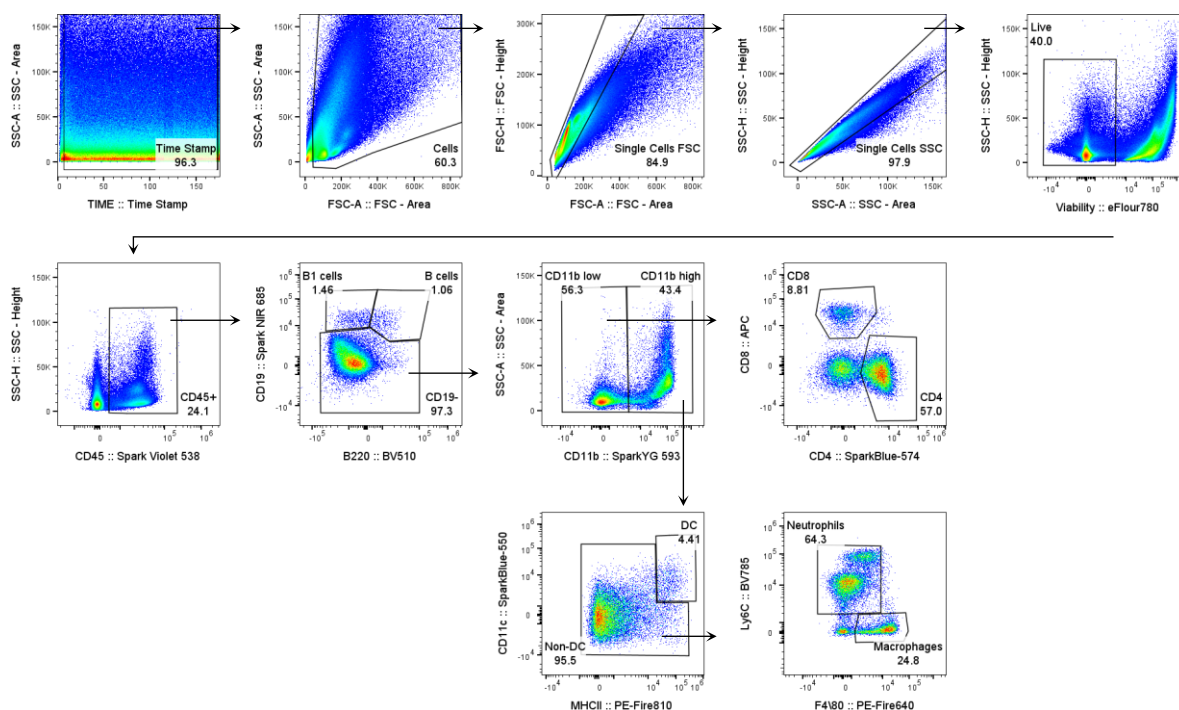
These changes indicate a transition from a suppressive, myeloid-dominated environment to one more permissive to antitumour immunity. The elevated presence of antigen-presenting dendritic cells, alongside higher CD4<sup>+</sup> and CD8<sup>+</sup> T cell frequencies, suggests enhanced antigen processing and T cell priming, potentially leading to improved effector function within the tumour. Similarly, increased B cell numbers could contribute to local antibody production.

In conclusion, ROBO4TTc vaccination reprograms the tumour immune microenvironment towards a more immunostimulatory profile. This shift likely underlies the observed tumour growth delay and highlights the vaccine's potential to elicit systemic immune responses and reshape local tumour immunity.

In the draining lymph node (Figure 4.20B) immune cell populations showed no significant differences, though dendritic and CD11b<sup>high</sup> cells exhibited a surprisingly decreasing trend.

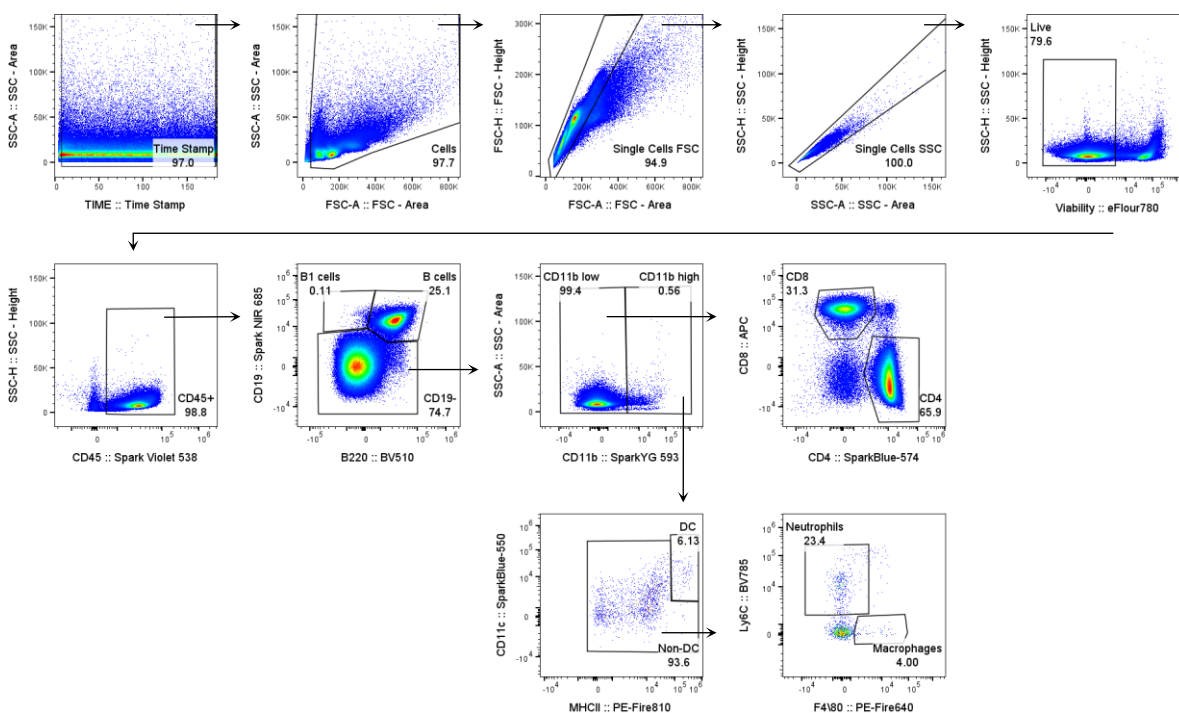
**A**

## 4T1 Tumour – Main Populations



**B**

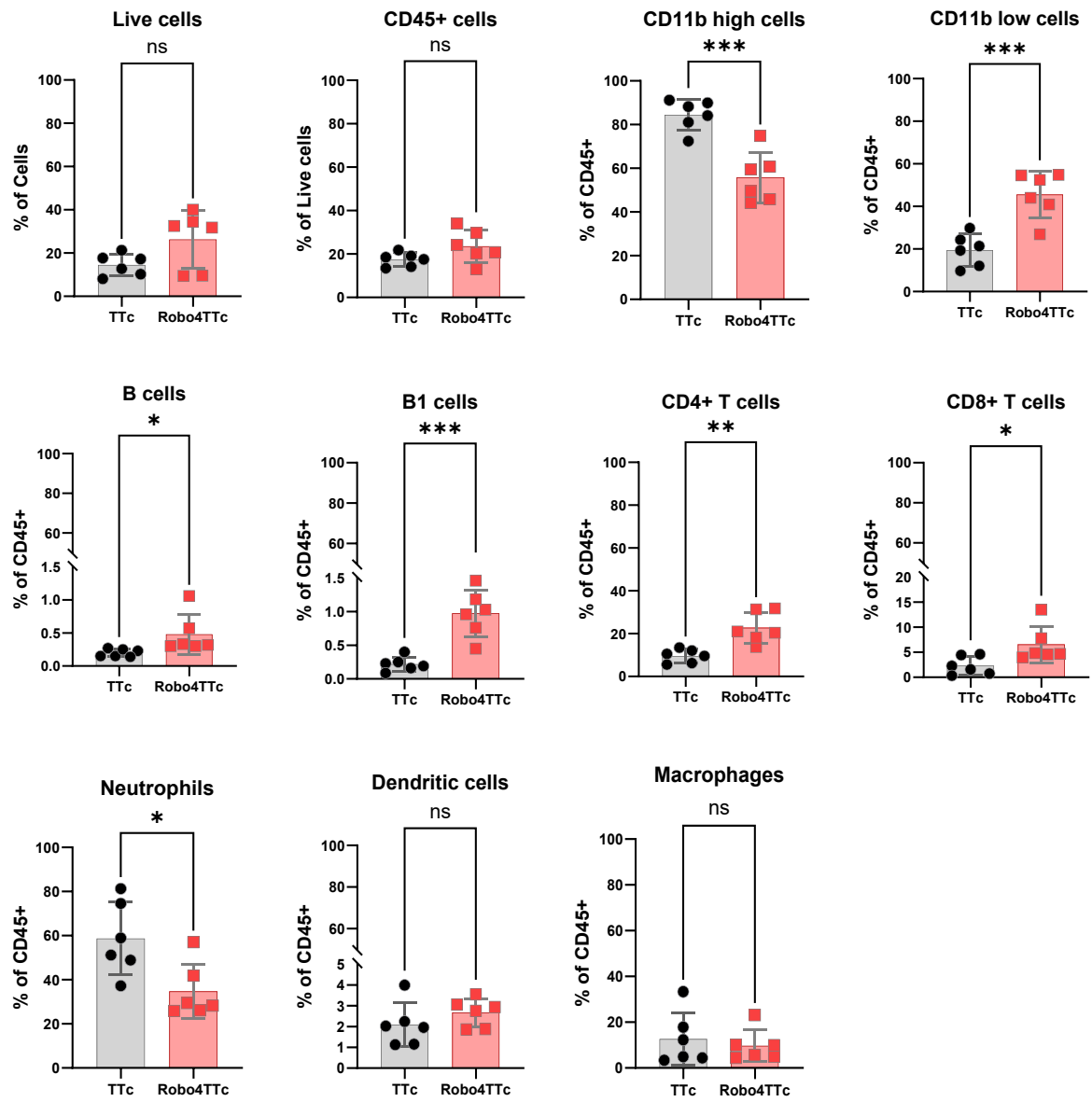
## Draining Lymph Node – Main Populations

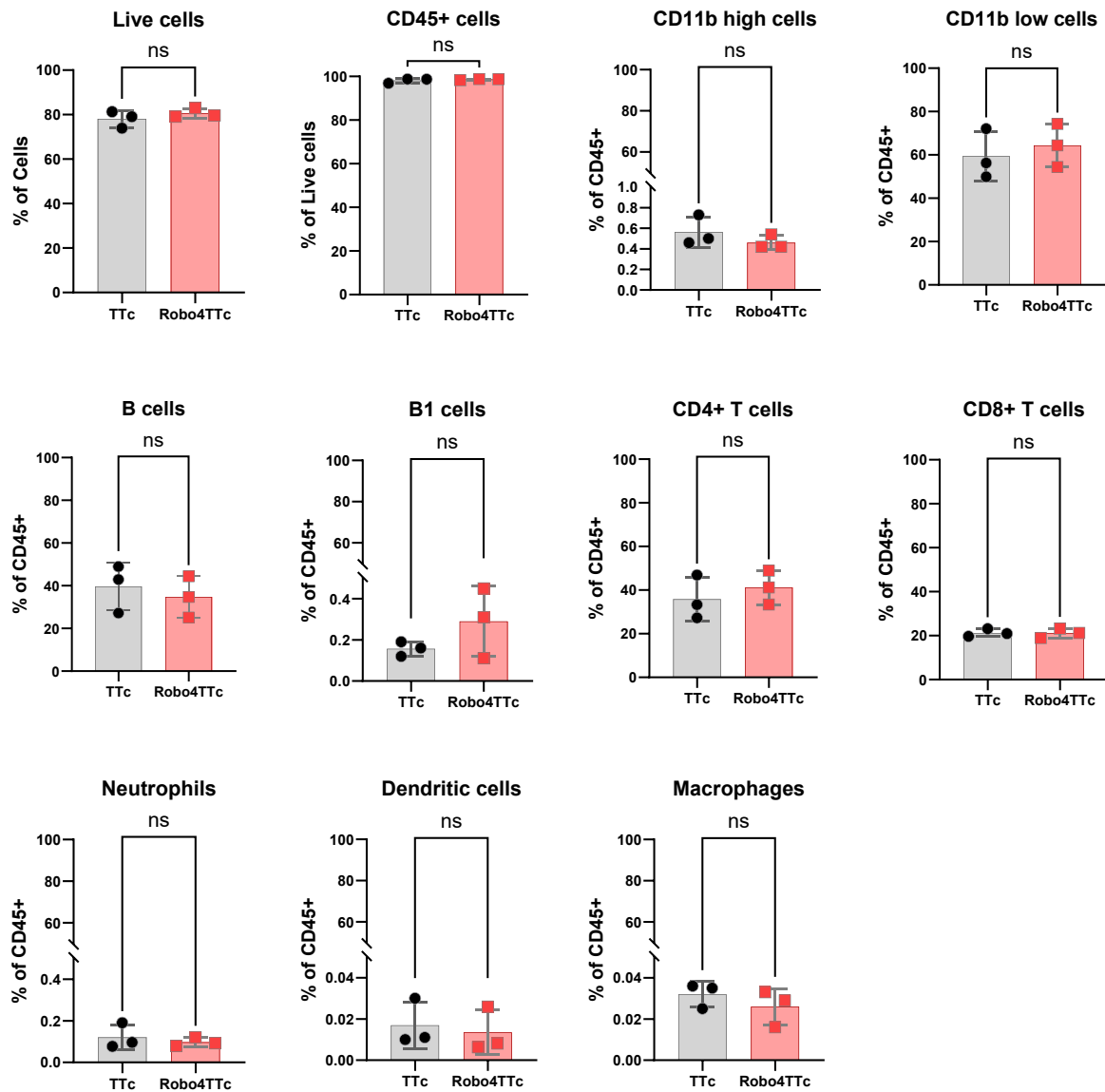


**Figure 4.19 Gating strategy for main immune populations in 4T1 tumours.** Gating strategy used to evaluate the immune response in ROBO4TTc vaccinated mice. 4T1 tumour (A) and draining lymph node (B) samples from control and ROBO4TTc-vaccinated groups were analysed by spectral flow cytometry. The gating strategy begins by excluding events from the first few seconds of acquisition to remove potential artefacts. Cells are then gated based on SSC and FSC to select singlets, followed by the exclusion of dead cells. Live CD45<sup>+</sup> immune cells are gated, after which CD19<sup>+</sup>B220<sup>+</sup> and CD19<sup>+</sup>B220<sup>-</sup> cells are identified as B and B1 cells, respectively. CD19<sup>-</sup> cells are further gated based on CD11b and CD11c expression. CD11b<sup>low</sup> cells are subdivided into CD4<sup>+</sup> and CD8<sup>+</sup> T cells. CD11b<sup>high</sup> cells are further analysed for MHC-II<sup>+</sup> and CD11c<sup>+</sup> dendritic cells (DCs). Ly6C<sup>-</sup>F4/80<sup>+</sup> macrophages and Ly6C<sup>+</sup>F4/80<sup>low</sup> neutrophils are classified.

**A**

## 4T1 Tumour – Main Populations



**B****Draining Lymph Node – Main Populations**

**Figure 4.20 Immune profiling of draining lymph nodes and 4T1 tumours in TTc- and ROBO4TTc-vaccinated mice.** Flow cytometry analysis quantifies live immune cell populations (CD45+ live cells) in the 4T1 tumour (A) and draining lymph node (B), including subsets such as CD11bhigh and CD11blow cells, CD4+ and CD8+ T cells, B cells (B220+ CD19+), dendritic cells, macrophages, and neutrophils. Data are presented as the percentage of CD45+ lymphocytes. Statistical comparisons between the two groups were conducted using an unpaired non-parametric t-test (two-tailed), with results shown as mean  $\pm$  SD. Significance levels are indicated as ns (not significant),  $p \geq 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ .

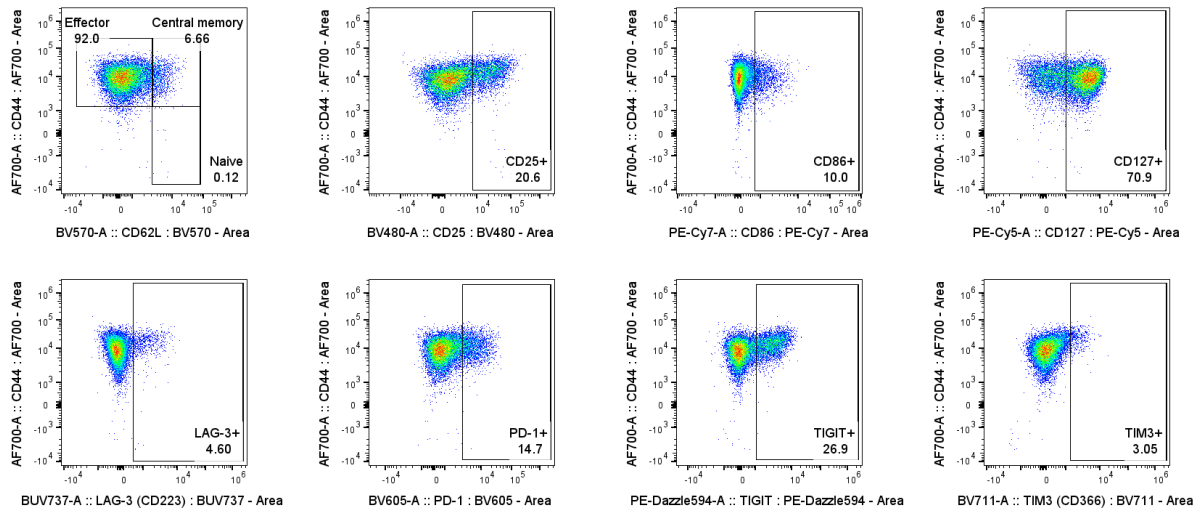
#### **4.3.6.1 ROBO4TTc vaccinated tumours show enhanced central memory and reduced exhaustion in CD4<sup>+</sup> and CD8<sup>+</sup> T cells.**

CD4<sup>+</sup> T cells were classified as central memory (CD44<sup>+</sup>CD62L<sup>+</sup>), effector (CD44<sup>+</sup>CD62L<sup>-</sup>), or naive (CD44<sup>-</sup>CD62L<sup>+</sup>) subsets. Exhaustion markers (PD-1, LAG-3, TIM-3, TIGIT) were assessed to determine T cell dysfunction. In contrast, activation markers (CD25, CD86, CD127) were analysed to evaluate regulatory activity, co-stimulation, and T-cell survival (detailed gating strategy shown in (Figure 4.21). In ROBO4TTc tumours, central memory CD4<sup>+</sup> T cells were significantly increased (2–7% of CD4<sup>+</sup> T cells) compared to controls (1–3%), while effector and naive subsets remained unchanged. Interestingly, PD-1 expression on CD4<sup>+</sup> T cells was markedly lower in ROBO4TTc tumours (10–25% of CD4<sup>+</sup> T cells) than in controls (25–40%). TIM-3 expression showed a decreasing trend, whereas LAG-3 levels remained unchanged. No significant differences were observed for CD127 and CD86, while CD25 expression was slightly lower in ROBO4TTc tumours (28%) compared to controls (35%) (Figure 4.22A).

Similarly, CD8<sup>+</sup> T cells were analysed for central memory, effector, and naive subsets, along with exhaustion and activation markers (Figure 4.23). Central memory CD8<sup>+</sup> T cells were increased in ROBO4TTc tumours (5–8% of CD8<sup>+</sup> T cells) compared to controls (7–16%) in 4T1 tumour, while effector and naive subsets showed no significant differences (Figure 4.24A). These findings were further supported by UMAP plots, providing a comprehensive overview of cellular distribution and marker expression patterns (Figure 4.27). Except for the increased CD8 memory T cell population, no significant changes were observed in the CD4 or CD8 T cell subsets in the draining lymph nodes from the ROBO4TTc group compared to the TTc group.

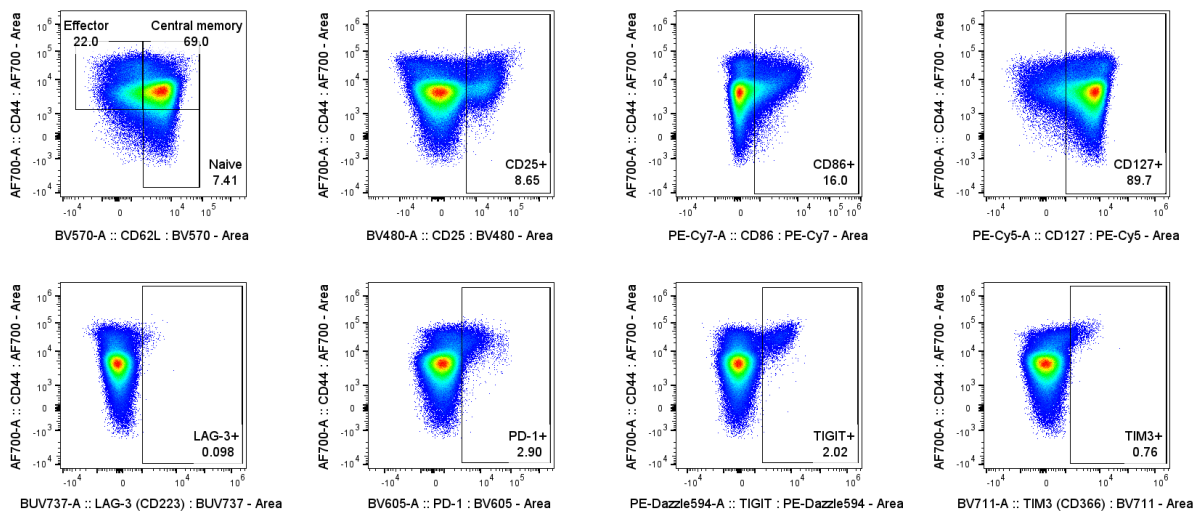
**A**

### 4T1 Tumour – CD4+ T cells



**B**

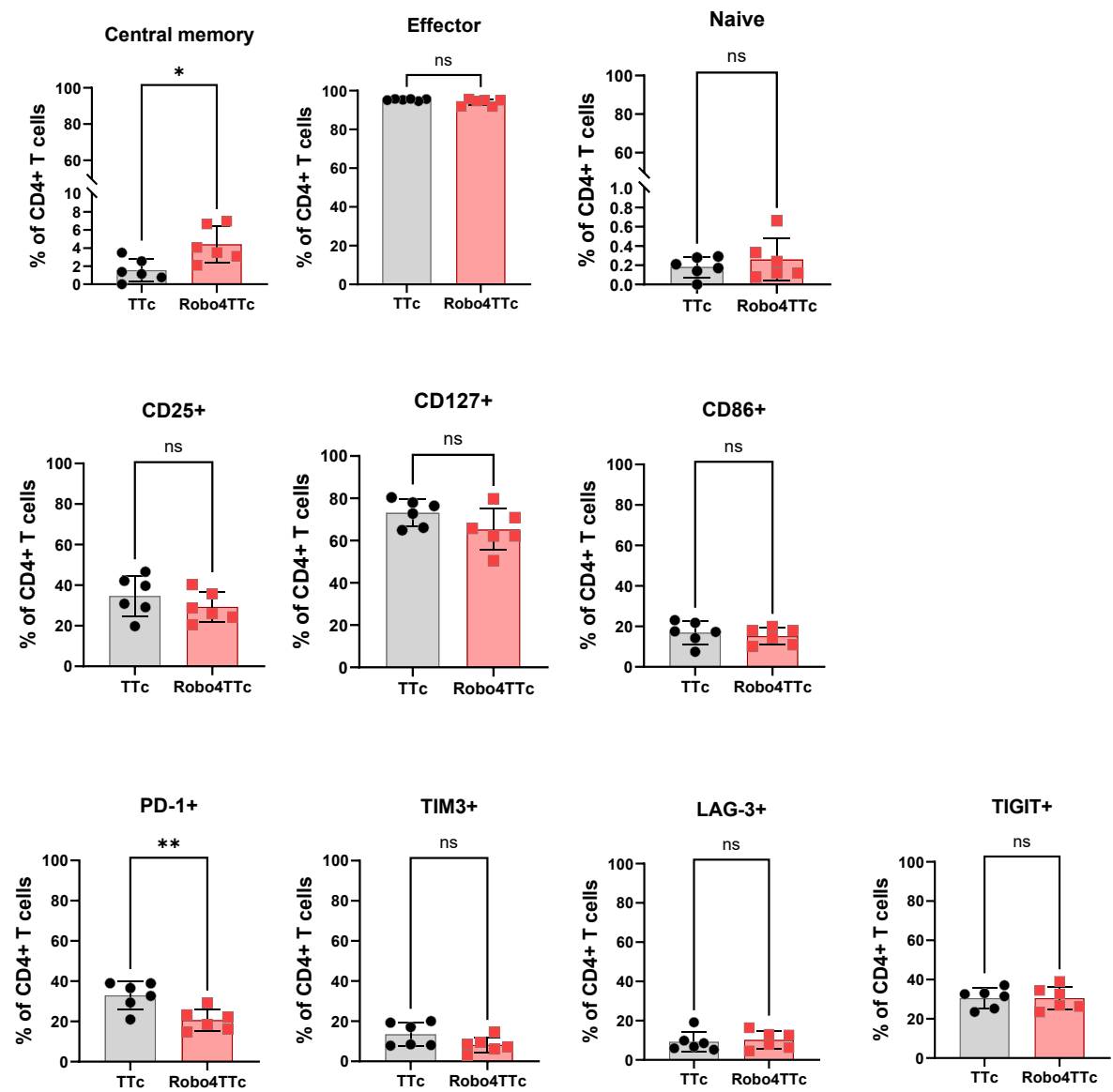
### Draining Lymph Node – CD4+ T cells

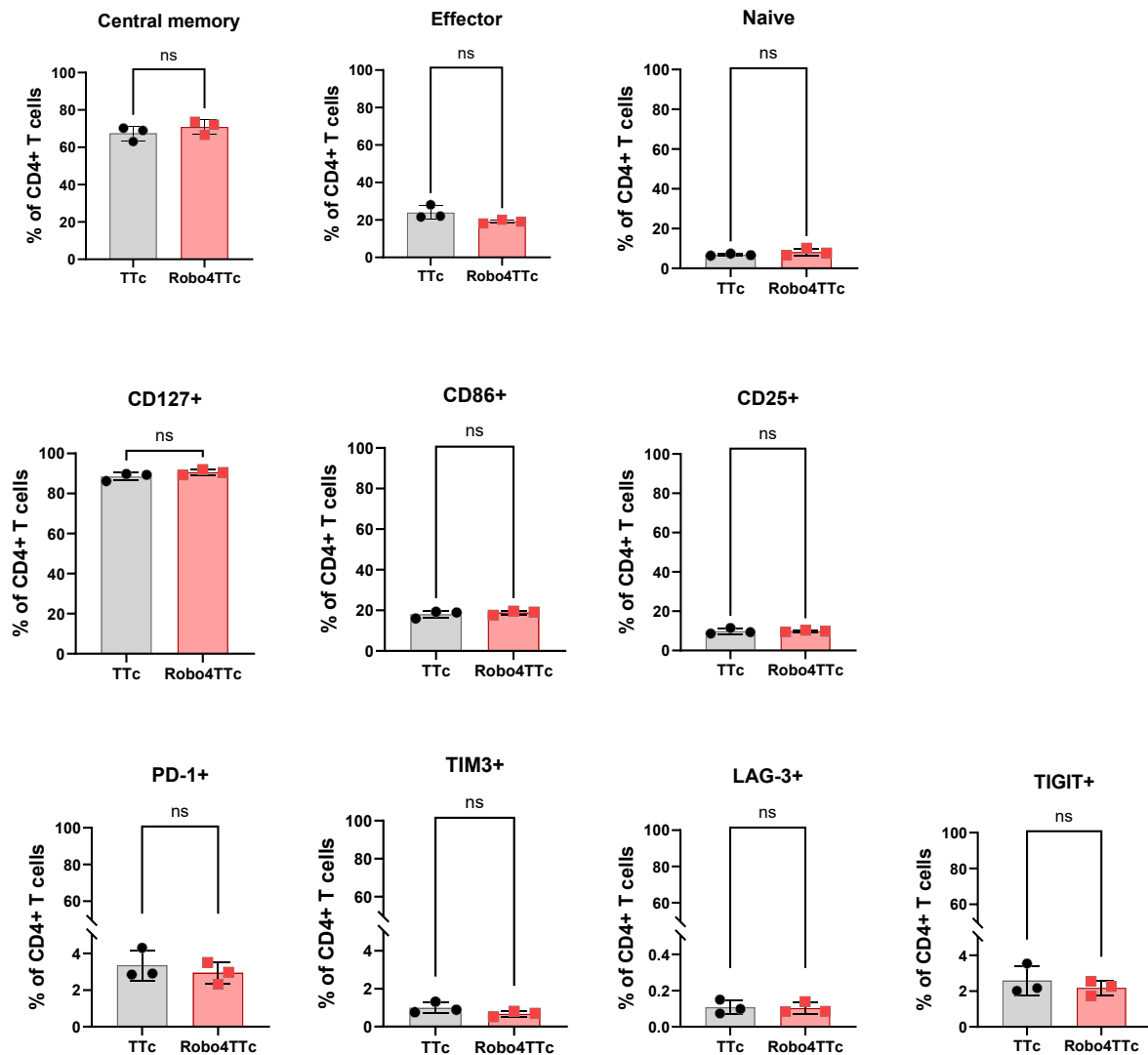


**Figure 4.21 Gating strategy for CD4+ T cell flow cytometry analysis.** 4T1 tumour (A) and draining lymph node (B) samples from TTc- and ROBO4TTc-vaccinated groups were analysed using spectral flow cytometry. The analysis begins by excluding acquisition artefacts, followed by gating for singlets and live CD45<sup>+</sup> immune cells. CD4<sup>+</sup> T cell subsets were identified as central memory (CD44<sup>+</sup>CD62L<sup>+</sup>), effector (CD44<sup>+</sup>CD62L<sup>-</sup>), and naive (CD44<sup>-</sup>CD62L<sup>+</sup>) T cells. Exhaustion markers (PD-1, LAG-3, TIM-3, TIGIT), while activation markers (CD25, CD86, CD127), co-stimulation, and T-cell survival.

**A**

# **4T1 Tumour – CD4+ T cells**

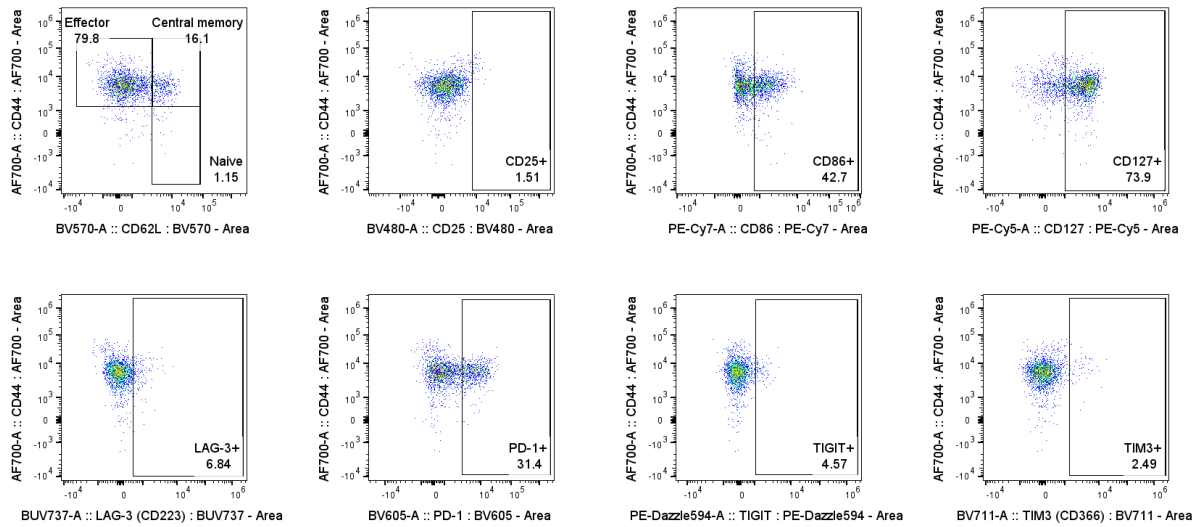


**B****Draining Lymph Node – CD4+ T cells**

**Figure 4.22 CD4+ T cell flow cytometry analysis.** 4T1 tumour (A) and draining lymph node (B) samples from TTc- and ROBO4TTc-vaccinated groups were analysed using spectral flow cytometry. The analysis begins by excluding acquisition artefacts, followed by gating for singlets and live CD45<sup>+</sup> immune cells. CD4<sup>+</sup> T cell subsets were identified as central memory (CD44<sup>+</sup>CD62L<sup>+</sup>), effector (CD44<sup>+</sup>CD62L<sup>-</sup>), and naive (CD44<sup>-</sup>CD62L<sup>+</sup>) T cells. Exhaustion markers (PD-1, LAG-3, TIM-3, TIGIT), while activation markers (CD25, CD86, CD127), co-stimulation, and T-cell survival. Data are presented as the percentage of CD4<sup>+</sup> T cells. Statistical comparisons between the two groups were conducted using an unpaired non-parametric t-test (two-tailed), with results shown as mean  $\pm$  SD. Significance levels are indicated as ns (not significant),  $p \geq 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ .

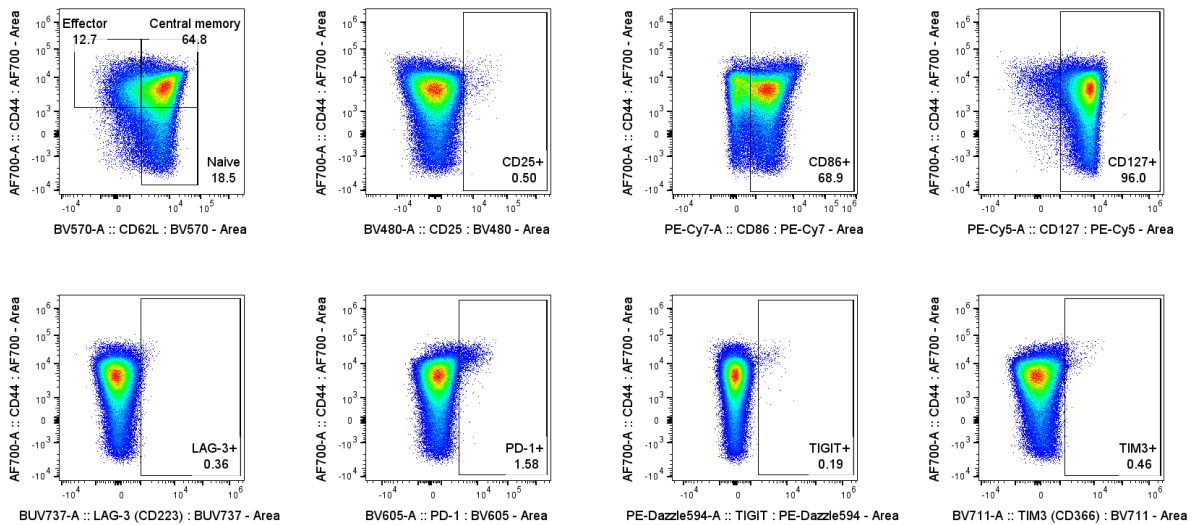
**A**

### 4T1 Tumour – CD8+ T cells



**B**

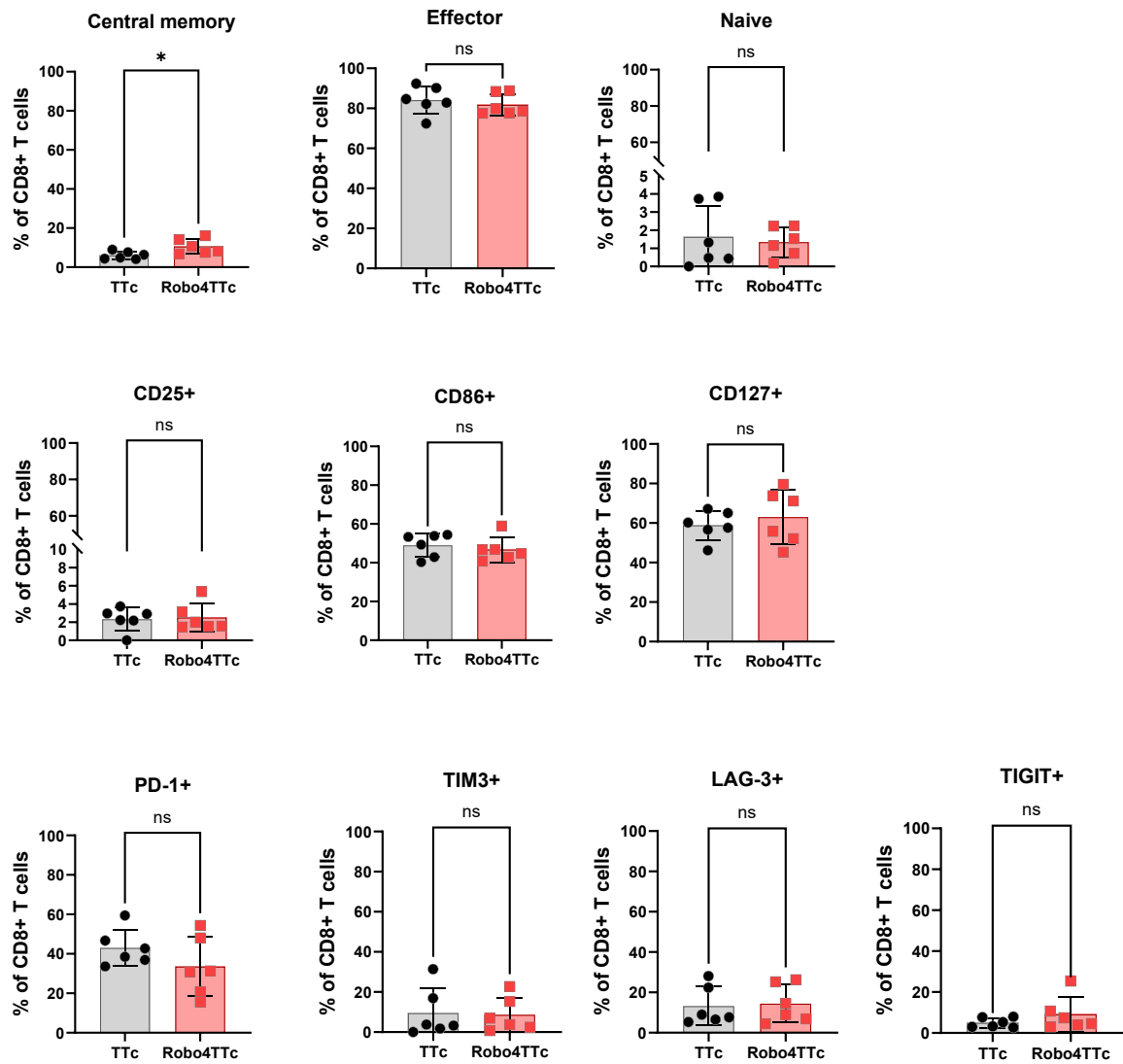
### Draining Lymph Node – CD8+ T cells

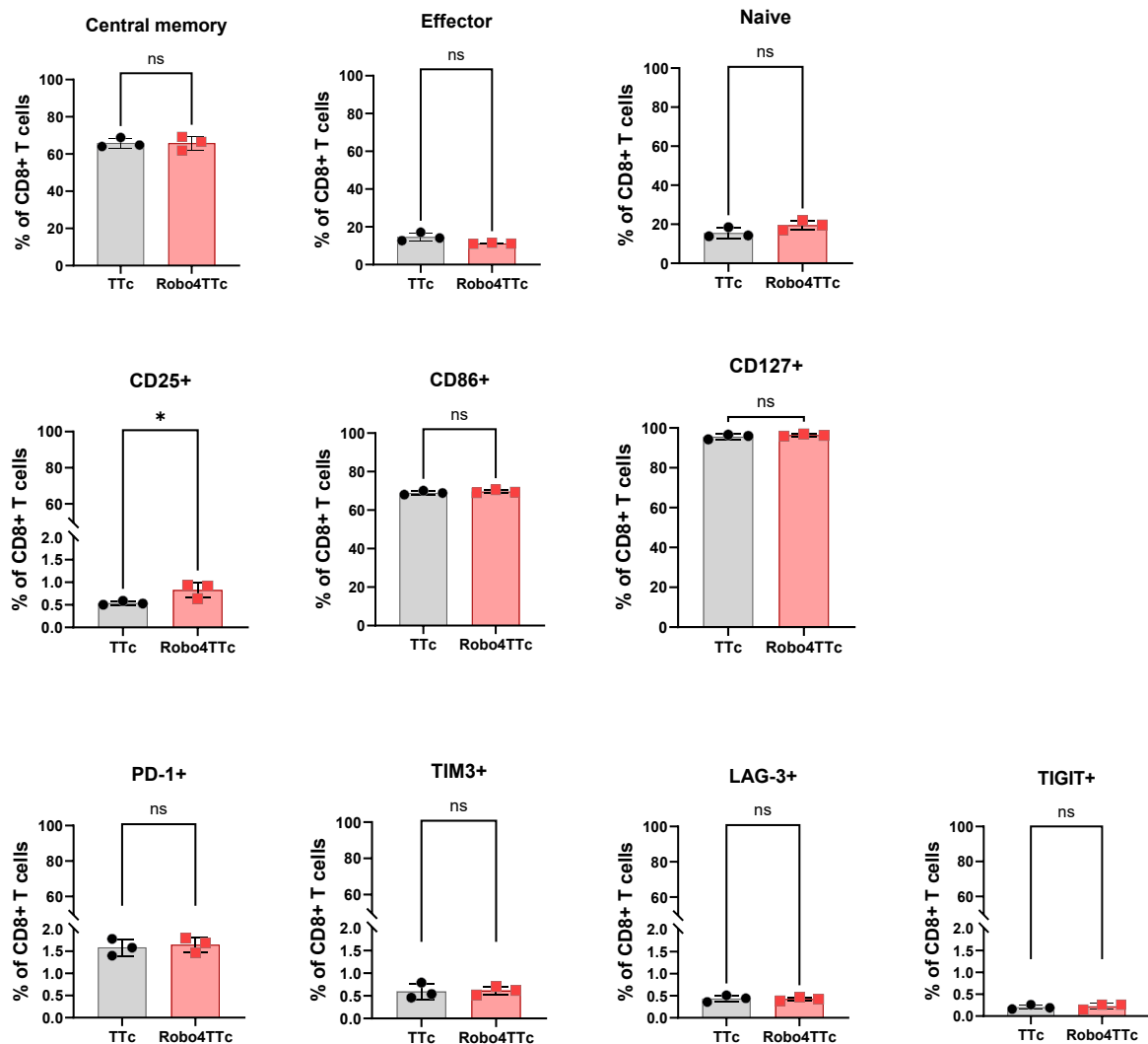


**Figure 4.23 Gating strategy for CD8+ T cell flow cytometry analysis.** Draining lymph node (A) and tumour (B) samples from TTc- and ROBO4TTc-vaccinated groups were analysed using spectral flow cytometry. The analysis begins by excluding acquisition artefacts, followed by gating for singlets and live CD45<sup>+</sup> immune cells. CD8<sup>+</sup> T cell subsets were identified as central memory (CD44<sup>+</sup>CD62L<sup>+</sup>), effector (CD44<sup>+</sup>CD62L<sup>-</sup>), and naive (CD44<sup>-</sup>CD62L<sup>+</sup>) T cells. Exhaustion markers (PD-1, LAG-3, TIM-3, TIGIT), activation markers (CD25, CD86, CD127), co-stimulation, and T cell survival.

**A**

# **4T1 Tumour – CD8+ T cells**



**B****Draining Lymph Node – CD8+ T cells**

**Figure 4.24 CD8+ T cell flow cytometry analysis.** 4T1 Tumour (A) and draining lymph node (B) samples from TTc- and ROBO4TTc-vaccinated groups were analysed using spectral flow cytometry. The analysis begins by excluding acquisition artefacts, followed by gating for singlets and live CD45<sup>+</sup> immune cells. CD8<sup>+</sup> T cell subsets were identified as central memory (CD44<sup>+</sup>CD62L<sup>+</sup>), effector (CD44<sup>+</sup>CD62L<sup>-</sup>), and naive (CD44<sup>-</sup>CD62L<sup>+</sup>) T cells. Exhaustion markers (PD-1, LAG-3, TIM-3, TIGIT), while activation markers (CD25, CD86, CD127), co-stimulation, and T-cell survival. Data are presented as the percentage of CD8<sup>+</sup> T cells. Statistical comparisons between the two groups were conducted using an unpaired non-parametric t-test (two-tailed), with results shown as mean  $\pm$  SD. Significance levels are indicated as ns (not significant),  $p \geq 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ .

#### **4.3.6.2 ROBO4TTc vaccinated tumours exhibit reduced ICOS-L and increased MHC-II expression in neutrophils.**

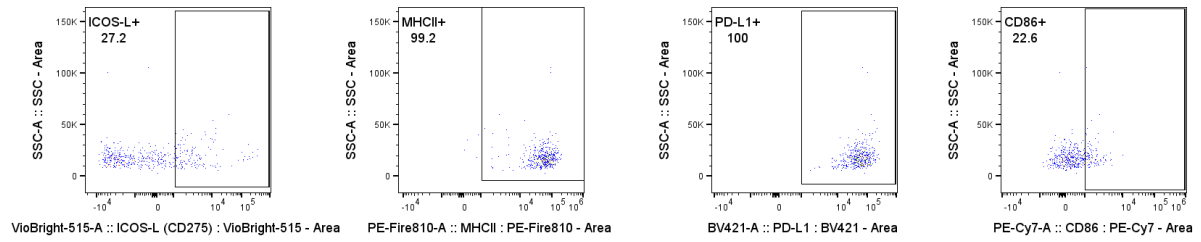
B cells, macrophages, neutrophils, and dendritic cells were also further analysed (detailed gating strategy shown in Figure 4.25). B and B1 cells were identified as CD19<sup>+</sup>B220<sup>+</sup> and CD19<sup>+</sup>B220<sup>-</sup> respectively, while CD19<sup>-</sup> cells were distinguished based on CD11b and CD11c expression. CD11b<sup>high</sup> cells were further characterised as MHC-II<sup>+</sup>CD11c<sup>+</sup> dendritic cells (DCs), Ly6C<sup>-</sup>F4/80<sup>+</sup> macrophages, and Ly6C<sup>+</sup> neutrophils (Figure 17). PD-L1, ICOS-L, MHC-II, and CD86 expression were analysed on B cells, macrophages, neutrophils and DCs.

In tumours, ICOS-L expression was significantly lower across all cell types (B cells, macrophages, neutrophils, and DCs) in the ROBO4TTc group than in controls (Figure 4.26). Although the total neutrophil population was diminished in the ROBO4TTc group, these neutrophils demonstrated a markedly enhanced expression of MHC-II. These findings suggest that the ROBO4TTc vaccination may alter the tumour microenvironment by reducing immunosuppressive neutrophil infiltration and promoting a more activated neutrophil phenotype, as evidenced by increased MHC-II expression.

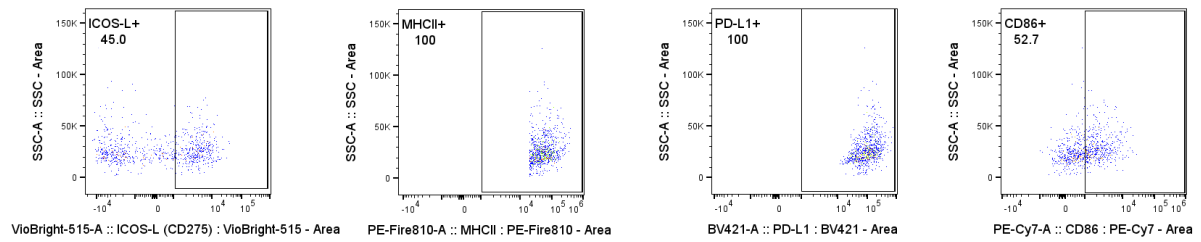
**A**

## 4T1 Tumour – Immune cell populations

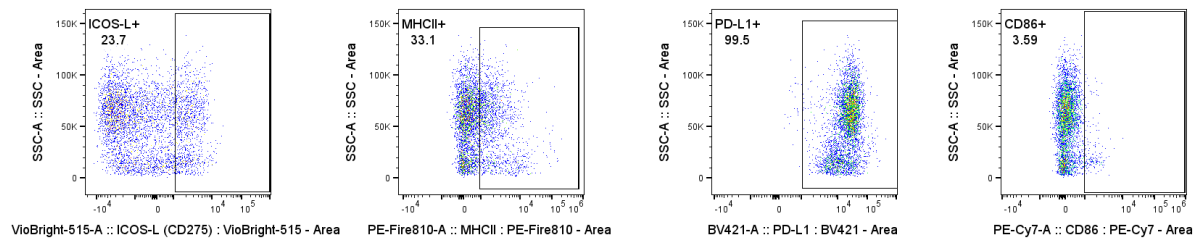
### B cells



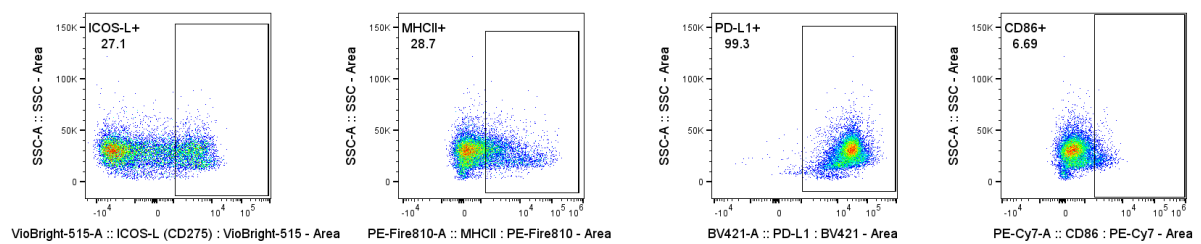
### Dendritic cells



### Macrophages

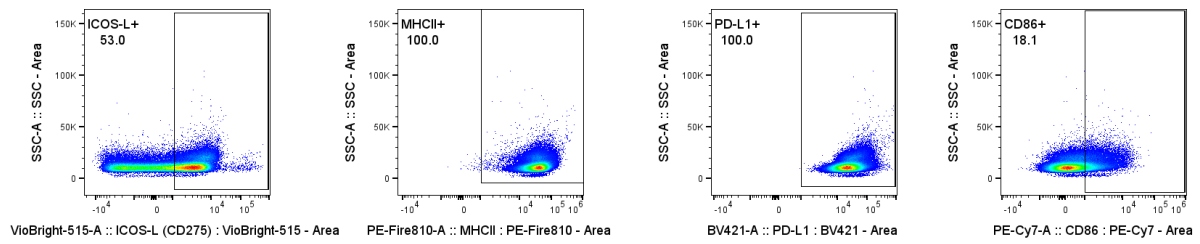


### Neutrophils

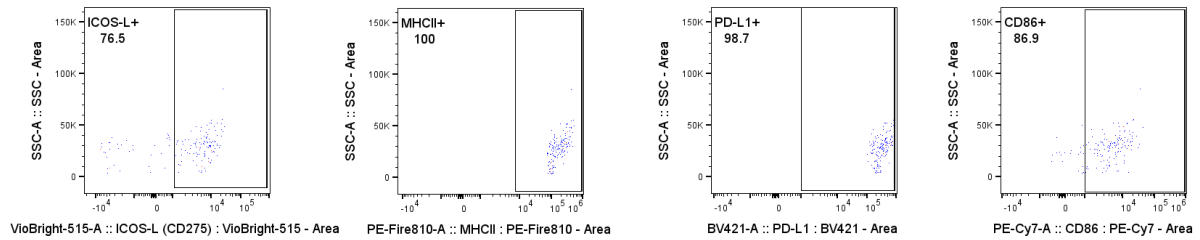


## B Draining Lymph Node – Immune cell populations

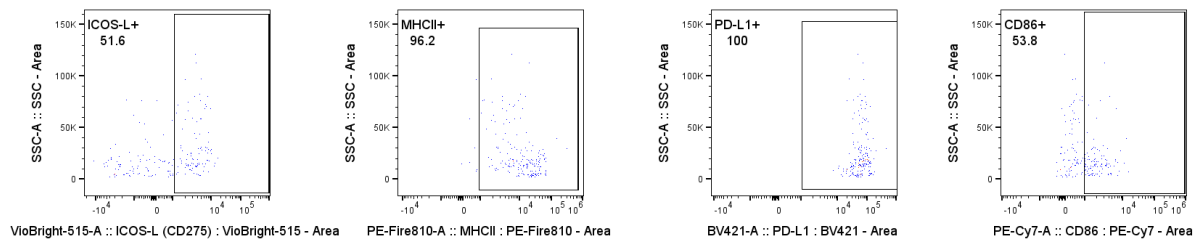
### B cells



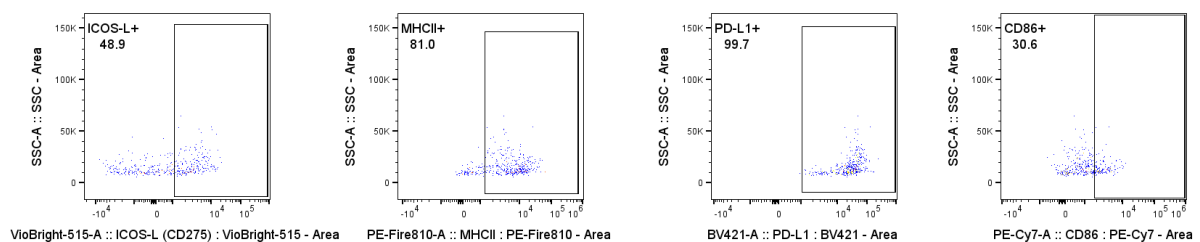
### Dendritic cells



### Macrophages



### Neutrophils

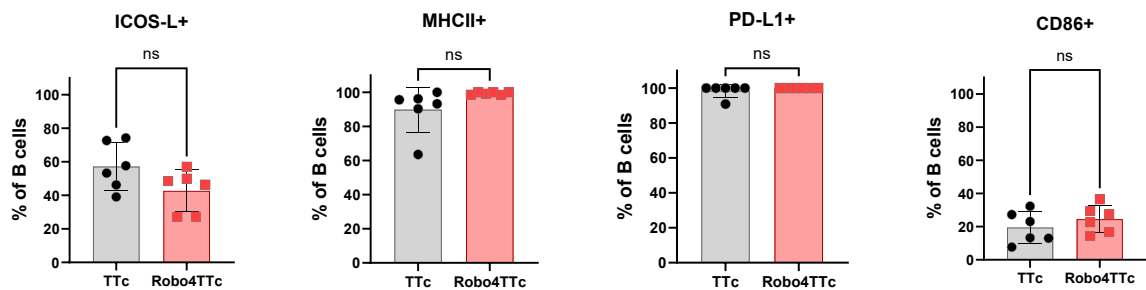


**Figure 4.25 Gating strategy for immune cell population analysis by flow cytometry.** Spectral flow cytometry was used to analyse immune cell populations in (A) 4T1 tumours and (B) draining lymph nodes from TTc- and ROBO4TTc-vaccinated mice. The gating strategy involved initial exclusion of acquisition artefacts, followed by doublet discrimination and gating on live CD45<sup>+</sup> immune cells. B and B1 cells were identified as CD19<sup>+</sup>B220<sup>+</sup> and CD19<sup>+</sup>B220<sup>-</sup>, respectively. CD19<sup>-</sup> cells were then differentiated based on CD11b and CD11c expression. CD11b<sup>high</sup> cells were further characterised as MHC-II<sup>+</sup>CD11c<sup>+</sup> dendritic cells (DCs), Ly6C<sup>-</sup>F4/80<sup>+</sup> macrophages, and Ly6C<sup>+</sup>F4/80<sup>low</sup> neutrophils. ICOS-L, MHCII, PD-L1 and CD86 expression was assessed on B cells, macrophages, neutrophils and DCs.

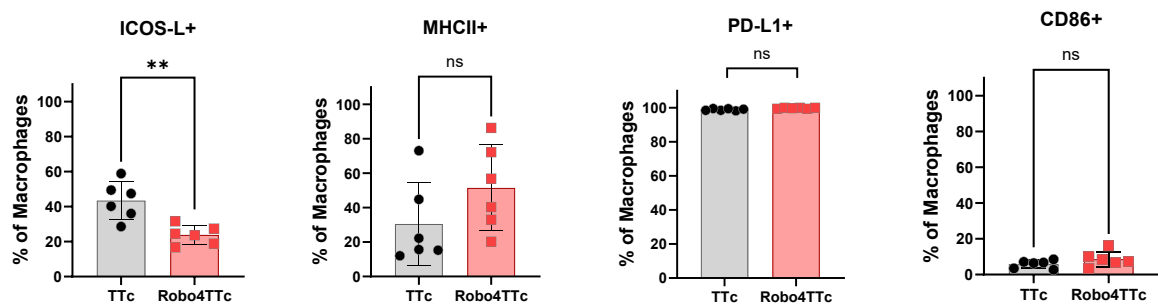
**A**

## 4T1 Tumour – Immune cell populations

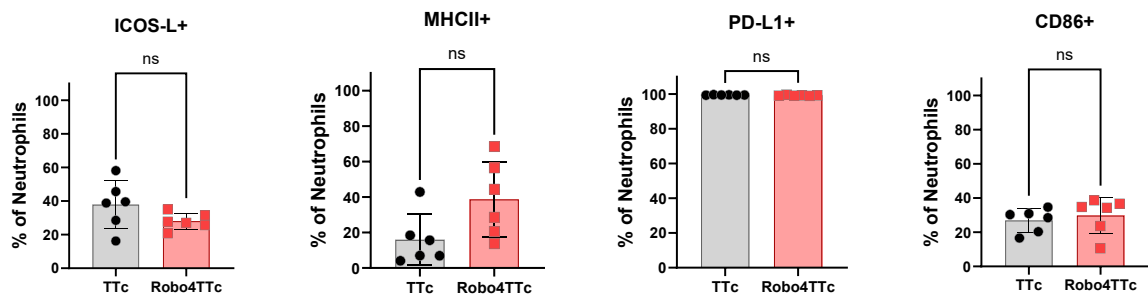
### B cells



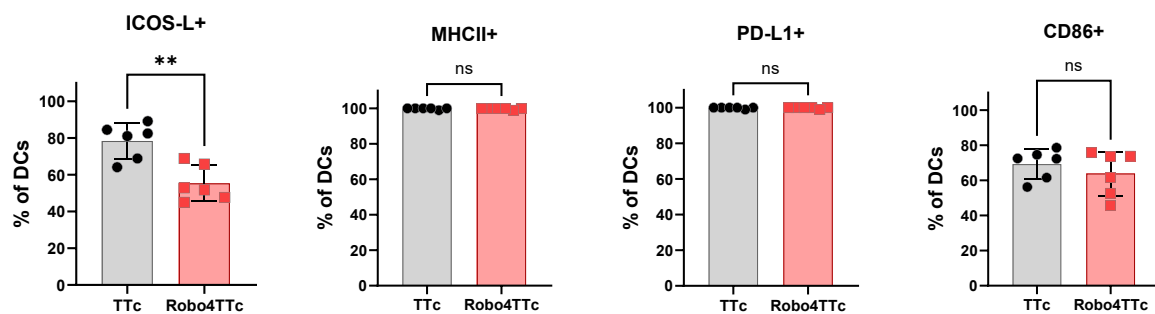
### Macrophages

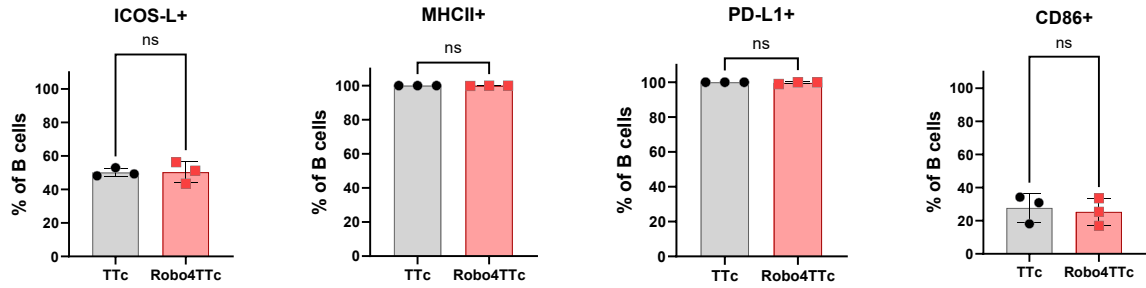
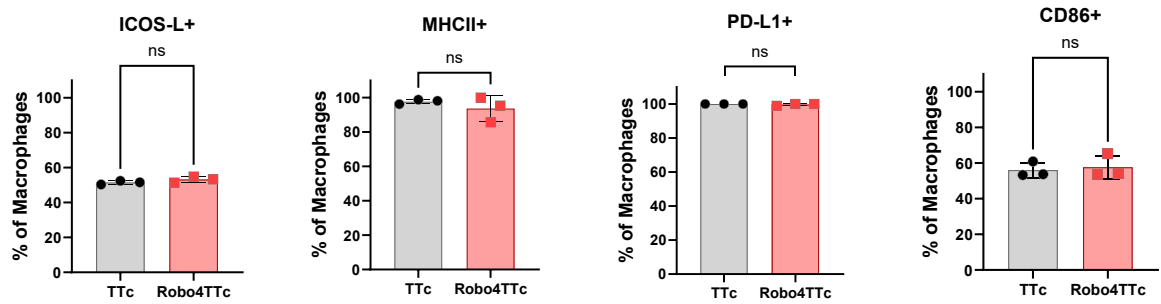
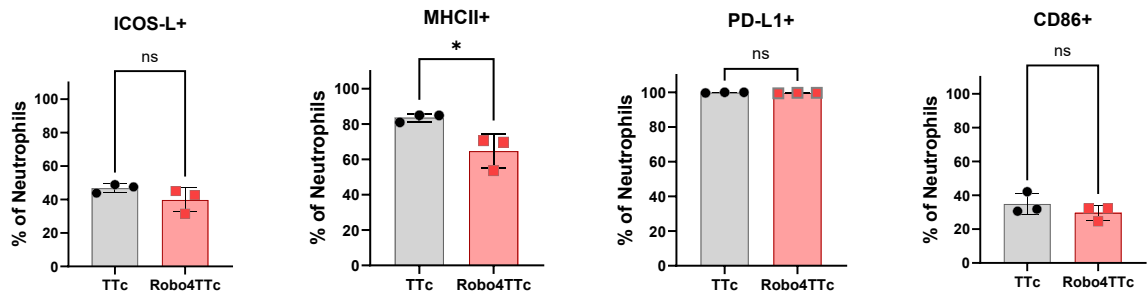
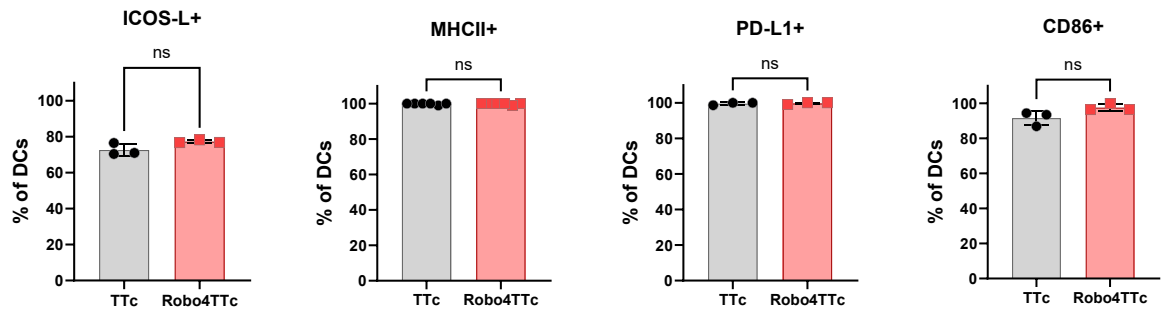


### Neutrophils



### Dendritic cells



**B****Draining Lymph Node – Immune cell populations****B cells****Macrophages****Neutrophils****Dendritic cells**

**Figure 4.26 Immune cell population analysis by flow cytometry.** Spectral flow cytometry was used to analyse immune cell populations in (A) 4T1 tumours and (B) draining lymph nodes from TTc- and ROBO4TTc-vaccinated mice. The gating strategy involved initial exclusion of acquisition artefacts, followed by doublet discrimination and gating on live CD45<sup>+</sup> immune cells. B and B1 cells were identified as CD19<sup>+</sup>B220<sup>+</sup> and CD19<sup>+</sup>B220<sup>-</sup>, respectively. CD19<sup>-</sup> cells were then differentiated based on CD11b and CD11c expression. CD11b<sup>high</sup> cells were further characterised as MHC-II<sup>+</sup>CD11c<sup>+</sup> dendritic cells (DCs), Ly6C<sup>-</sup>F4/80<sup>+</sup> macrophages and Ly6C<sup>+</sup>F4/80<sup>low</sup> neutrophils. PD-L1, ICOS-L, MHCII, and CD86 expression was assessed on B cells, macrophages, neutrophils and DCs. Statistical comparisons between the two groups were conducted using an unpaired non-parametric t-test (two-tailed), with results shown as mean  $\pm$  SD. Significance levels are indicated as ns (not significant),  $p \geq 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ .

#### 4.3.6.3 UMAP analysis

Figure 4.27 presents a UMAP analysis of the 30-colour dataset, highlighting CD45<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, and neutrophil populations. Events were evenly distributed between ROBO4TTc and TTc tumours, and classical gating strategies (Figure 4.19) were used for defining populations.

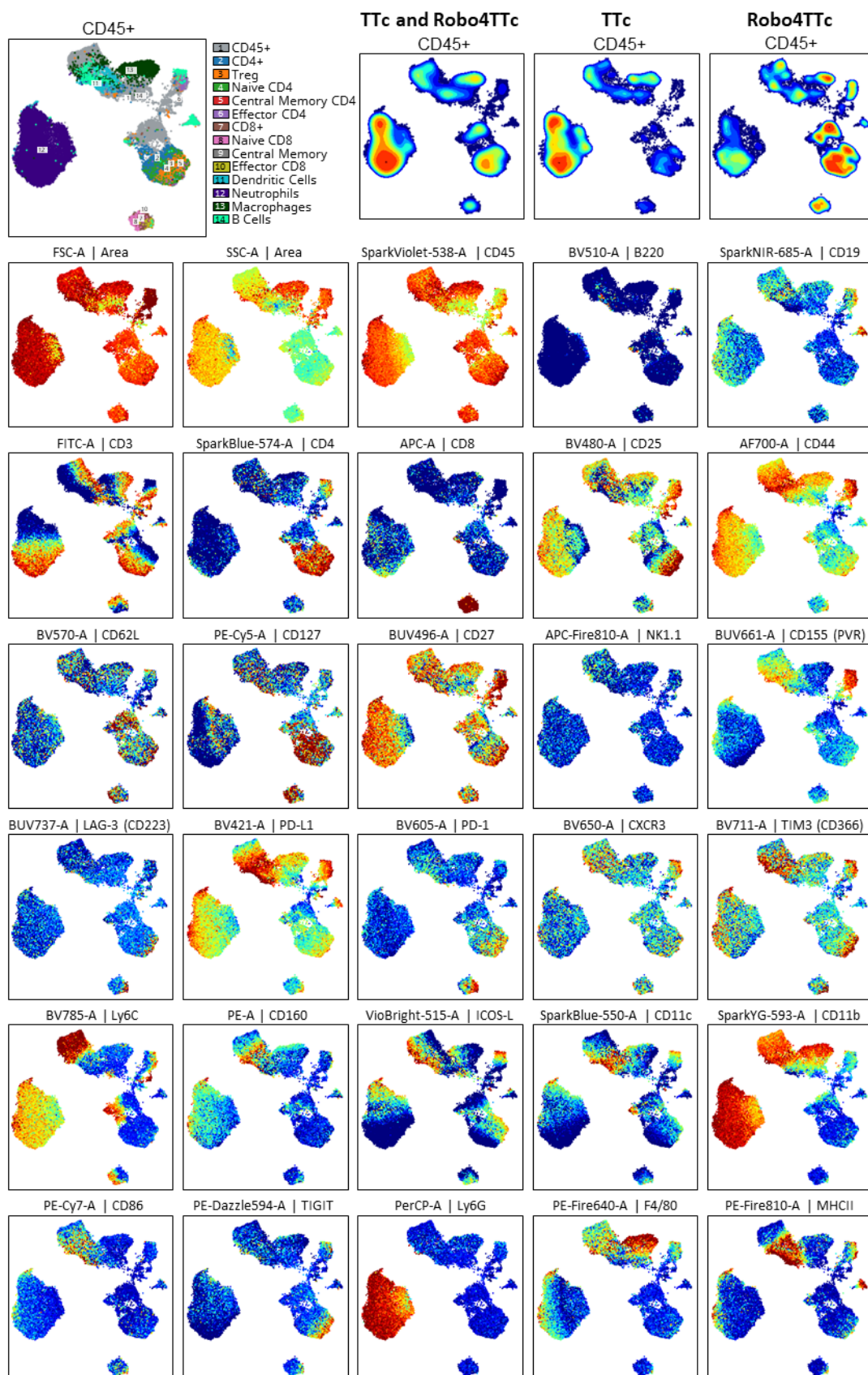
These findings underscore the immunomodulatory effects of the ROBO4TTc vaccine, demonstrating enhanced T cell and dendritic cell responses while reducing myeloid cell infiltration within the tumour microenvironment. Notably, the increased proportion of central memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells, coupled with reduced PD-1 expression (Figure 4.27B and C) suggest a more robust and sustained anti-tumour immune response. Central memory T cells (TCMs) are characterized by their self-renewing capacity and their ability to rapidly proliferate and differentiate into effector cells upon re-encounter with their cognate antigen, providing long-term immune surveillance and swift recall responses against persistent or recurrent tumours (Takahashi et al., 2022). The concurrent reduction in PD-1 expression, a key inhibitory receptor associated with T cell exhaustion, indicates a less exhausted and more functional T cell phenotype. This shift from an exhausted to a more effector-like or memory-driven state enables T cells to maintain their cytotoxic activity and cytokine production, thereby sustaining an effective anti-tumour response over time.

The elevated dendritic cell population may also enhance antigen presentation, further supporting adaptive immunity. Conversely, the significant reduction in CD11b<sup>high</sup> myeloid cells indicates a shift from an immunosuppressive environment. These cells, often comprising myeloid-derived suppressor cells (MDSCs) and certain macrophage subsets, are known to actively suppress anti-tumour immune responses through

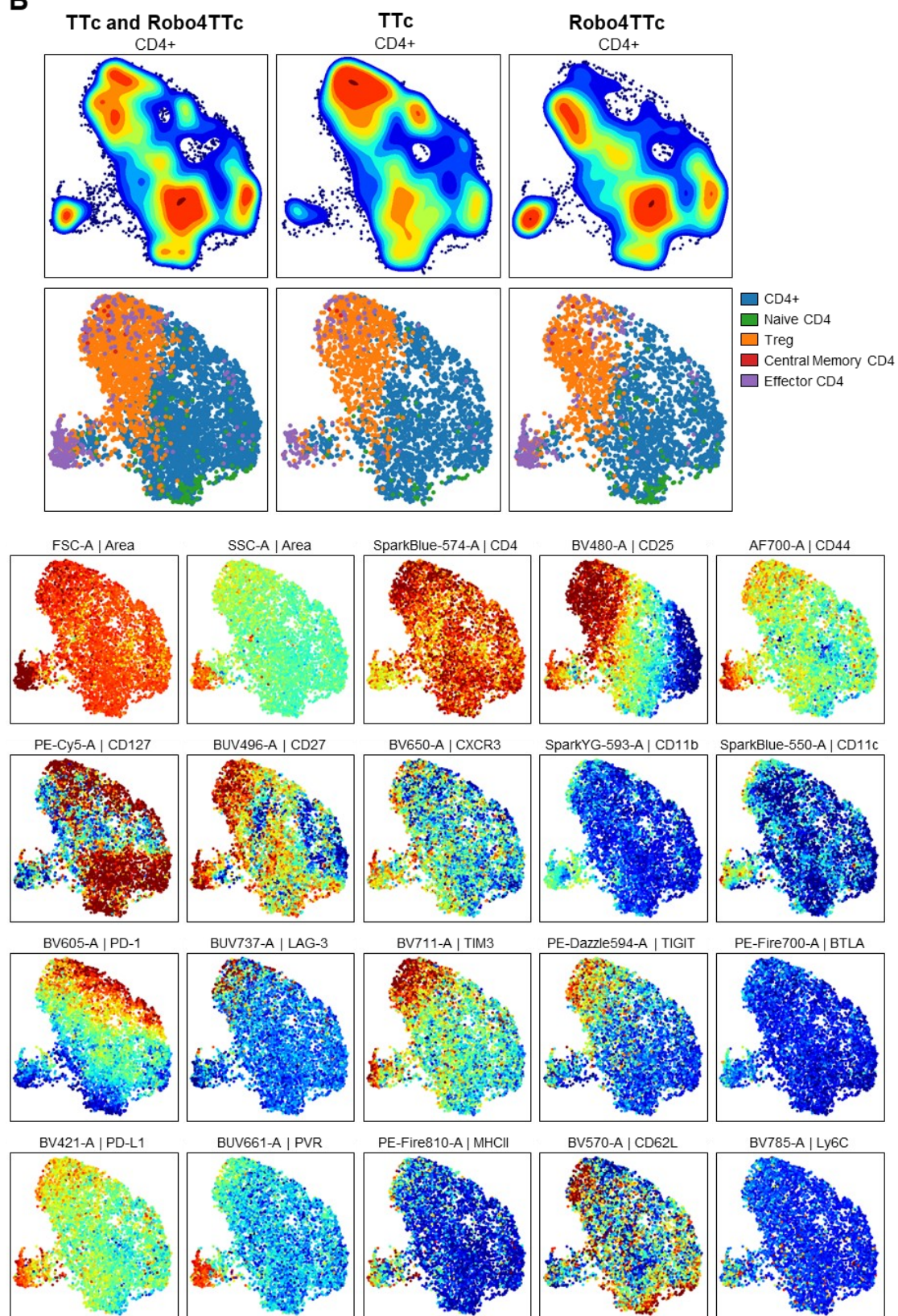
various mechanisms, including inhibiting T cell proliferation and function, promoting angiogenesis, and fostering an immune-tolerant milieu (Marvel & Gabrilovich, 2015). Their decrease suggests a substantial reduction in this suppressive activity, thereby creating a more permissive and favourable microenvironment for the induction and efficacy of anti-tumour immunity.

UMAP analysis provided a comprehensive overview of immune cell distributions, reinforcing these findings through unbiased clustering. Collectively, these results highlight the potential of the ROBO4TTc vaccine to reprogram the tumour immune landscape, promoting durable anti-tumour immunity while mitigating immune exhaustion.

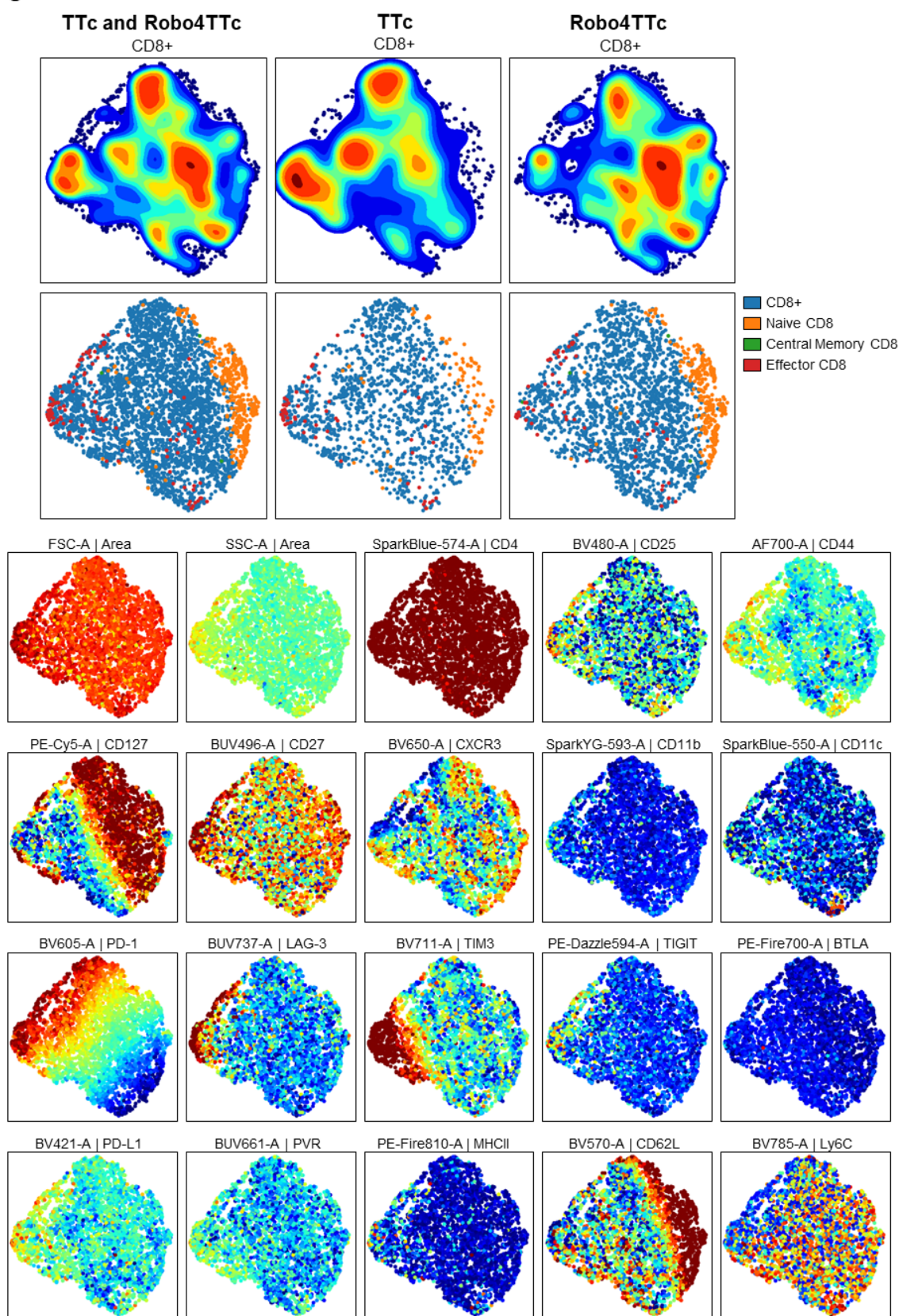
**A**

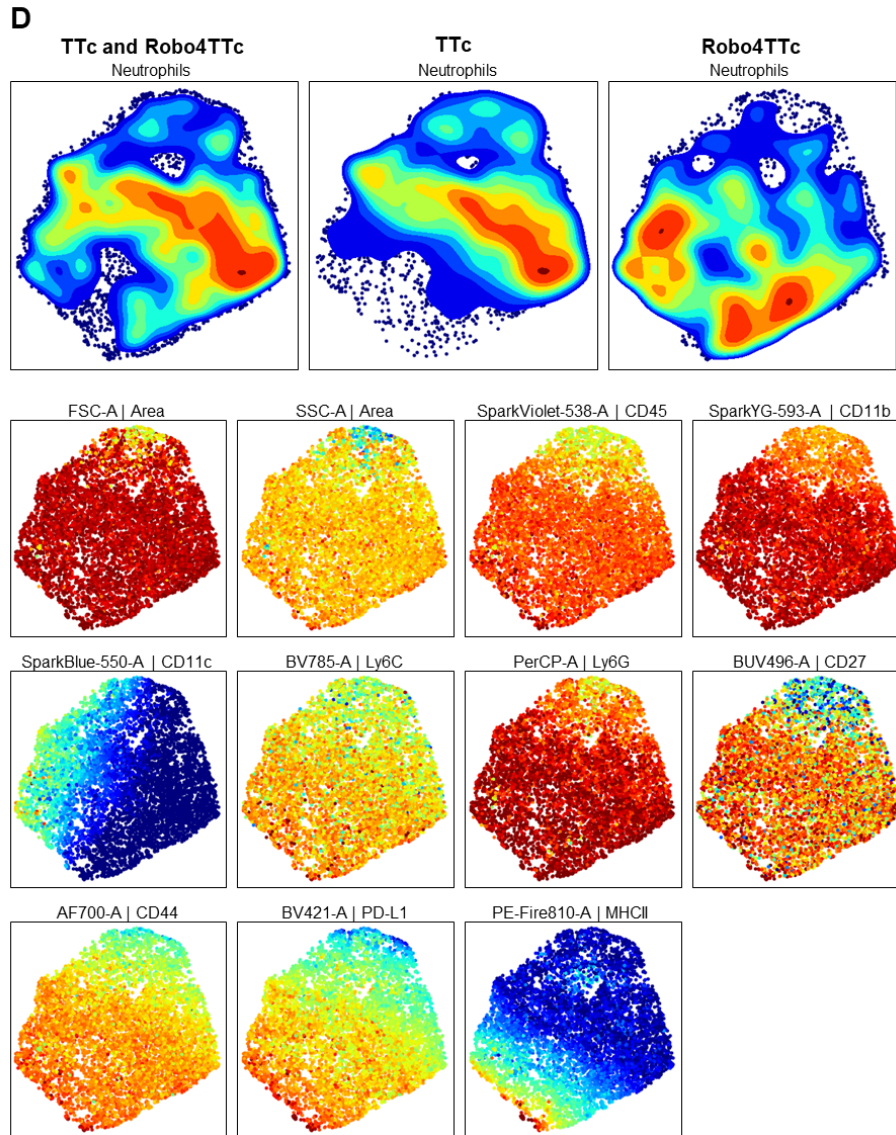


**B**



C





**Figure 4.27 UMAP analysis of a 30-colour dataset in (A) CD45<sup>+</sup>, (B) CD4<sup>+</sup>, (C) CD8<sup>+</sup> T cells, and (D) neutrophils, showing the distribution of events in 4T1 tumours from ROBO4TTC and TTC groups. Lower panels display combined samples from both groups, with density distributions representing an equal number of events per tissue. Cell populations were defined using the classical gating strategy shown in Figure 4.19 and visualised on the UMAP. Relative expression of selected markers is overlaid on the respective plots. UMAP parameters (OMIQ): Distance metric – Euclidean; Nearest Neighbours – 15; Minimum Distance – 0.5; Number of Components – 2.**

#### 4.4 Discussion

This study aimed to target and inhibit ROBO4 in mouse tumour models to investigate its role in tumour angiogenesis and progression. To achieve this, I first validated ROBO4 expression in suitable in vivo tumour models. I selected a widely used triple-negative breast cancer (TNBC) mouse model, 4T1, for its well-characterized tumour microenvironments and relevance to human disease.

Mura et al. (2012) previously demonstrated high ROBO4 expression in human tumours, including breast cancer, where ROBO4 co-localized with endothelial markers such as UEAI (*Ulex europaeus agglutinin*). Other studies have demonstrated ROBO4's essential role in endothelial cell stabilisation and tumour progression (Dai et al., 2019; Huminiecki et al., 2002). To confirm similar expression patterns in our mouse models, I performed immunofluorescence staining for ROBO4. Our data revealed that ROBO4 is notably expressed in the 4T1 tumour. Specifically, ROBO4 (red) was localised near endothelial cells marked by MECA-32 (green), with frequent co-localization (yellow). This consistent expression pattern underscores the relevance of ROBO4 as a therapeutic target in angiogenesis and tumour progression. Our results align with human tumour data, confirming the validity of these mouse models for ROBO4-targeting studies.

Following this, ROBO4 is a self-antigen, which limits its ability to elicit an immune response. To overcome this, we used a conjugate vaccination strategy. Conjugate vaccines have been shown to significantly improve immune responses against low immunogenic antigens by facilitating stronger T-cell activation (Astronomo & Burton, 2010; Costantino et al., 2011). To develop this conjugated vaccine for clinical use, TTc

(tetanus toxin non-toxic fragment C) is selected as a carrier protein. TTc is a well-characterized carrier used in vaccine development to enhance immune responses against weakly immunogenic antigens (Bayart et al., 2022). By conjugating ROBO4 to TTc, we aimed to break immune tolerance and induce a ROBO4-specific antibody response.

In our lab, we produced ROBO4TTc and TTc proteins using stably transfected HEK293 cells, generously provided by Dr Fernanda Nicole Escobar-Riquelme. The proteins were purified, and their quality was assessed through SDS-PAGE and western blot analyses. Purified TTc protein showed no signs of contamination or degradation, as indicated by a single distinct band at ~80 kDa on SDS-PAGE (Figure 4.3). Western blot analysis confirmed its identity with anti-TTc (green) and anti-human Fc (red) antibodies.

For ROBO4TTc, SDS-PAGE revealed three bands at ~100, 130, and 150 kDa. Western blot analysis showed that the higher molecular weight bands (130 and 150 kDa) contained both ROBO4 and TTc components (yellow signal), confirming the presence of intact conjugates. The smaller band (~100 kDa) reacted only with anti-human Fc antibody (red), suggesting partial denaturation or degradation of ROBO4TTc. Further analysis identified multiple protease cleavage sites in the extracellular region of ROBO4 used for the conjugate (see Appendix D). To minimise potential side effects in vivo, protease inhibitors were not used during protein production, which may have contributed to the observed degradation. Additionally, we used the entire extracellular domain of ROBO4 to ensure coverage of potential immunogenic epitopes and to induce a polyclonal antibody response. Despite partial degradation, most of the ROBO4TTc protein retained the essential components, making it suitable for immunisation.

Next, I tested the ability of ROBO4TTc to induce ROBO4-specific antibody responses in vivo. Using a previously optimised and established carrier-priming immunisation strategy (Escobar-Riquelme, 2024), ROBO4-specific antibody response was detected in mice. As expected, ROBO4-specific antibodies were detectable 14 days after the first immunisation with ROBO4-TTc, aligning with the expected priming and boosting effects. A second boost significantly increased antibody titres, demonstrating that the ROBO4TTc vaccine successfully overcame immune tolerance and induced robust antibody production.

Moreover, the effect of ROBO4-specific antibody response on 4T1 tumour growth was investigated. Tumour growth was monitored after the transplantation of tumour cells.

Tumours were detectable approximately 10 days post-transplantation of 4T1 cells (n = 16 mice). The ROBO4TTc group exhibited significantly slower tumour growth than the control group, with a notable difference in growth rates after day 21 (Figure 4.4). At the study endpoint (day 28), tumours in the control group were significantly more significant than those in the ROBO4TTc group. These results were consistently replicated in a second experiment with the same model (n=16 mice).

The qRT-PCR analysis revealed that ROBO4TTc vaccination upregulated *Ifng* expression while downregulating *Klf8*, suggesting an immunostimulatory effect that may contribute to tumour suppression. *Ifng* is a key cytokine (derived from CD4 T cells) involved in antitumour immunity, promoting macrophage activation, antigen presentation, and cytotoxic CD8 T-cell responses (Ikeda et al., 2002). The observed increase in *Ifng* expression aligns with the enhanced presence of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, indicating a robust adaptive immune response. The downregulation of *Klf8*, a

transcription factor linked to epithelial-to-mesenchymal transition (EMT) and tumour invasiveness, suggests that the vaccine may reduce TNBC metastatic potential (Lu et al., 2012). Considering *Ifng*'s elevation, future research should examine if the shift towards a Th1-type response correlates with higher levels of antigen-specific IgG2a antibodies, commonly linked with IFN- $\gamma$ -driven class switching (Jorgovanovic et al., 2020). This analysis could enhance understanding of the complete spectrum of immune activation initiated by ROBO4TTc and clarify the interplay between cellular and humoral responses that aid tumour control.

Spectral flow cytometry analysis further supports the immunogenic effects of ROBO4TTc vaccination. The increased infiltrations of CD4<sup>+</sup> and CD8<sup>+</sup> T cells may be the cause of the elevated *Ifng* levels to enhance tumour immunity (Ostroumov et al., 2018). Additionally, dendritic cells (DCs) enrichment indicates improved antigen presentation, which is critical for sustained T cell activation (MacNabb et al., 2022). The reduction in CD11b<sup>high</sup> myeloid cells, which are often associated with immunosuppressive functions, suggests a shift towards a more immune-permissive tumour microenvironment (Marvel & Gabrilovich, 2015).

A particularly noteworthy finding is the phenotypic shift in neutrophils following the ROBO4TTc vaccination. While total neutrophil numbers declined, those that remained exhibited increased MHC-II expression, indicating a transition towards an antigen-presenting phenotype (Gungabeesoon et al., 2023; Vono et al., 2017). This suggests that the vaccine probably reprograms tumour-associated neutrophils, promoting immune activation rather than immune evasion. The increased frequency of central memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells further supports the hypothesis that ROBO4TTc

vaccination contributes to long-term immune surveillance and sustained tumour control (Gray et al., 2018).

Multiplex imaging provided additional insights into the structural changes within the tumour microenvironment. The observed redistribution of CD4<sup>+</sup> and CD8<sup>+</sup> T cells towards tumour vasculature suggests improved vascular integrity and immune accessibility, crucial for effective immune infiltration in TNBC (Peske et al., 2015). The reduction in pericyte coverage, particularly NG2<sup>+</sup> pericytes, indicates a shift towards vascular normalisation, which probably decreases hypoxia-driven immunosuppression and enhances immune cell trafficking (Ferland-McCollough et al., 2017; Jiang et al., 2023). The group of B cells, T cells, and DCs near tumour vasculature suggests that ROBO4TTc vaccination fosters an environment conducive to long-term immune activation, possibly enhancing durable tumour control.

The significant reduction in tumour vascularisation further supports the hypothesis that ROBO4TTc vaccination disrupts tumour vessel development. ROBO4 has roles in endothelial cell migration and vascular stability; its blockade possibly impairs TNBC-associated angiogenesis, limiting tumour progression (Dai et al., 2019; Potente et al., 2011).

These findings indicate that ROBO4TTc vaccination exerts multifaceted antitumour effects by enhancing adaptive immunity, reprogramming neutrophil populations and possibly normalising tumour vasculature. Additionally, it promotes the infiltration of T and B cells. By reshaping the tumour microenvironment, this vaccine holds significant promise for improving immunotherapeutic strategies for TNBC.

## 4.5 Conclusions

Triple-negative breast cancer (TNBC) remains a highly aggressive malignancy with limited treatment options, necessitating the development of novel therapeutic strategies. In this study, we explored the efficacy of ROBO4TTc vaccination in a preclinical 4T1 TNBC model, which demonstrated its potential to enhance antitumor immunity, modulate cytokine expression, and alter tumour vasculature.

We confirmed that ROBO4 is highly expressed in the 4T1 mouse tumour model, establishing the foundation for targeting studies using ROBO4. We successfully produced and purified ROBO4TTc and TTc proteins for immunisation. The vaccination with ROBO4TTc effectively broke self-tolerance and induced a robust and specific antibody response against ROBO4. Importantly, this immune response significantly inhibited tumour growth in the BALB/c (4T1) mouse model.

ROBO4TTc vaccination increased expression of *Ifng* and downregulation of *Klf8*, which promotes immune activation and may reduce tumour progression. Spectral cytometry analysis revealed an enhanced infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, increased central memory subsets, and reduced PD-1 expression, indicating improved immune functionality. Additionally, the vaccine increased the presence of dendritic cells while reducing immunosuppressive CD11b<sup>+</sup> myeloid cells, shifting the tumour microenvironment towards a more immune-stimulatory state.

Multiplex imaging confirmed significant structural changes indicative of vascular normalisation, including reduced vessel area and altered pericyte coverage. CD4<sup>+</sup> and CD8<sup>+</sup> T cells were found closer to tumour-associated vasculature, enhancing immune accessibility.

Overall, the ROBO4TTc vaccination presents a promising immunotherapeutic strategy, effectively combining immune activation with vascular modulation to improve treatment outcomes in TNBC. Future research should investigate its potential synergy with checkpoint inhibitors and other immunotherapies to optimise therapeutic efficacy and enhance clinical outcomes for TNBC patients. Notably, a reduction in PD-1 expression following vaccination could boost immune responses and potentially improve the effectiveness of immune checkpoint blockade therapy, either by directly decreasing PD-1 expression on T cells or by priming the immune system to better respond to checkpoint inhibition.

## Chapter 5: Conclusion

Vaccination is the primary treatment for many diseases, designed to induce a specific immune response and provide long-term protection against infections (Pollard & Bijker, 2021). By stimulating the immune system to recognize and respond to harmful pathogens, vaccines have significantly reduced the global burden of infectious diseases (Pollard & Bijker, 2021). However, there are limitations in generating a strong immune response against weak antigens, such as polysaccharides, which are poorly immunogenic on their own (Khan, 2006). To address this challenge, conjugate vaccination is a widely used approach, where weak antigens are covalently linked to immunogenic carrier proteins that can recruit T cells to help (Rappuoli, 2018; Rappuoli et al., 2019). This strategy leads to a more robust and long-lasting immune response (Rappuoli, 2018). Conjugate vaccines stimulate both T cell-dependent and B cell-mediated immunity, resulting in affinity maturation, plasma cell differentiation, and the formation of memory B cells (Rappuoli, 2018).

In cancer immunotherapy, conjugate vaccines have gained interest in targeting tumour-associated antigens, many of which are self-antigens (Chotprakaikiat et al., 2016; Zhuang et al., 2015). Due to immune tolerance, self-antigen-specific B cells struggle to receive adequate T cell help, leading to restricted autoantibody secretion and weak immune activation (Bonasia et al., 2021). Two such self-antigens are Roundabout 4 (ROBO4) and C-type lectin domain containing 14A (CLEC14A), endothelial markers selectively expressed in tumour vasculature (Mura et al., 2012; Park et al., 2003; Weitzman et al., 2008). Both markers play critical roles in pathological angiogenesis and are found in the blood vessels of various tumour types, making them

an attractive candidate for therapeutic targeting (Dai et al., 2019; Robinson et al., 2020). Research has shown that targeting CLEC14A and ROBO4 can disrupt tumour vascularisation, reducing vessel density, blood supply, and oxygen availability, inhibiting tumour growth (Hirobe et al., 2022; Zhuang et al., 2020). Conjugate vaccination offers a potential strategy to break immune tolerance by providing the necessary T cell help to self-antigen-specific B cells, thereby enhancing anti-tumour immunity and selectively targeting the tumour vasculature (Escobar-Riquelme, 2024; Zhuang et al., 2015).

Zhuang et al. (2015) demonstrated that immunisation with the extracellular domain of mouse ROBO4 fused to the Fc domain of human immunoglobulin can suppress angiogenesis and inhibit tumour growth in LLC1 models. The vaccinated mice developed an antibody response against ROBO4 without significant health complications. Immunised mice exhibited impaired fibrovascular invasion and angiogenesis. Notably, the anti-tumour effects persisted in CD8-deficient mice. Still, they were absent in B cells or IgG1 knockout mice, indicating that antibody-dependent cell-mediated cytotoxicity (ADCC) was the primary mechanism of action. These findings align with our research investigating ROBO4 as a target for tumour angiogenesis inhibition.

In this study, we validated the expression of CLEC14A in tumour endothelial cells across various mouse models, including 4T1, E0771, and LLC1. Immunofluorescence staining and qRT-PCR analyses confirmed that CLEC14A is highly expressed within tumour vasculature while being absent in normal tissues, reinforcing its potential as a selective therapeutic target (Figure 3.1 and Figure 3.2). These findings are consistent

with prior research, which identified CLEC14A as a tumour endothelial marker with limited involvement in physiological angiogenesis (Mura et al., 2012; Noy et al., 2016).

CLEC14A is a self-antigen and has a low immunogenicity (Mura et al., 2012). Thus, we designed a conjugate vaccine using TTc as a carrier protein to enhance immune responses. By genetically fusing the extracellular domain of CLEC14A (a tumour endothelial marker) to the non-toxic fragment C of tetanus toxin (TTc), we aimed to enhance immune recognition and antibody-mediated tumour suppression. The successful cloning and expression of CLEC14A-TTc in HEK293 cells facilitated its purification and validation through SDS-PAGE and Western blotting, confirming its structural integrity (Figure 3.18). Prior research has demonstrated that TTc can significantly amplify immune responses against weak antigens by stimulating robust CD4<sup>+</sup> T cell activation (Chotprakaikiat et al., 2016; Xu et al., 2021). Our strategy leveraged this capability to enhance CLEC14A-specific immunogenicity, to direct immune responses against CLEC14A-expressing tumour vasculature. Stable cell line generation provided a reliable system for large-scale protein production. Protein purification via affinity chromatography using Protein A columns yielded sufficient CLEC14A-TTc protein for experimental applications. However, minor degradation bands suggested proteolytic cleavage of the CLEC14A domain during expression. This aligns with previous observations that CLEC14A proteins can undergo partial proteolytic processing (Khan, 2016). Future studies should explore strategies to mitigate this degradation, potentially by modifying culture conditions, employing protease inhibitors, or implementing protein engineering solutions.

Despite the ability of the CLEC14A-TTc vaccine to induce IgG1 responses against CLEC14A in mice, antibody titres were lower than expected (Figure 3.19C). Adding

CpG oligodeoxynucleotides (ODNs) as an adjuvant could enhance this response. CpG ODNs mimic bacterial DNA and activate Toll-like receptor 9 (TLR9), boosting innate and adaptive immunity (Krieg, 2007). Specifically, CpG ODN types B (ODN1826) and C (ODN2395) have demonstrated efficacy in enhancing immune responses against NP (4-hydroxy-3-nitrophenyl acetyl) (Escobar-Riquelme, 2024). Additionally, in my experiments, combining CpG B with the ROBO4TTc vaccine in alum significantly increased ROBO4-specific IgG1 titres compared to alum alone (see Appendix C), suggesting that CpG ODNs could similarly enhance the immune response against CLEC14A.

The successful generation and purification of CLEC14A-TTc mark a crucial step toward in vivo immunisation studies to evaluate its therapeutic efficacy. Leveraging TTc's strong immunogenic properties could effectively counteract CLEC14A's weak immunogenicity and elicit a potent anti-tumour response.

In parallel, this study also investigated ROBO4 as a target for tumour angiogenesis inhibition. Due to its well-characterised tumour microenvironment and relevance to human disease, ROBO4 expression was validated in tumour models, particularly the 4T1 triple-negative breast cancer (TNBC) model. Previous studies have reported high ROBO4 expression in human tumours, where it co-localizes with endothelial markers such as UEAI (Mura et al., 2012). Additionally, ROBO4 has been implicated in endothelial cell stability and tumour progression (Dai et al., 2019; Huminiecki et al., 2002). Our immunofluorescence data confirmed ROBO4's expression in the 4T1 tumour microenvironment, co-localising with the endothelial marker MECA-32. This supports ROBO4's potential as a therapeutic target in tumour angiogenesis.

Given that ROBO4 is a self-antigen, immune tolerance presents a challenge. To address this, our lab developed a conjugate vaccine strategy (Escobar-Riquelme, 2024). The conjugate vaccines have enhanced immune responses against weakly immunogenic antigens by promoting stronger T-cell activation (Astronomo & Burton, 2010; Costantino et al., 2011). TTc was selected as the carrier protein due to its well-established role in improving immunogenicity (Bayart et al., 2022). By conjugating ROBO4 to TTc, we aimed to induce a ROBO4-specific antibody response and break immune tolerance. ROBO4TTc and TTc proteins were produced using stably transfected HEK293 cells, generously provided by Dr. Fernanda Nicole Escobar-Riquelme. SDS-PAGE and Western blot analyses confirmed these proteins' successful purification and integrity.

The ROBO4TTc vaccination successfully elicited a ROBO4-specific antibody response in BALB/c mice. A strong priming response was detectable 14 days post-immunisation, with a significant increase in antibody titres following a booster dose. Furthermore, tumour growth assessments indicated that ROBO4TTc vaccination resulted in significantly slower tumour progression in 4T1-bearing mice compared to controls (TTc-vaccinated mice), with noteworthy differences emerging after day 21 (Figure 4.4). These findings were validated independently in a second experiment.

qRT-PCR analysis showed that the ROBO4TTc vaccination upregulated *Ifng* expression while downregulating *Klf8* in 4T1 tumours, suggesting an immunostimulatory effect contributing to tumour suppression. *Ifng* is a key cytokine involved in anti-tumour immunity, facilitating macrophage activation, antigen presentation, and cytotoxic CD8 T-cell responses (Ikeda et al., 2002). The reduction in *Klf8*, a transcription factor associated with epithelial-to-mesenchymal transition (EMT)

and tumour invasiveness, suggests a potential decrease in TNBC metastatic capacity (Lu et al., 2012).

Spectral flow cytometry further confirmed that ROBO4TTc vaccination enhanced immune cell infiltration into tumours, remarkably increasing CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations. Additionally, dendritic cell enrichment suggested improved antigen presentation, critical for sustained T-cell activation (MacNabb et al., 2022). Notably, vaccinated mice exhibited a phenotypic shift in neutrophils, with increased MHC-II expression suggesting a transition to an antigen-presenting phenotype (Gungabeesoon et al., 2023; Vono et al., 2017). This indicates that ROBO4TTc vaccination may reprogram tumour-associated neutrophils to promote immune activation rather than immune evasion.

Additionally, the ROBO4TTc vaccine was tested in the oestrogen receptor-negative (ER-) breast cancer model (E0771) on C57BL/6 mice (see Appendix E). Similar to BALB/c mice, the vaccination successfully broke the self-tolerance against ROBO4 in C57BL/6 mice, inducing a robust anti-ROBO4 IgG1 antibody response in C57BL/6, consistent with findings from Escobar-Riquelme (2024). This led to significant inhibition of angiogenesis in E0771 tumours and limited tumour growth.

Similar to findings in the 4T1 model, ROBO4TTc-vaccinated mice bearing E0771 tumours exhibited increased CD4<sup>+</sup> and CD8<sup>+</sup> T cell infiltration. Notably, these T cells expressed significantly lower levels of exhaustion markers such as PD-1, TIM-3, and LAG-3 compared to those in TTc-vaccinated mice, potentially enhancing responsiveness to immune checkpoint blockade (ICB) therapy (Chow et al., 2022). Furthermore, the ROBO4TTc group showed a marked increase in  $\gamma\delta$  T cells

(CD3<sup>+</sup>TCR $\gamma$  $\delta$ <sup>+</sup>) and NKT cells (CD3<sup>+</sup>NK1.1<sup>+</sup>), indicating enhanced activation of both innate and adaptive immunity. Spectral flow cytometry analysis also revealed an increased population of MHC-II<sup>+</sup> neutrophils in vaccinated mice, suggesting a phenotypic shift toward antigen presentation. This transition may contribute to the observed enhancement in T-cell activation. Interestingly, while E0771 tumours showed higher CD8<sup>+</sup> T cell infiltration, 4T1 tumours demonstrated more significant B cell accumulation and dendritic cell enrichment (see Appendix E). These results highlight the impact of genetic background on vaccine efficacy and suggest that ROBO4TTc vaccination elicits tumour model-specific immune responses.

The immune response mechanism induced by ROBO4TTc vaccination appears to be multifaceted. Like Zhuang et al. (2015), our data indicate that ADCC is a primary mode of tumour suppression, where vaccine-induced IgG1 antibodies facilitate the recruitment of natural killer (NK) cells and macrophages to eliminate ROBO4-expressing endothelial cells. Additionally, the increased CD4<sup>+</sup> and CD8<sup>+</sup> T cells suggest that ROBO4TTc vaccination may enhance antigen presentation, leading to a stronger adaptive immune response. Upregulation of *Ifng* and antibody production post-vaccination suggests an immunostimulatory effect that promotes macrophage activation and enhances antigen presentation. Moreover, the reduction in *Klf8* expression implies a potential suppression of epithelial-to-mesenchymal transition (EMT), which could reduce tumour invasiveness and metastatic potential.

Overall, this study highlights the potential of CLEC14A-TTc and ROBO4-TTc vaccines as novel immunotherapeutic strategies targeting tumour angiogenesis. By leveraging TTc as a carrier protein, we successfully elicited antigen-specific antibody responses against CLEC14A and ROBO4, overcoming immune tolerance and demonstrating anti-

tumour efficacy in preclinical models. While CLEC14A-TTc vaccination induced an immune response, further optimisation is needed to enhance antibody titres, potentially by including CpG ODN adjuvants or alternative formulations. Similarly, ROBO4TTc vaccination effectively slowed tumour progression and promoted immune cell infiltration, suggesting a broad impact on the tumour microenvironment. Future directions should focus on optimising vaccine formulations, exploring combination therapies with immune checkpoint inhibitors, and conducting mechanistic studies to further elucidate the immune pathways involved. Extending these findings to humanised tumour models and patient-derived xenografts will also be essential for clinical translation. By refining these vaccines, we aim to develop a robust and targeted approach to disrupt tumour vascularisation and enhance anti-tumour immunity in solid malignancies.

## Chapter 6: References

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## Appendix

### Appendix A

#### Mouse CLEC14A with Fc-tag (CLEC14A-Fc)

##### DNA sequence

GCTAGCAAGCTTGCCACCATGGCCGGCCCACTGGCCCTGTGTCTGCTGTGTCCCTGCCTTCTGGCCCAGACCTGGCA  
ACGGCGAGCACCCACAGCTGATAGAGCCGCCTGTAGCGCCAGCGGCGCCTGCTACAGCCTGCACCACGCCACATTCA  
AGCGGAGAGCCGCAGAAGAAGCCTGCAGCCTGCGAGGCGGCACTCTGAGCACCGTGCATAGCGGAAGCGAATTCCAG  
GCTGTGCTGCTGCTCCTGCGGGCCGGCCCTGGCCCCGGCGGCGGATCTAAGGACCTGCTGTTTTGGGTGGCCCTGGAAA  
GAAGCATCAGCCAGTGCACACAGGAGAAGGAACCGCTGCGGGGCTTTAGCTGGCTGCACCCCGACAGCGAGGACTCTG  
AAGATAGCCCCCTGCCCTGGGTCGAGGAGCCCCAAGAAGCTGTACCGTGCAGGAAGTGCGCCGCCCTGCAAGCCACCA  
GAGGCGTGAACCTGCCGGCTGGAAGGAGATGCGGTGTACCTGAGAACCGATGGTTATCTGTGCAAGTACCAGTTCGA  
GGTGTGTGCCCTGCCCCCTAGACCTGGAGCCGCTTCTAATCTGTCTTTTCAGGCCCTTTCCGGCTGAGCAGCAGCGCCC  
TGGATTTACAGCCCCCAGGCACAGAGGTGTCTGCTATGTGCCCGGGGACCTGAGCGTGAGCTCCACCTGTATCCAAGA  
GGAAACATCTGCTCACTGGGACGGCCTTTTTCTGGCACCGTCTGTGCCCTGTTCGGGAAGATACCTGCTGGCCGGAA  
AGTGCCTGGAGCTGCCTGACTGCCTGGACCACCTGGGCGACTTCACCTGCGAGTGCGCCGTGGGCTTCGAGCTGGGCA  
AGGACGGCAGAAGCTGCGAGACAAAAGTGGAGGAACAGCTGACGCTGGAAGGCACCAAGCTGCCCACAAGAAACGTG  
ACCGCCACCCCTGCCGGAGCTGTGACGAACAGAACCTGGCCTGGCCAGGTGTACGACAAGCCTGGCGAAATGCCCAA  
GTGACCGAGATCCTGCAGTGGGGCACCCAGTCCACACTGCCAACAATCCAGAAGACCCCCAGACGAAGCCTAAGGTG  
ACGGGCACCCCAAGCGGCAGCGTGGTGTGAATTACACCAAGTAGTCTCTGTGTCCCTGACCTTCGACACAAGCTCCA  
CCGGAGGCAGCGGCGGCGGCTCTGGCGGAGAACCTAAGTCTGCGACAAGACACACACCTGTCTCCATGCCCTGCTC  
CTGAGCTCCTTGGCGGCCCTAGCGTTTTCTGTTCGCCCCCAAGCCCAAGGATACCTGATGATCTCTAGGACCCAGAG  
GTGACATGCGTGGTCTGTGGACGTGAGCCACGAGGATCCTGAGGTGAAATTCAACTGGTACGTGGACGGCGTGGAAAGT  
CACAACGCCAAGACCAACCCAGAGAAGAGCAGTACAATAGCACCTACAGAGTGGTGAAGCTGCTGACAGTGTGCATC  
AGGACTGGCTGAACGGCAAAGAGTACAAATGCAAGGTGAGCAACAAGGCCCTTCCTGCCCTATCGAGAAAACCATCAG  
CAAAGCCAAGGGCCAGCCTCGGGAACCTCAGGTGTACACCTGCCTCCTCTCGCGACGAGCTGACCAAGAACCAGGT  
GAGCCTGACATGTCTGGTGAAGGGATTCTATCCCTCTGATATTGCCGTGGAGTGGGAGAGCAACGGACAGCCTGAAAAC  
AACTACAAGACCACCCCTCCAGTGTCTGGATAGCGACGGTTCCTTCTCTCTGTAAGCTGACAGTTGATAAGAGCAG  
ATGGCAGCAGGGCAATGTGTTCTCTTGCAGCGTTATGCACAGAGGCCCTACACAACCACTACACCCAGAAAAGCCTGTCCC  
TGAGCCCTGGCAAGTAATAGCTCGAGTCTAGAGGGCC

Legend: NheI + Start codon + CLEC14A + Linker 1 + Fc + Stop codon + ApaI

##### Protein sequence

MGRPALALCLLCPAFWPRPGNGEHPTADRAACSASGACYSLHHATFKRRAAEEACSLRGTLSTVHSGSEFQAVLLLLRAGP  
GPGGGSKDLLFWVALERSISQCTQEKEPLRGFSWLHPDSEDSPLPWVEEPQRSCTVRKCAALQATRGVEPAGWKEMRC  
HLRTDGYLCKYQFEVLCAPRPGAASNLFSQAPFRLSSSALDFSPPGTEVSAMCPGDLVSSTCIQEETSAHWDGLFPGTVLC  
PCSGRYLLAGKCVLPDCLDHLGDFTECAVGFELGKDGRSCETKVEEQTLLEGTKLPTRNVTATPAGAVTNRTWPGQVYDK  
PGEMPQVTEILQWGTQSTLPTIQKTPQTKPKVTGTPSGSVVLNYTSSPPVSLTFDTSSTGGSGGGSGGEPKSCDKHTCPPCP  
APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW  
LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPV  
LDSGGSFFLYSKLTVDKSRWQQGNVFSQSVMEALHNHYTQKSLSLSPGK\*

Legend: CLEC14A + Linker 1 + Fc

## Mouse CLEC14A plus Fragment C of Tetanus Toxin (TTc) (CLEC14A-TTc) with Fc-tag

### DNA sequence

GCTAGCAAGCTTGCCACCATGGGATGGTCTTGATTATCTTCTTTCTGGTCGCCACTGCCACTGGGGTCTACTCAGAGCAC  
CCCACAGCTGATAGAGCCGCTGTAGCGCCAGCGGCGCCTGCTACAGCCTGCACCACGCCACATTCAAGCGGAGAGCC  
GCAGAAGAAGCCTGCAGCCTGCGAGGCGGCACCTCTGAGCACCTGCATAGCGGAAGCGAATTCAGGCTGTGCTGCTG  
CTCCTGCGGGCCGGCCCTGGCCCCGGCGGCGGATCTAAGGACCTGCTGTTTTGGGTGGCCCTGGAAAGAAGCATCAGC  
CAGTGCACACAGGAGAAGGAACCGCTGCGGGGCTTACCTGGCTGCACCCCGACAGCGAGGACTCTGAAGATAGCCCC  
CTGCCTTGGGTGCGAGGAGCCCCAAAGAAGCTGTACCGTGCAGGAAGTGCGCCGCCCTGCAAGCCACCAGAGGCGTCGAA  
CCTGCCGGCTGGAAGGAGATGCGGTGTACCTGAGAACCGATGGTTATCTGTGCAAGTACCAGTTCGAGGTGCTGTGCC  
CTGCCCCCTAGACCTGGAGCCGCTTCTAATCTGTCTTTTCAGGCCCTTTCCGGCTGAGCAGCAGCGCCCTGGATTTCAGC  
CCCCCAGGCACAGAGGTGTCTGCTATGTGCCCCGGGGACCTGAGCGTGAGCTCCACCTGTATCCAAGAGGAAACATCTG  
CTCACTGGGACGGCCTTTTCTGTCACCGTCTGTGCCCTTGTTCGGGAAGATACCTGCTGGCCGGAAAGTGCCTGGA  
GCTGCCTGACTGCCTGGACCACCTGGGCGACTTCACCTGCGAGTGCGCCGTGGGCTTCGAGCTGGGCAAGGACGGCAG  
AAGCTGCGAGACAAAAGTGGAGGAACAGCTGACGCTGGAAGGCACCAAGCTGCCCAAGAAGACGTGACCGCCACCC  
CTGCCGGAGCTGTGACGAACAGAACCTGGCCTGGCCAGGTGTACGACAAGCCTGGCGAAATGCCCCAAGTGACCGAGA  
TCCTGCAGTGGGGCACCCAGTCCACACTGCCAACATCCAGAAGACCCCCCAGACGAAGCCTAAGGTGACGGGCACCC  
CAAGCGGCAGCGTGGTGTGAATTACACCAAGTAGTCTCTGTGTCCCTGACCTTCGACACAAGCTCCACCAGAGGAG  
CGGGCGGGCTCTGGCGGAATAAATCTGGACTGTTGGGTGATAACGAAGAGGACATCGATGTGATCCTGAAGAAGTCC  
ACAATCCTGAATCTGGACATCAACAATGATATCATCTCCGACATCTCTGGCTTCAACAGCTCCGTGATCACCTACCCTGAC  
GCCCAGCTGGTGCCAGGCATCAATGGCAAGGCCATCCACCTGGTGAACAATGAGTCTAGCGAAGTGATCGTGACAAGG  
CCATGGATATCGAGTATAACGACATGTTCAACAATTCACCGTGAGCTTCTGGCTGCGGGTGCCCAAGGTGTCTGCCAGC  
CACCTGGAGCAGTACGGCACCAATGAGTATTCATCATCTCTCTATGAAGAAGCACTCCCTGTCTATCGGCTCTGGCTGG  
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GACCTGCCTGATAAGTTAATGCCTACCTGGCCAACAAGTGGGTGTTTCATCACCATCACAAACGACCGGCTGAGCTCCGC  
CAATCTGTATATCAACGGCGTGCTGATGGGCTCTGCCGAGATCACAGGCCTGGGAGCCATCAGGGAGGATAACAATATCA  
CCCTGAAGCTGGACCGCTGCAACAATAACAATCAGTACGTGAGCATCGATAAGTTCAGAATCTTTGTAAGGCCCTGAATC  
CTAAGGAGATCGAGAAGCTGTACACCAGCTATCTGTCCATCACATTCTGAGGGATTTTGGGGCAACCCACTGCGCTAC  
GACACCGAGTACTATCTGATCCCCGTGGCCTCTAGCTCCAAGGATGTGCAGCTGAAGAATATCACCGACTACATGTATCTG  
ACAAACGCCCTTCTCTATACCAATGGCAAGCTGAACATCTACTATCGGAGACTGTACAACGGCCTGAAGTTTCATCATCAAG  
CGGTATACCCCAACAATGAGATCGATTCTTCTGTAAGTCTGGCGACTTTATCAAGCTGTACGTGAGCTATAACAATAAC  
GAGCACATCGTGGGCTACCCAAAGGATGGCAATGCCTTAATAACCTGGACAGAATCCTGAGGGTGGGATACAACGCAC  
CAGGCATCCCTCTGTATAAGAAGATGGAGGCCGTGAAGCTGAGGGACCTGAAGACATACTCCGTGCAAGCTGAAGCTGTAT  
GACGATAAGAATGCCTCTCTGGGCTGGTGGGCACCCACAACGGACAGATCGGCAATGATCCCAACCGCGACATCCTGA  
TCGCCAGCAATTGGTACTTCAACCACCTGAAGGATAAGATCCTGGGCTGCGACTGGTATTTTGTGCCAACCGATGAGGGC  
TGGACAAATGACGGAGGCAGCGGAGGAGGCTCCGGAGGAGATAAGACCCACACATGCCACCTTGTCCAGCACCAGAG  
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CATGCGTGGTGGTGGACGTGAGCCACGAGGACCCTGAGGTGAAGTTCAATTGGTACGTGGATGGCGTGGAGGTGCACA  
ACGCCAAGACCAAGCCCCGGGAGGAGCAGTACAACCTACATATAGAGTGGTGGAGCGTGCTGACCGTGCTGCACCAGG  
ACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTGTCTACAAGGCCCTGCCTGCCCAATCGAGAAGACAATCAGCAA  
GGCAAAGGGACAGCCACGGGAGCCACAGGTGTACACCTGCCTCCAGCCGGGACGAGCTGACAAAGAATCAGGTGA  
GCCTGACCTGTCTGGTGAAGGGCTTCTATCTAGCGACATCGAGTGGAGTGGGAGTCCAACGGACAGCCAGAGAATAA  
CTACAAGACCACACCCCTGTGCTGGACTCTGATGGCAGCTTCTTTCTGTATTCCAAGCTGACCGTGGACAAGTCTCGCT  
GGCAGCAGGGCAACGTGTTTTCTGTAGCGTGATGCATGAAGCACTGCACAACCATTACACCCAGAAAAGCCTGTCACTG  
TCCCCGGAAGTAATAGCTCGAGTCTAGAGGGCC

Legend: NheI + Start codon + CLEC14A + Linker 1 + TTc + Linker 2 + Fc + Stop codon + ApaI

## Protein sequence

MGWSCIIFFLVATATGVYSEHPTADRAACSASGACYSLHHATFKRRAAEEACSLRGGTLSTVHSGSEFQAVLLLLRAGPGPGG  
GSKDLLFWVALERSISQCTQEKEPLRGFSWLHPDSEDSPLPWVEEPQRSCTVRKCAALQATRGVEPAGWKEMRCHLRT  
DGYLCKYQFEVLCAPAPRPGAASNLSFQAPFRLSSSALDFSPPGTEVSAMCPGDLVSSTCIQEETSAHWDGLFPGTVLCPCS  
GRYLLAGKCVELPDCLDHLGDFTCECAVGFEKGDRSCETKVEEQLTLEGTKLPTRNVTATPAGAVTNRTWPGQVYDKPGE  
MPQVTEILQWGTQSTLPTIQKTPQTKPKVTGTPSGSVVLNYTSSPPVSLTFDTSSTGGSGGGSGGKNLDCWVDNEEDIDVILKK  
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NEYSIISSMKKHLSLSIGSGWSVSLKGNNLIWTLKDSAGEVRQITFRDLPDKFNAYLANKWVFITITNDRSSANLYINGVLMGSAE  
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TDYMYLTNAPSYTNGKLNIIYRRLYNGLKFIKRYTPNNEIDSFVKSGDFIKLYVSYNNNEHIVGYPKDGNAFNNLDRIIRVGYNA  
PGIPLYKKMEAVKLRDLKTYSVQLKLYDDKNASLGLVGTNNGQIGNDPNRDILIASNWYFNHLKDKILGCDWYFVPTDEGWTND  
GGSGGGSGGDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE  
EQYNSTYRVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI  
AVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSPGK\*

Legend: CLEC14A + Linker 1 + TTc + Linker 2 + Fc

## Mouse ROBO4 with Fc-tag (ROBO4-Fc)

The DNA and protein sequences of ROBO4 and Fc are adapted from Escobar-Riquelme (2024).

### DNA sequence

**GCTAGCGCCACC**ATGGGCTCAGGGGGGACTGGGCTGCTGGGAACCGAATGGCCTCTGCCTCTGCTGCTGCTGTTATTAT  
GGGGGGAGAAAGCTCTGGATTCTCCCCCTCAGATCCTGGTGCACCCACAGGACCAGCTGCTGCAGGGAAGCGGCCCTGC  
AAAGATGAGGTGCAGAAGCTCCGGACAGCCACCACCTACCATCAGGTGGCTGCTGAACGGCCAGCCACTGAGCATGGC  
CACACCCGACCTGCACTACCTGCTGCCTGATGGCACCCCTGCTGCTGCACAGGCCTTCCGTGCAGGGCCGCCACAGGAC  
GATCAGAACATCTGTCCGCCATCTGGGCGTGTATACATGCGAGGCCCTCTAATAGGCTGGGAACCGCCGTGCTAGGGG  
CGCCAGACTGAGCGTGGCCGTGCTGCAGGAGGACTCCAGATCCAGCCAAGGGATACAGTGGCAGTGGTGGGAGAGAG  
CCTGGTGTGAGTGTGGCCACCATGGGGATACCCAAAGCCCTCTGTGAGCTGGTGGAAAGGACGGCAAGCCTCTGGT  
GCTGCAGCCAGGCCGAGAACCCTGAGCGGCGACTCCCTGATGGTGTCTAGGGCCGAGAAGAATGATAGCGGCACCTA  
TATGTGCATGGCCACAAACAATGCAGGACAGAGGGAGAGCAGGGCAGCACGGGTGTCCATCCAGGAGTCTCAGGATCA  
CAAGGAGCACCTGGAGCTGCTGGCCGTGCGCATCCAGCTGGAGAACGTGACACTGCTGAATCCTGAGCCAGTGAAAGG  
CCCCAAGCCTGGCCCAAGCGTGTGGCTGTCTTGAAGGTGTCTGGCCCTGCAGCACCAGCAGAGAGCTACACCGCCCT  
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CCCCGAGGTGACATGCGTGGTGGTGGACGTGAGCCACGAGGATCCTGAGGTGAAGTTAACTGGTACGTGGACGCGCT  
GGAGGTGCACAATGCCAAGACCAAGCCCCGGGAGGAGCAGTACAACAGCACATATAGAGTGGTGTCCGTGCTGACCGT  
GCTGCACCAGGATTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTGTCCAATAAGGCCCTGCCAGCCCCCATCGAGAA  
GACAATCTCTAAGGCAAAGGGACAGCCAAGGGAGCCTCAGGTGTACACCCTGCCTCCATCTAGAGACGAGCTGACAAAG  
AACCAGGTGAGCCTGACCTGTCTGGTGAAGGGCTTCTATCCTAGCGATATCGCCGTGGAGTGGGAGTCCAATGGCCAGC  
CAGAGAACAATTACAAGACCACACCCCCTGTGCTGGACTCTGATGGCAGCTTCTTTCTGTATTCCAAGCTGACCGTGGAT  
AAGTCTAGATGGCAGCAGGGCAACGTGTTCAAGTGTCTGTGATGCACGAAGCACTGCATAACCACTACACTCAGAAGA  
GCCTGTCACTGTACCTGGGAAAT**AAGGGCCC**

Legend: **NheI** + **Start codon** + ROBO4 + **Linker 3** + **TTc** + **Linker 2** + **Fc** + **Stop codon** + **Apal**

### Protein sequence

MGSGGTGLLGTEWPLPLLLLFIMGGEALDSPPQILVHPQDQLLQGSGPAKMRCRSSGQPPPTIRWLLNGQPLSMATPDLHYLL  
PDGTLHLHRPSVQGRPDQDQNILSAILGVYTCEASNRLGTAVSRGARLSVAVLQEDFQIQPRDTVAVVGESLVLECGPPWGYPK  
PSVSWWKDGKPLVLQPGRRTVSGDSLMVSRAEKNDSTYMCMATNNAGQRESRAARVSIQESQDHKEHLELLAVRIQLENV  
TLLNPEPVKGPKPGPSVWLSWKVSGPAAPAESYALFRTQSPRDQGSPTWTEVLLRGLQSAKLGGLHWGQDYEFKVRPSSG  
RARGPDSNVLLRLPEQVPSAPPQVTLRSGNGSVFVSWAPPPAESHNQVIRGYQVWSLGNASLPAANWTVVGEQTQLEIAT  
RLPGSYCVQAAVTGAGAGELSTPVCLLLEQAMEQSARDPRKHVPWTLEQLRATLRRPE**GGRGSSSEPKSCDKTHTCPPC**  
**PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD**  
**WLNQKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPP**  
**VLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK\***

Legend: ROBO4 + **Linker 3** + **TTc** + **Linker 2** + **Fc**

## Mouse ROBO4 plus Fragment C of Tetanus Toxin (TTc) (ROBO4-TTc) with Fc-tag

The DNA and protein sequences of ROBO4-TTc with Fc-tag are adapted from Escobar-Riquelme (2024).

### DNA sequence

GCTAGCGCCACCATGGGCTCAGGGGGGACTGGGCTGCTGGGAACCGAATGGCCTCTGCCTCTGCTGCTGCTGTTTATTAT  
GGGGGGAGAAAGCTCTGGATTCTCCCCCTCAGATCCTGGTGCACCCACAGGACCAGCTGCTGCAGGGAAGCGGCCCTGC  
AAAGATGAGGTGCAGAAGCTCCGGACAGCCACCACCTACCATCAGGTGGCTGCTGAACGGCCAGCCACTGAGCATGGC  
CACACCCGACCTGCACTACCTGCTGCCTGATGGCACCCTGCTGCTGCACAGGCCCTCCGTGCAGGGCCGCCACAGGAC  
GATCAGAACATCTGTCCGCCATCTGGGCGTGATACATGCGAGGCCCTCTAATAGGCTGGGAACCGCCGTGCTAGGGG  
CGCCAGACTGAGCGTGGCCGTGCTGCAGGAGGACTCCAGATCCAGCCAAGGGATACAGTGCCAGTGCTGGGAGAGAG  
CCTGGTGTGAGTGTGGCCACCATGGGGATACCCAAAGCCCTCTGTGAGCTGGTGGAAGGACGGCAAGCCTCTGGT  
GCTGCAGCCAGGCCGAGAACCGTGAGCGGCGACTCCCTGATGGTGTCTAGGGCCGAGAAGAATGATAGCGGCACCTA  
TATGTGCATGGCCACAAACAATGCAGGACAGAGGGAGAGCAGGGCAGCACGGGTGTCCATCCAGGAGTCTCAGGATCA  
CAAGGAGCACCTGGAGCTGCTGGCCGTGCGCATCCAGCTGGAGAACGTGACACTGCTGAATCCTGAGCCAGTGAAAGG  
CCCCAAGCCTGGCCCAAGCGTGTGGCTGTCTGGAAGGTGTCTGGCCCTGCAGCACCAGCAGAGAGCTACACCGCCCT  
GTTCAGAACACAGAGGTCCCCCGCGACCAGGGCTCTCCTTGGACCGAGGTGCTGCTGAGGGGCCCTGCAGTCCGCCAA  
GCTGGGAGGCCTGCACTGGGGACAGGACTATGAGTTCAAGGTGCGGCCCTCTAGCGGACGGGCCAGAGGCCCCGATAG  
CAACGTGCTGCTGCTGAGGCTGCCAGAGCAGGTGCCATCCGCCCTCCACAGGGAGTGACCTGCGCAGCGGAAACGG  
ATCCGTGTTCTGTCTTGGGCTCCCCCTCCAGCCGAGAGCCACAATGGCGTGATCCGGGGCTACCAAGTGTGGAGCCTG  
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GGATCCTATTGCGTGCAGGTGGCAGCAGTGACAGGAGCAGGAGCAGGCGAGCTGAGCACCCCGTGTGCCTGCTGCTG  
GAGCAGGCAATGGAGCAGTCCGCCCGGGATCCAGAAAGCACGTGCCTTGGACACTGGAGCAGCTGAGGGCCACCCTG  
AGGCGCCCAAGAGGGAGGACCGCGCTCTCTAGCTCCGGATGGTCTTGTATTATCTTCTTTCTGGTCGCCACTGCCACTGG  
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ATCTGGACATCAACAATGATATCATCTCCGACATCTCTGGCTTCAACAGCTCCGTGATCACCTACCCTGACGCCCAGCTGG  
TGCCAGGCATCAATGGCAAGGCCATCCACCTGGTGAACAATGAGTCTAGCGAAGTGATCGTGACAAGGCCATGGATATC  
GAGTATAACGACATGTTCAACAACCTTACCCTGAGCTCTGGCTGCGGGTGCCCAAGGTGTCTGCCAGCCACCTGGAGC  
AGTACGGCACCAATGAGTATTCCATCATCTCCTCTATGAAGAAGCACTCCCTGTCTATCGGCTCTGGCTGGAGCGTGTCCC  
TGAAGGGCAACAATCTGATCTGGACACTGAAGGATAGCGCCGAGAGGTGCGGCAGATCACCTTCCGGGACCTGCCTGA  
TAAGTTTAATGCCTACCTGGCCAAACAAGTGGGTGTTTCATCACCATCACAAACGACCGGCTGAGCTCCGCCAATCTGTATAT  
CAACGGCGTGCTGATGGGCTCTGCGGAGATCACAGGCCTGGGAGCCATCAGGGAGGATAACAATATCACCTGAAGCTG  
GACCGCTGCAACAATAACAATCAGTACGTGAGCATCGATAAGTTCAGAATCTTTTGAAGGCCCTGAATCCTAAGGAGATC  
GAGAAGCTGTACACCAGCTATCTGTCCATCACATTCTGAGGGATTTTGGGGCAACCCACTGCGCTACGACACCGAATA  
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TGTATAAGAAGATGGAGGCCGTGAAGCTGAGGGACCTGAAGACATACTCCGTGCAGCTGAAGCTGTATGACGATAAGAAT  
GCCTCTCTGGGCTGGTGGGCACCCACAACGGACAGATCGGCAATGATCCCAACCGCGACATCCTGATCGCCAGCAATT  
GGTACTTCAACCACCTGAAGGATAAGATCCTGGGCTGCGACTGGTATTTGTGCCAACCAGTGAAGGGCTGGACAAATGAC  
GGAGGCAGCGGAGGAGGCTCCGGAGGAGAGCCCAAGAGCTGTGATAAGACCCACACATGCCACCTTGTCCAGCACCA  
GAGCTGCTGGGCGGCCCTTCCGTGTTCTGTTCCACCCAAGCCTAAGGACACACTGATGATCAGCAGGACCCCGAGG  
TGACATGCGTGGTGGTGGACGTGAGCCACGAGGACCCTGAGGTGAAGTTCAATTGGTACGTGGATGGCGTGGAGGTGC  
ACAACGCCAAGACCAAGCCCCGGGAGGAGCAGTACAACCTACATATAGAGTGGTGAGCGTCTGACCGTGTGACCA  
GGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTGTCTAACAAGGCCCTGCCTGCCCAATCGAGAAGACAATCAGC  
AAGGCAAGGGACAGCCACGGGAGCCACAGGTGTACACCCTGCCTCCAGCCGGGACGAGCTGACAAAGAATCAGGT  
GAGCCTGACCTGTCTGGTGAAGGGCTTCTATCCTAGCGACATCGCAGTGGAGTGGGAGTCCAACGGACAGCCAGAGAAT  
AACTACAAGACCACACCCCTGTGCTGGACTCTGATGGCAGCTTCTTTCTGTATTCCAAGCTGACCGTGGACAAGTCTCG  
CTGGCAGCAGGGCAACGTGTTTTCTGTAGCGTGATGCATGAAGCACTGCACAACCATTACCCAGAAAAGCCTGTCAC  
TGTCCCCCGGAAAGTAATAGCTCGAGTCTAGAAGGCCC

Legend: NheI + Start codon + ROBO4 + Linker 3 + TTc + Linker 2 + Fc + Stop codon + ApaI

## Protein sequence

MGSGGTGLLGTEWPLPLLLLFIMGGEALDSPPQILVHPQDQLLQSGPAKMRCRSSGQPPPTIRWLLNGQPLSMATPDLHYLL  
PDGTLHLHRPSVQGRPQDDQNILSAILGVYTCEASNRLGTAVSRGARLSVAVLQEDFQIQPRDTVAVVGESLVLECGPPWGYPK  
PSVSWWKDGKPLVLQPGRRTVSGDSLMSVRAEKNDSTYMCATNNAGQRESRAARVSIQESQDHKEHLELLAVRIQLENV  
TLLNPEPVKGPKGPSVWLSWKVSGPAAPAESYALFRTQSRPDQGSPTVEVLLRGLQSAKLGGLHWGQDYEFKVRPSSG  
RARGPDSNVLLRLPEQVPSAPPQGVTLRSGNGSVFVSWAPPAESHNGVIRGYQVWSLGNASLPAANWTVVGEQTQLEIAT  
RLPGSYCVQVAAVTGAGAGELSTPVCLLLEQAMEQSARDPRKHVPWTLEQLRATLRRPEGGRGSSSGWSCIIFLVATATGV  
YSKNLDCWVDNEEDIDVILKKSTILNLDINNDIISDISGFNSSVITYPDAQLVPGINGKAIHLVNNESEVIVHKAMDIEYNDMFNNF  
TVSFWRVPKVSASHLEQYGTNEYSISSMKKHSLSIGSGWSVSLKGNNLIWTLKDSAGEVRQITFRDLPDKFNAYLANKWVFI  
ITNDRSSANLYINGVLMGSAEITGLGAIEDNNITLKDRCNNNNQYVSIDKFRIFCKALNPKEIEKLYTSYLSITFLRDFWGNPL  
RYDTEYYLIPVASSSKDVQLKNITDYMILNAPSNTNGKLNIIYRRLYNGLKFIKRYTPNNEIDSFVKSGDFIKLYVSYNNNEHIVG  
YPKDGNFNNLDRILRVGYNAPGIPLYKKMEAVKLRLDKTYSVQLKLYDDKNASLGLVGTNNGQIGNDPNRDILIASNWFNHL  
KDKILGCDWYFVPTDEGWTNDGSGGGSGGEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVS  
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY  
TLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEAL  
HNHYTQKSLSLSPGK\*

Legend: ROBO4 + Linker 3 + TtC + Linker 2 + Fc

## Fragment C of Tetanus Toxin (TTc) with Fc-tag

The DNA and protein sequences of Fragment C of Tetanus Toxin (TTc) with human Fc-tag are adapted from Escobar-Riquelme (2024).

### DNA sequence

GCTAGCAAGCTTGCCACCATGGGATGGTCTTGATTATCTTCTTTCTGGTCGCCACTGCCACTGGGGTCTACTCAAAAAAT  
CTGGACTGTTGGGTCGATAACGAAGAGGACATCGATGTGATCCTGAAGAAGTCCACAATCCTGAATCTGGACATCAACAA  
TGATATCATCTCCGACATCTCTGGCTTCAACAGCTCCGTGATCACCTACCTGACGCCAGCTGGTGCCAGGCATCAATGG  
CAAGGCCATCCACCTGGTGAACAATGAGTCTAGCGAAGTGATCGTGACAAGGCCATGGATATCGAGTATAACGACATGT  
TCAACAACCTTACCCTGAGCTTCTGGCTGCGGGTGCCCAAGGTGTCTGCCAGCCACCTGGAGCAGTACGGCACCAATGA  
GTATTCATCATCTCCTCTATGAAGAAGCACTCCCTGTCTATCGGCTCTGGCTGGAGCGTGTCCCTGAAGGGCAACAATCT  
GATCTGGACACTGAAGGATAGCGCCGAGAGGTGCGGCAGATCACCTTCCGGGACCTGCCTGATAAGTTTAATGCCTACC  
TGGCCAACAAGTGGGTGTTTCATCACCATCACAAACGACCGGCTGAGCTCCGCCAATCTGTATATCAACGGCGTGTCTGATG  
GGCTCTGCCGAGATCACAGGCCCTGGGAGCCATCAGGGAGGATAACAATATCACCTGAAGCTGGACCGCTGCAACAATA  
ACAATCAGTACGTGAGCATCGATAAGTTCAGAATCTTTGTAAGGCCCTGAATCCTAAGGAGATCGAGAAGCTGTACACCA  
GCTATCTGTCCATCACATTCCTGAGGGATTTTTGGGGCAACCCACTGCGCTACGACACCGAATACTATCTGATCCCCGTGG  
CCTCTAGCTCCAAGGATGTGCAGCTGAAGAATATCACCGACTACATGTATCTGACAAACGCCCTTCTCTATACCAATGGCA  
AGCTGAACATCTACTATCGGAGACTGTACAACGGCCTGAAGTTCATCATCAAGCGGTATACCCCAAAACAATGAGATCGATT  
CCTTCGTGAAGTCTGGCGACTTTATCAAGCTGTACGTGAGCTATAACAATAACGAGCACATCGTGGGCTACCCAAAGGATG  
GCAATGCCTTTAATAACCTGGACAGAATCCTGAGGGTGGGATACAACGCACCAGGCATCCCTCTGTATAAGAAGATGGAG  
GCCGTGAAGCTGAGGGACCTGAAGACATACTCCGTGCAGCTGAAGCTGTATGACGATAAGAATGCCTCTCTGGGCCTGG  
TGGGCACCCACAACGGACAGATCGGCAATGATCCCAACCGCGACATCCTGATCGCCAGCAATTGGTACTTCAACCACCT  
GAAGGATAAGATCCTGGGCTGCGACTGGTATTTGTGCCAACCGATGAGGGCTGGACAAATGACGGAGGCAGCGGAGGA  
GGCTCCGGAGGAGAGCCCAAGAGCTGTGATAAGACCCACACATGCCACCTTGTCAGCAGCAGAGCTGCTGGGCGGC  
CCTTCCGTGTTCTGTTCCACCAAGCCTAAGGACACACTGATGATCAGCAGGACCCCCGAGGTGACATGCGTGGTGGT  
GGACGTGAGCCACGAGGACCCTGAGGTGAAGTTCAATTGGTACGTGGATGGCGTGGAGGTGCACAACGCCAAGACCAA  
GCCCCGGGAGGAGCAGTACAACCTTACATATAGAGTGGTGAGCGTGCTGACCGTGCTGCACCAGGACTGGCTGAATGGC  
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GTGAAGGGCTTCTATCCTAGCGACATCGCAGTGGAGTGGGAGTCCAACGGACAGCCAGAGAATAACTACAAGACCACAC  
CCCCTGTGCTGGACTCTGATGGCAGCTTCTTCTGTATTCCAAGCTGACCGTGGACAAGTCTCGCTGGCAGCAGGGCAAC  
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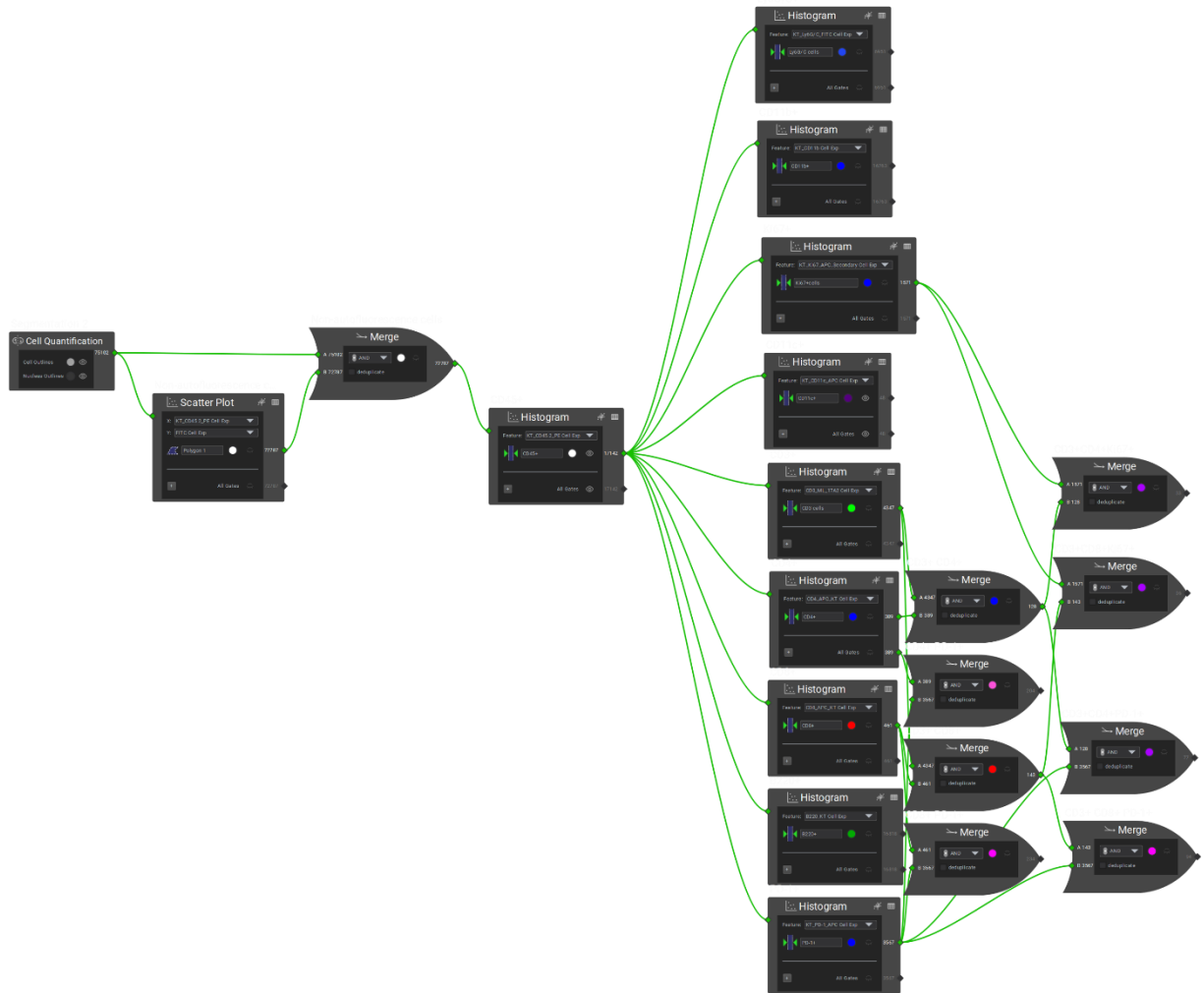
Legend: NheI + Start codon + TTc + Linker 2 + Fc + Stop codon + ApaI

### Protein sequence

MGWSCIIFFLVATATGVYSKNLDCWVDNEEDIDVILKKSTILNLDINNDIISDISGFNSSVITYPDAQLVPGINGKAIHLVNNESSEVIV  
HKAMDIEYNDMFNNFTVSFWRVLPKVSASHLEQYGTNEYSIISSMKKHSLSIGSGWSVSLKGNNLIWTLKDSAGEVRQITFRDL  
PDKFNAYLANKWVITITNDRLSSANLYINGVLMGSAEITGLGAIREDNNTLKLDRCNQNNQYVSIDKFRIFCKALNPKEIEKLYT  
SYLSITFLRDFWGNPLRYDTEYLLIPVASSSKDVQLKNITDYMILTNPSTYNGKLNIIYRRLYNGLKFIKRYTPNNEIDSFVKSG  
DFIKLYVSYNNNEHIVGYPKDGNAFNLDRLRVGYNAPGIPLYKKMEAVKLRLDKTYSVQLKLYDDKNASLGLVGTNNGQIGND  
PNRDILIASNWYFNHLKDKILGCDWYFVPTDEGWTNDGGSGGSGGEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL  
MISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK  
TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQ  
QGNVFSCSVMHEALHNHYTQKSLSLSPGK\*

Legend: TTc + Linker 2 + Fc

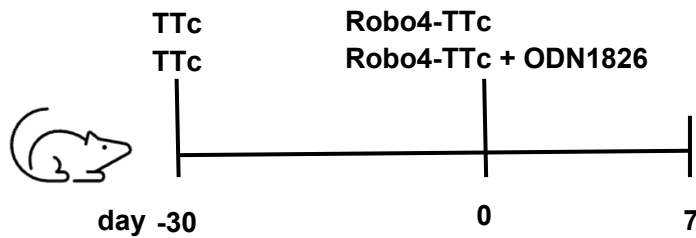
## Appendix B



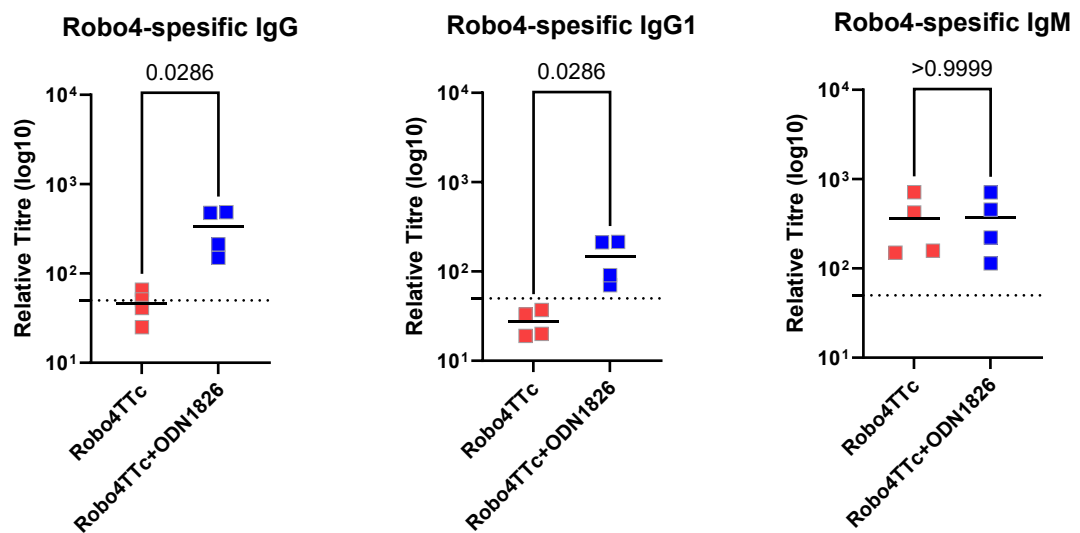
**Workflow used for the spatial analysis of multiplex imaging on MACS iQ View.** Cell type annotation began with segmenting the selected regions of interest using specific settings outlined in the accompanying table. Highly auto-fluorescent cells were excluded from the analysis. CD45<sup>+</sup> cells were gated and subsequently sub-gated into B220<sup>+</sup>, CD11b<sup>+</sup>, Ly6G/C<sup>+</sup>, CD3<sup>+</sup>, CD11c<sup>+</sup>, Ki67<sup>+</sup>, and PD-1<sup>+</sup> populations. CD3<sup>+</sup> T cells were further subdivided into CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which were then analysed for Ki67 and PD-1 expression. Data are presented as the percentage of CD45<sup>+</sup> lymphocytes. Statistical comparisons between groups were performed using the Mann–Whitney test. Results are shown as mean  $\pm$  SD. Significance is indicated as follows: ns (not significant,  $p \geq 0.05$ ), \* $p < 0.05$ .

## Appendix C

**A**



**B**



### ODN1826 adjuvant enhances ROBO4-specific antibody response after ROBO4TTc vaccination.

(A) Experimental strategy to investigate the effect of the ODN1826 adjuvant on enhancing ROBO4-specific antibody response. Mice were intraperitoneally primed with 50 µg of TTc formulated in a 1:1 ratio with alum. Thirty days later, the ROBO4TTc group received a boost with 50 µg of ROBO4TTc, while the ROBO4TTc+ODN1826 group received 50 µg of ROBO4TTc combined with ODN1826, both in a 1:1 ratio with alum. Blood samples were collected on Days 0 and 7. (B) ROBO4-specific IgG, IgG1 and IgM titres following immunisation. Each symbol represents an individual mouse (ROBO4TTc: n=4, ROBO4TTc+ODN1826: n=4). Statistical analysis was performed using a two-tailed Mann-Whitney test. The bar represents the median.

## Appendix D

### Mouse ROBO4 amino acid sequence

```

10      20      30      40      50      60
MSGGGTGLLG TEWPLPLLLL FIMGGEALDS PPQILVHPQD QLLQGSGPAK MRCRSSGQPP

70      80      90      100     110     120
PTIRWLLNGQ PLSMATPDLH YLLPDGTL LL HRPSVQGRPQ DDQNILSAIL GVYTCEASNR

130     140     150     160     170     180
LGTAVSRGAR LSAVLQEDF QIQPRDTVAV VGESLVLECG PPWGYPKPSV SNNKDGKPLV

190     200     210     220     230     240
LQPGRRTVSG DSLMVSRAEK NDSGTVMCMA TNNAGQRESR AARVSIQESQ DHKEHLELLA

250     260     270     280     290     300
VRIQLENTVL LNPEPVKGPK PGPSVWLSWK VSGPAAPAES YTALFRTQRS PRDQGS PWTE

310     320     330     340     350     360
VLLRGLQSAK LGGLHWGQDY EFKVRPSSGR ARGPDSNVLL LRLPEQVPSA PPQGVTLRSV

370     380     390     400     410     420
NGSVFVSWAP PPAESHNGVI RGYQVWSLGN ASLPAANWTV VGEQTQLEIA TRLPGSYCVQ

430     440     450     460     470
VAAVTGAGAG ELSTPVCLLL EQAMEQSARD PRKHVPWTL EQLRATLRRPE

```

*Table: Cleavage in mouse ROBO4 protein sequence.*

| Name of enzyme | No. of cleavages | Positions of cleavage sites                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
|----------------|------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Proteinase K   | 214              | 6 8 9 11 12 13 15 17 18 19 20 21 22 26 27 28 34 35 36 42 43<br>49 62 63 65 66 67 72 75 76 79 81 82 83 87 88 89 90 95 105<br>106 108 109 110 112 113 114 116 117 121 123 124 125 129<br>131 133 134 135 136 138 140 142 147 148 149 150 151 153<br>155 156 157 158 163 165 170 172 173 179 180 181 187 188<br>193 195 198 199 205 206 210 211 214 218 221 222 224 226<br>228 234 236 237 238 239 240 241 243 245 246 248 249 250<br>251 254 256 265 266 267 269 271 275 276 278 279 281 282<br>283 284 285 287 298 299 300 301 302 303 306 309 311 314<br>316 320 321 322 324 331 338 339 340 341 343 345 347 350<br>355 356 357 364 365 366 368 369 373 374 379 380 383 385<br>386 388 391 393 395 396 398 399 400 401 403 405 407 408<br>409 410 411 413 417 419 421 422 423 424 425 427 429 431<br>432 434 436 438 439 440 441 443 445 448 455 457 458 459<br>460 462 464 465 466 470 |
| Trypsin        | 37               | 50 52 54 64 120 127 130 145 174 185 186 197 200 217 220<br>223 233 242 257 270 286 289 292 304 310 323 330 332 342<br>358 381 412 449 452 453 463 467                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |

**Cleavage sites of mouse ROBO4 protein identified by enzymes and chemicals.** The amino acid sequence of the mouse ROBO4 protein was analysed using the PeptideCutter tool available at [https://web.expasy.org/peptide\\_cutter/](https://web.expasy.org/peptide_cutter/). Predicted cleavage sites for various proteolytic enzymes and chemical reagents are shown.

## Mouse CLEC14A amino acid sequence

```

10      20      30      40      50      60
MGWSCIIFFL VATATGVYSE HPTADRAACS ASGACYSLHH ATFKRRAAEE ACSLRGGTLS

70      80      90      100     110     120
TVHSGSEFQA VLLLLRAGPG PGGGSKDLLF WVALERSISQ CTQEKEPLRG FSWLHPDSED

130     140     150     160     170     180
SEDSPLPWVE EPQRSCTVRK CAALQATRGV EPAGWKEMRC HLRTDGYLCK YQFEVLC PAP

190     200     210     220     230     240
RPGAASNL SF QAPFRLSSSA LDFSPPGTEV SAMCPGDL SV SSTCIQEETS AHWDGLFP GT

250     260     270     280     290     300
VLCPCSGRYL LAGKCVLPD CLDHLGDFTC ECAVGFE LGK DGRSCETKVE EQLTLEGTKL

310     320     330     340     350     360
PTRNVTATPA GAVTNRTWPG QVYDKPGEMP QVTEILQWGT QSTLPTIQKT PQTKPKVGTG T

370     380
PSGSVVLNYT SSPPVSLTFD TSST

```

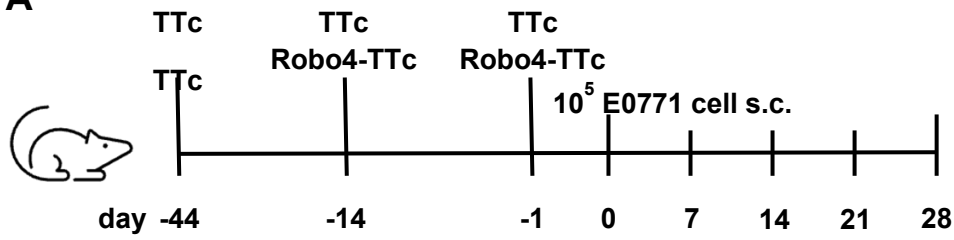
*Table: Cleavage in mouse CLEC14A protein sequence.*

| Name of enzyme | No. of cleavages | Positions of cleavage sites                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
|----------------|------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Caspase1       | 1                | 165                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |
| Proteinase K   | 186              | 3 6 7 8 9 10 11 12 13 14 15 17 18 20 23 24 27 28 31 34 36 38<br>41 42 43 47 48 49 50 51 54 58 59 61 62 67 68 70 71 72 73 74<br>75 77 88 89 90 91 92 93 94 95 98 102 104 106 108 111 113<br>114 119 122 126 128 129 130 131 137 138 142 143 144 146<br>147 150 151 153 155 157 162 164 167 168 171 173 174 175<br>176 179 184 185 188 190 192 194 196 200 201 203 208 209<br>210 212 218 220 223 225 227 228 229 231 233 236 237 240<br>241 242 249 250 251 252 256 257 258 262 265 268 269 271<br>273 274 276 277 278 286 287 289 290 291 293 294 295 296<br>298 300 302 305 306 307 308 310 312 313 314 317 318 322<br>323 328 332 333 334 335 336 338 340 343 344 346 347 350<br>353 357 358 360 365 366 367 369 370 375 377 378 379 381<br>384 |
| Trypsin        | 28               | 50 52 54 64 120 127 130 145 174 185 186 197 200 217 220<br>223 233 242 257 270 286 26 44 45 46 55 76 86 96 105 109<br>134 139 140 148 156 159 163 195 248 254 280 283 288 299<br>303 316 349 356292 304 310 323 330 332 342 358 381 412<br>449 452 453 463 467                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |

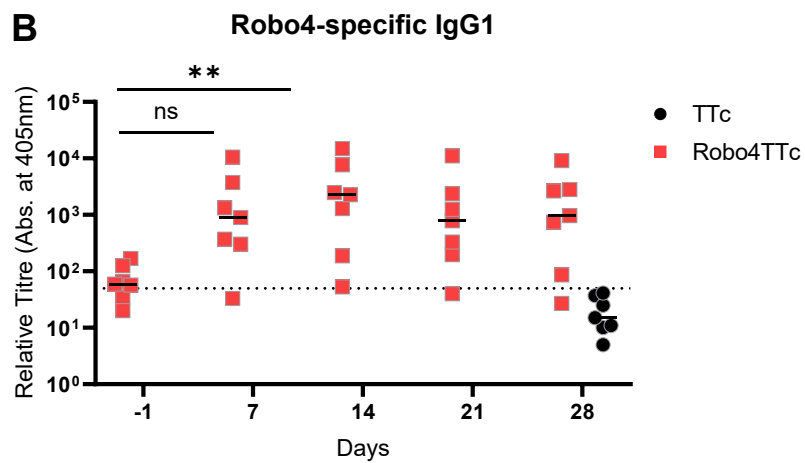
**Cleavage sites of mouse CLEC14A protein identified by enzymes and chemicals.** The amino acid sequence of the mouse CLEC14A protein was analysed using the PeptideCutter tool available at [https://web.expasy.org/peptide\\_cutter/](https://web.expasy.org/peptide_cutter/). Predicted cleavage sites for various proteolytic enzymes and chemical reagents are shown.

## Appendix E

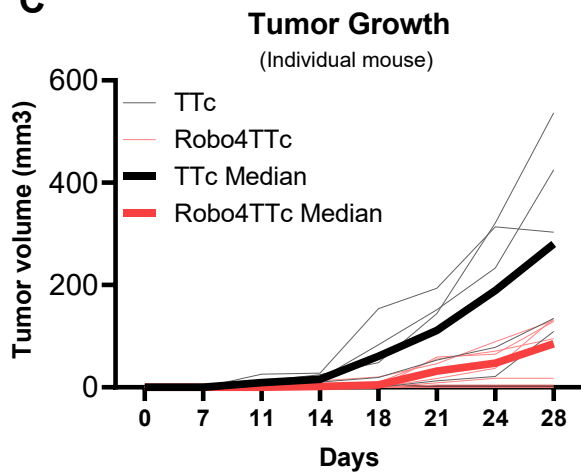
**A**



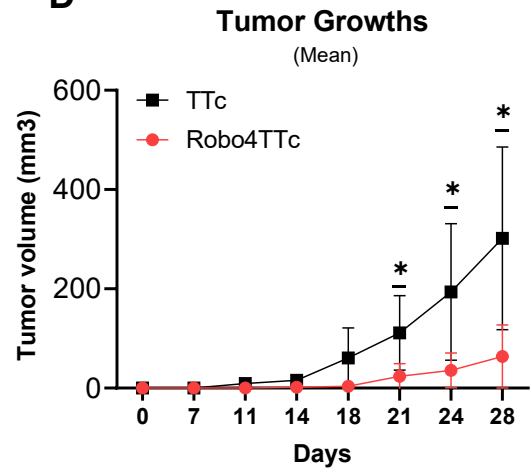
**B**



**C**



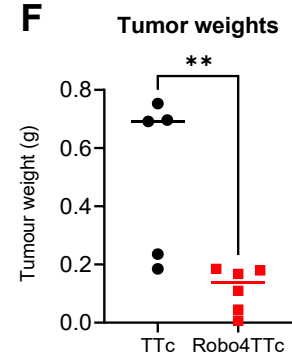
**D**



**E**



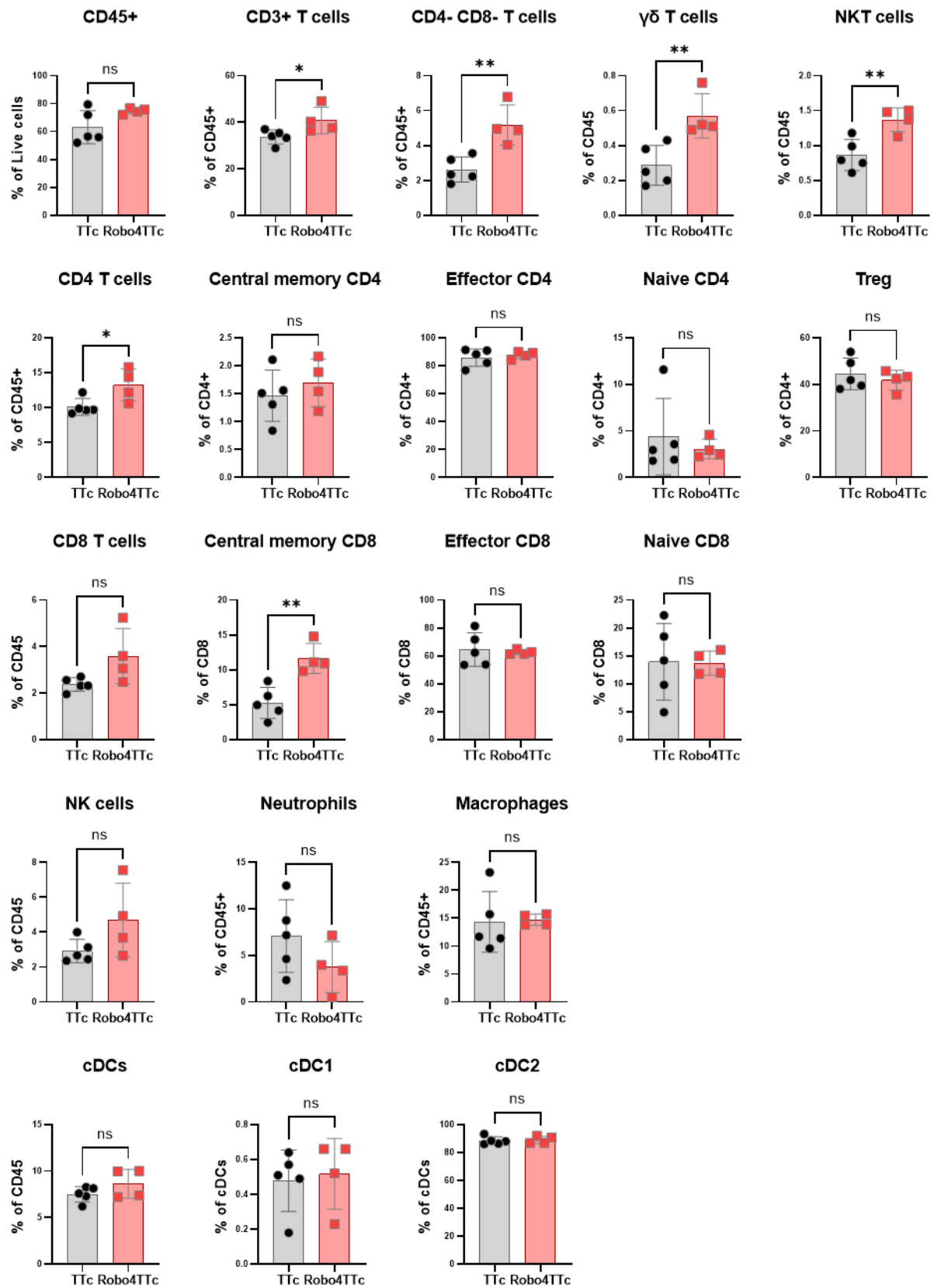
**F**



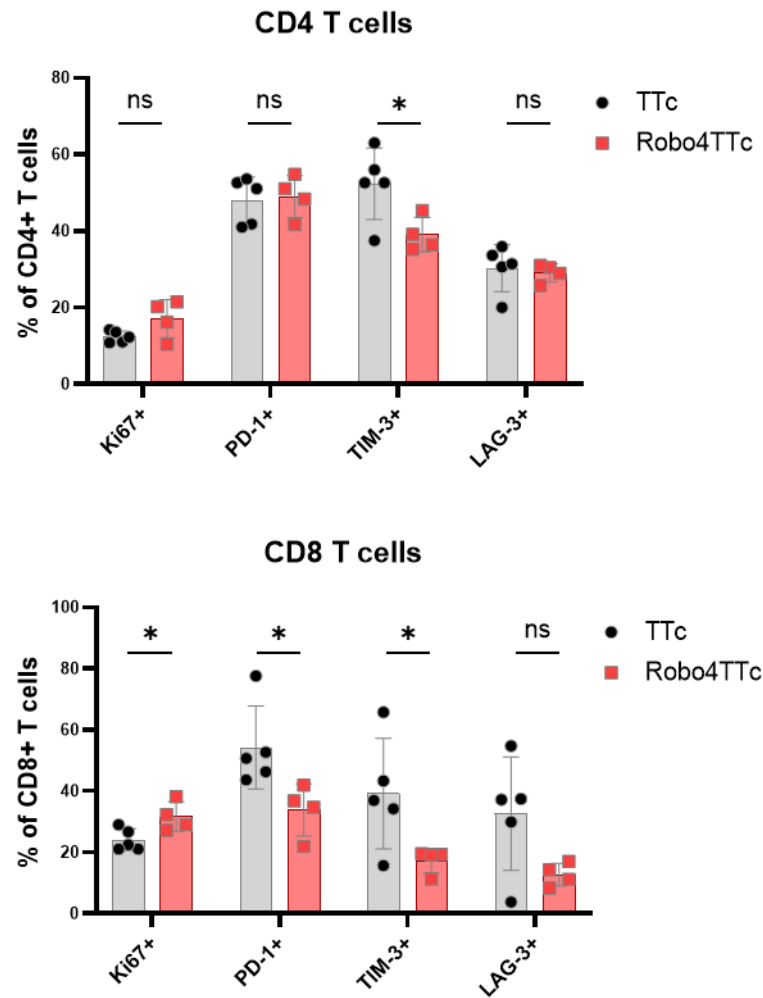
**ROBO4TTc vaccine induces ROBO4-Specific IgG1 response and restricts tumour growth in ER-negative breast cancer mouse model (E0771).** (A) Experimental strategy for ROBO4TTc vaccine immunisation in vivo. Mice were intraperitoneally primed with 50 µg of TTc or ROBO4TTc formulated in a 1:1 ratio with alum. This priming was followed by two booster doses of 50 µg of the respective antigen on Days 30 and 45 post-initial immunisations. One day post the final booster, all mice were subcutaneously implanted in the fourth mammary fat pad with 4T1 cells. All mice were sacrificed on Day 28. (B) ROBO4TTc immunisation induces ROBO4-specific IgG1 response. The time course of ROBO4-specific IgG1 titres following immunisation for TTc (n=7) and ROBO4TTc (n=7) mice. Each symbol represents an individual mouse, with bars showing the median. The threshold for significance is set at 50. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test (ns > 0.5; \*\*p < 0.01). (C) Individual and median tumour growth curves. Data for TTc (n=5) and ROBO4TTc (n=6) mice are shown. (D) Mean tumour growth curves. Error bars represent the standard deviation. Data were analysed using a two-tailed Mann-Whitney test (\*p < 0.05). (E) Representative tumour images: Images from the TTc and ROBO4TTc groups on Day 28. (G) Tumour weights. Data were analysed using a two-tailed Mann-Whitney test (\*\*p < 0.01).

A

## E0771 Tumour



**B**



**Immune cell profiling of E0771 tumours after ROBO4TTc vaccination.** (A) Flow cytometry analysis quantifies immune cell populations in E0771 tumours from ROBO4TTc and TTc groups. Statistical comparisons between groups were performed using a two-tailed Mann-Whitney test, with results presented as mean  $\pm$  SD. Significance levels are indicated as ns (not significant), \* $p \geq 0.05$ . (B) Quantification of activation (Ki67) and exhaustion markers (PD-1, TIM-3, and LAG-3) in CD4<sup>+</sup> and CD8<sup>+</sup> T cells.