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Targeting CD5 to enhance immune T cell activation and function in treatment of solid tumours

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology and Immunology

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Abstract

CD5 is a member of scavenger receptor cysteine-rich superfamily that is expressed primarily on T cells. It can attenuate T-cell receptor signaling and impair cytotoxic T lymphocyte (CTL) activation and is a therapeutic targetable tumour antigen expressed on leukemic T and B cells. However, the potential therapeutic effect of functionally blocking CD5 to increase T cell anti-tumour activity against tumours (including solid tumours) has not been explored. CD5⁻ solid tumours in CD5 knockout mice display increased in anti-tumour immunity. Hence, blocking CD5 function may have a potential therapeutic effect by enhancing CTL function. Here, I assessed CD5 levels in T cell subsets in different organs in mice bearing syngeneic 4T1 breast tumour homografts and determined the association between CD5 and increased CD69 and PD-1 (markers of T cell activation and exhaustion) by flow cytometry. I report that CD5 levels in T cells was higher in peripheral organs than in lymphocytes infiltrated into tumours, and that CD5^{high} T cells from peripheral organs exhibited higher levels of activation and exhaustion than CD5^{low} T cells from the same organs. Interestingly, among the population of tumour-infiltrated lymphocyte subtypes, CD8⁺ T cells with low CD5 were activated to a higher level than CD8⁺ T cells with high CD5, with concomitantly increased exhaustion markers. Thus, differential CD5 levels among T cells in tumours and lymphoid organs can be associated with different levels of T cell activation and exhaustion, suggesting that CD5 could be a therapeutic target for immunotherapeutic activation in cancer therapy. I then studied the effect on primary T cell effector function of targeting CD5 *ex vivo* using an anti-CD5 MAb. The result showed enhanced cytotoxic T cell capacity to respond to activation and enhanced the capacity of CD8⁺ T cells to kill 4T1-mouse tumour cells in an *ex vivo* assay. These data support the potential of blockade of CD5 function to enhance T cell-mediated anti-tumour immunity. Based on these results I tested anti-CD5 *in vivo* as a single agent and in combination with other drugs and found a significant increase in activation and effector function of T cells: an effect that resulted in decreased 4T1 tumour homograft growth *in vivo*. These data suggest potential use of anti-CD5 MAb to enhance immune activation to poorly immunogenic tumour antigens and reduce tumour growth.

Keywords

Cancer immunotherapy, CD5, CTL, CD8⁺ T cell, anti-CD5 MAb, anti-PD-1 MAb, PD-1, immune cell, breast cancer, 4T1 tumour, immunotherapy, TILs, poorly immunogenic tumour antigen, T cell exhaustion, T cell activation

Summary for lay audience

T cells are an important part of the immune system, containing molecules that participate in recognizing foreign substances (antigens) including antigens that arise in cancer cells. When antigens stimulate those molecules, they activate T cells to recognize and kill the foreign organisms that contain those antigens (including cancer cells that contain unusual molecules that act as antigens). On the other hand, T cells also contain proteins that control their degree of activation by limiting their response to antigen recognitions: those proteins are termed “inhibitory receptors”. CD5 is such a receptor, expressed mainly on T cells, that limits T cell capacity to undergo activation in response to recognition of tumour antigen. Other studies have shown that mice with deleted CD5 and intact immune systems had delayed growth of transplanted tumours, suggesting that loss of CD5 can lead to increased immune activation in response to cancer cell antigen recognition, and immune cell-mediated attack and destruction of cancer cells resulting in slower cancer growth. I propose that blocking CD5 will coax T cells into better anti-cancer action, both as a treatment applied alone and in combination with already-approved immunotherapies. In this thesis, I describe my study of the correlation between CD5 level and T cell activation and exhaustion, and my assessment of dynamic CD5 expression among peripheral organs. I also inhibited CD5 on T cells obtained from mouse spleens using an anti-CD5 blocking antibody, a step taken to make them more active in recognizing and killing cancer cells. I found a significant increase in T cell effector function following treatment with a blocking anti-CD5 antibody and, based on these results, I then moved on to test the efficacy of this drug to treat mice with fully functional immune systems and transplanted mouse breast tumours. Treating mice with anti-CD5 antibody increased T cell activation function in spleen, lymph node and tumour. Furthermore, tumour growth was delayed by the treatment. These findings suggest that treatment with anti-CD5 MAbs can result in increased T cell effector function and reduced tumour homograft growth. Further investigation of reduction of CD5 in combination with other immunotherapy approaches may enhance anti-cancer immune T cells.

Co-authorship statement

All work presented in this thesis and submitted and published in peer-reviewed journals were completely done by Faizah Alotaibi. All laboratory members who contributed through constructive criticism, providing additional information, and assisting in animal experiments assistance are named in the acknowledgements section.

Dedications

*To my Parents (Mesfer and Sara), my husband Bandar and
my siblings Majed and Hawazin*

Thank you for your unconditional love and support throughout the years

To my little angel Ryan

*Who joined in during the first year of my PhD, it is a pleasure watching you grow
along with my PhD research project. You have taught me multitasking,
determination, patience, love and joy which I needed to thrive in graduate school*

To my grandma Dede

*Growing up by her side she used to tell me with a warm smile on her face, “Faizah,
you are one person but equal to seven brave men”. I always laughed when she said
that, and I said but why only seven grandma!*

To Dr. Jim Koropatnick and my M.Sc. supervisor Dr. Peter Greer

*When I joined your labs, I wanted nothing but the fact that I want to fight cancer.
Thank you for opening the gate for me.*

Both of you will always be part of my continued future success

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“A determined woman is unstoppable and can finish on time”

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List of abbreviations

Abbreviation	Meaning
5FU	5-fluorouracil
APCs	Antigen-presenting cells
ACK	Ammonium-Chloride-Potassium
ATC	Adoptive T cell
ADC	Antibody-drug conjugates
AICD	Activation-induced death
BCR	B cell receptor
CARs	Chimeric antigen receptors
CTL	Cytotoxic T-lymphocyte
CTLA-4	T lymphocyte antigen 4
CFSE	Carboxyfluorescein succinimidyl ester
CTCL	Cutaneous T-cell lymphoma
CLL	Chronic lymphocytic leukemia
CK2	Casein kinase II
cDCs	Conventional DCs
DCs	Dendritic cells
DMEM	Dulbecco's Modified Eagle Medium
DLBCLs	Diffuse large B-cell lymphomas
DCs	Dendritic cells
DOX	Doxorubicin
Erk1/2	Extracellular signal-regulated protein kinase

FOXP3	Forkhead box P3
FBS	Fetal bovine serum
i.p.	Intraperitoneal
i.v.	Intravenous
IFN	Interferon
IFN γ	Interferon gamma
IL-2	Interleukin-2
ICS	Intracellular cytokine staining
ICBs	Immune checkpoint blockers
Lag3	Lymphocyte-activation gene 3
MHC	Major histocompatibility complex
MDSC	Myeloid-derived suppressor cell
mDCs	Myeloid DCs
MCL	Hairy cell leukemia, mantle cell lymphoma
NK	Natural killer
NK-92	IL-2-dependent NK cell line
NHL	Chronic non-Hodgkin B cell lymphoma
PD-1	Programmed death 1
PD-L2	Programmed death ligand 1
PAMP	Pathogen-associated molecular pattern
PI	Propidium iodide
pDCs	Plasmacytoid DCs
p.t	Peritumoural
RNA	Ribonucleic acid

RPMI	Roswell Park Memorial Institute
SRCR	Scavenger receptor cysteine-rich
SLL	Small cell leukemia/lymphoma
SHP-1	Src homology-2 protein phosphatase-1
SD	Standard deviation
SCID	Severe combined immunodeficiency
TNF	Tumour necrosis factor
T _{regs}	T regulatory cells
Tigit	T cell immunoreceptor with Ig and ITIM domains
TLR	Toll-like receptor
TAA _s	Tumour-associated antigens
TCR _s	T cell receptors
T-ALL	T-cell acute lymphoblastic leukaemia
VEGF	Vascular endothelial growth factor
ZAP70	Zeta-chain-associated protein kinase 70

Chapter 1

1. Introduction

Part of this chapter has been submitted for consideration as a mini-review article titled “*CD5 as a Targetable Antigen in Solid and Non-Solid Tumours*” in the *European Journal of Immunology*.

1.1 Cancer immunity

Early evidence suggesting a role for the human immune system in response to, and treatment of tumours was reported in 1891 when William Coley, known today as the “Father of Immunotherapy”, injected live bacteria as an immune activator in treatment of cancer patients (1). He used streptococcal bacteria that causes a cellulitis infection of the superficial layer of skin known as erysipelas (St. Anthony’s Fire)(2). In response to that injection, some patients showed spontaneous remission from their cancers (1). Coley began injecting live and weakened bacteria, including *Streptococcus pyogenes* and *Serratia marcescens*, into the tumours of patients: the first immune-based treatments for cancer (3). Despite promising results including long-term survival in some (but certainly not all) cancer patients, Coley’s toxins were widely forgotten for over fifty years due to the risk of infecting cancer patients with potentially life-threatening bacteria, lack of clear understanding of the mechanism of action for these toxins, and the rise of radiation-based treatments (with comparatively clear and consistent results) at approximately the same time as Coley reported responses to bacterial injections. In the late 1950s, Burnet and Thomas published a series of papers

proposing a theory of cancer immunosurveillance (*i.e.*, that lymphocytes act as sentinels in recognizing and eliminating continuously arising, nascent transformed cells): a role for the immune system in defending against tumour initiation and growth (4, 5). However, the enthusiasm for developing immunotherapeutic drugs was, thereafter, slow due to limited understanding of immune mechanisms and the increase interest in chemotherapeutic approaches. Cancer immunosurveillance theory re-emerged in 1974 when more immune cell subsets and their function were discovered.

1.2 Cancer immunosurveillance

It is clear now that the interaction between the immune system of host organisms and cancers arising in, or transplanted into, those host organisms is complex. Several studies conducted using mouse models and human samples suggest a role for the immune system in cancer formation (6). The immune system not only carries out cancer immunosurveillance to eliminate cancer cells but also, paradoxically, promotes the growth of poorly immunogenic tumour cells that can escape immune surveillance (6, 7). Tumours are composed of a population of transformed cells that are morphologically, phenotypically, and genetically heterogeneous (8). Because immune cells rely on specific tumour cell antigens and susceptibility to innate and humoral immune events to induce death (described below), that heterogeneity renders tumour cells variably susceptible to immune recognition and attack (9). These immune cells become alerted to the stromal remodeling by tumour cells and production of proinflammatory cytokines and chemokines (10). This results in recruitment of immune cells such as

monocytes and natural killer T cells and cytotoxic T cells. The recruited tumour-infiltrating lymphocytes (TILs) and monocytes eliminate tumour cells by cytotoxic mechanisms such as production of interferon gamma (11), in addition to production of perforin (12), and pro-inflammatory cytokines such as tumour necrosis factor (TNF) (13) which modulate cells in the tumour and was shown to exert potent anti-tumour effect through inducing tumour cell cycle arrest, apoptosis and necroptosis (14). However, these cytotoxic mechanisms result in identification and killing of some, but not all, transformed cells that comprise tumour.

Tumour cells that escape the early stage of immunosurveillance can then be recognized by immune cells (T lymphocytes) that secrete IFN- γ to eliminate escaping tumour cells. Although many tumour cells that escaped immune-cell elimination are destroyed during this phase, some non-immunogenic tumour cells and cells that are well-recognized by immune cells and antibodies but are resistant to the mechanisms that induce death survive this stage (15). This stage can last for many years and can result in a heterogeneous parental population that is highly capable of escaping the immune system. There are several mechanisms, selected for tumour cells in this stage which undergoing continuous exposure to immune cells and immune cell products, contribute to increasing tumour cell immune resistance. These include production of tumour-driven soluble factors such as vascular endothelial growth factor (VEGF) and Fas and Fas ligand which are produced by many cell types including tumour cells (16, 17) and immune cells (18, 19) and can initiate T cell death and mediate immune

resistance (20). In addition, inhibitory signaling molecules such as PD-L1 (21) and CTLA-4 (22) and the presence of inhibitory immune cells such as myeloid-derived-suppressor cells (MDSCs) (23) and T regulatory (T_{reg}) cells (24) can suppress T cell effector function. Our understanding of cancer immunosurveillance has led to identification of mechanisms by which cancer cells can escape immune recognition and elimination (25) and therapeutic approaches to prevent tumour cell escape from immune-mediated killing. Those therapeutic approaches are described below.

1.3 Current and developing immunotherapy strategies

1.3.1 Current immunotherapy strategies

Tumours with abundant T cell infiltration (“inflamed” tumours), unlike “T-cell desert” tumours (characterized by the absence of immune cells) or “T-cell excluded” tumours (with peripheral invasion of T cells around tumours but poor infiltration into tumours), are a good prognostic biomarker (26). However, the presence of T cells within tumours is not sufficient, in itself, to lead to tumour regression or elimination. Tumour cells can have multiple characteristics that lead to lack of T cell activity, including high levels of immune checkpoint molecules that inhibit T cell activation. These molecules are expressed by several immune cells including T cells and, after binding to their ligands, can induce inhibitory signaling in immune cells (27). Although immune checkpoint molecules are necessary to ameliorate and “fine tune” T cell function, their activity also reduces their ability to recognize and eliminate tumour cells. Treatment with immune checkpoint blockers (ICBs) that target immune

checkpoint molecules can enhance T cell activation potential and are among the most successful immunotherapeutic agents in current clinical use (28). ICBs currently used therapeutically antibodies that target T cell immune checkpoint molecules and have exhibited good results in enhancing overall survival in cancer patients. The first ICB to show a clinical benefit was the anti-CTLA-4 antibody Ipilimumab in patients with metastatic melanoma (29, 30), non-small-cell lung cancer (31), small-cell lung cancer (32), renal cell carcinoma (33), and prostate cancer (34). Following anti-CTLA-4 blockade, further evidence showed clinical benefit in response to treatment with antibodies nivolumab and pembrolizumab (35, 36) that target programmed death-1 (PD-1), and antibodies durvalumab, atezolizumab and avelumab (37) targeting its ligand PD-L1 (38). The use of these monoclonal antibodies to target immune checkpoints has been tested and approved in different cancers including advanced melanoma (39, 40), non-small cell lung cancer (41), and Hodgkin lymphoma (42), improving overall survival in metastatic settings (43). However, these drugs benefit only a minority of patients (44). Additional studies are required and under way to investigate treatment of other tumour types with ICBs, and the capacity of combining blockers with other treatment modalities for additive and/or synergistic therapeutic effectiveness.

Another current immunotherapeutic approach is the use of adoptive T cell (ATC) therapy which was first proposed in 1966 after demonstration that half the patients with advanced cancer exhibited tumour suppression after patient-derived leukocyte infusion into those patients (45). This approach can either use

TILs (46) which can be obtained from excised tumours and expanded *in vitro* before infusion back into the patients, or by obtaining peripheral blood lymphocytes that can be engineered to express tumour-specific receptor prior to expansion and infusion back to patients (47). The use of TILs was first reported in the late 1980s when lymphocytes isolated from a tumour biopsy and then expanded *in vitro* in the presence of IL-2 to generate large numbers of tumour-reactive lymphocytes were then reinfused back to the same patient, with continued *in vivo* treatment with IL-2 to maintain TIL survival in the body. The response rate of this treatment was 34% with a median time of 4 months (48). Although this approach yielded promising results, there were challenges in recovering and expanding lymphocytes *in vitro*. Response to these challenges led to development of TCR-engineered lymphocytes and, later, synthetic chimeric antigen receptors (CARs) genetically engineered to recognize tumour-specific or tumour-associated antigens presented by MHC molecules on antigen-presenting cells, including tumour cells. T cell receptor-engineered T cells are prepared from lymphocytes isolated from cancer patients, genetically engineered to express chimeric antigen receptors (CARs), and then infused back to the same patients. CARs contain an antigen-binding domain associated with the signaling domain of the TCR and various co-stimulatory molecules such as CD28 and CD40 which increase T cell activity *in vivo* (49, 50).

Cancer vaccines are another immunotherapeutic approach used in the clinic to enhance the immune system and stimulate anticancer immunity. They can be either “preventable vaccines” (for example, to prevent infection by

oncogenic viruses; Gardasil, targeting human papillomavirus subtypes is one such preventable vaccine (51)) or “therapeutic vaccines” aimed at activating immune cells to eliminate tumour cells (52). Bacillus Calmette–Guérin is an example of a vaccine to prevent tuberculosis that can also be used as therapeutic vaccine for bladder cancer (53), and sipuleucel (Provenge), a vaccine composed of activated antigen-presenting dendritic cells, is approved for use in treatment of advanced metastatic prostate cancer (54). Other anti-cancer vaccines for use in combination with agents targeting immune checkpoints (anti-CTLA-4 antibodies, for example) have been explored in the preclinical setting to treat melanoma (55) but have not yet received FDA or Health Canada approval for standard treatment of human patients.

1.3.2 Developing immunotherapy strategies

Scientists are also investigating newly-discovered strategies to enhance anti-tumour immunity. Following the clinical success of blockade of immune checkpoint blockade molecules (PD-1 and CTLA-4), therapeutic targeting of additional negative regulators of T cell activation is under investigation: candidate targets for therapeutic suppression include the lymphocyte activation gene 3 (LAG3), T cell immunoglobulin 3 (TIM3) and T cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domains (TIGIT)(56). LAG3 is an inhibitory ligand that binds to CD4 and blocks its binding to MHC class II molecules (57). Similar to PD-1, LAG3 is a marker for T cell exhaustion and can induce cell cycle arrest (57). Although monotherapy with an anti-LAG3 drug has not succeeded in reaching its declared targets in clinical

trials, 50% of patients with metastatic breast cancer responded to combination of LAG3 blockade and paclitaxel (58). Another negative regulator of the T cell response is TIM3: unlike LAG3, it regulates apoptosis following galectin 9 binding (59). Increased levels of TIM3 correlated with poor prognosis in non-small-cell lung cancer, suggesting a role for it in cancer progression (60). Furthermore, increased TIM3 could mediate resistance to anti-PD-1 blockade (61), making it a candidate for combination with anti-PD1 therapy. Lastly, TIGIT, which inhibits T cell hyperactivation, is highly expressed in TILs compared to peripheral lymphocytes. Therapeutic targeting of TIGIT has shown promising results in enhancing the effect of pre-existing checkpoint blockade and reversing T cell exhaustion (62). So far, the blockade of immune checkpoints other than PD-1 and CTLA-4 has not shown promising results as single agents but rather as adjuvant cancer drugs to increase the effectiveness of other treatments (63).

Immune suppression by inhibitory immune cells such as MDSCs can contribute to escape of cancer cells from immune detection and elimination. It has become clear that some immune checkpoint inhibitor-resistant immune cells can successfully respond to immunotherapeutic approaches when combined with elimination of immune-suppressor cells (64). MDSCs are among the main immune-suppressor cells and have been shown to suppress anti-tumour T cell immunity through several mechanisms, including production of arginase, reactive oxygen species, IL-10, and sequestration of cystine which is an essential amino acid for T cell activation (65, 66). There are a broad range of strategies that can be used to reduce the function of MDSCs or reduce their number *in vivo*, and

which are in pre-clinical trials but have not yet received FDA or Health Canada approval. For example, treatment with some chemotherapeutic drugs reduces intratumoural and splenic MDSCs (67). *In vivo* administration of gemcitabine into immunologically-competent mice bearing syngeneic large tumour homografts derived from five cancer lines grown in both C57Bl/6 and BALB/c mice resulted in reduction in the total number of MDSCs in the spleen (68). This elimination reduced tumour growth after treatment with immunotherapy (68). The depletion of cells was specific to MDSCs as the number of T cell and B cells in spleen was not changed in these mice (68). The exact mechanism of this specificity is not well understood, but it is possible that because gemcitabine reduced tumour growth, it similarly reduced the number of tumour-induced MDSCs. Other chemotherapeutic drugs (doxorubicin (69) and 5-FU (70)) administered to syngeneic tumour-bearing mice resulted in a similar selective reduction in splenic and tumour MDSCs. This strategy and others such as use of a small-molecule inhibitor of CXCR1 and CXCR2 and the use of histone deacetylase inhibitors (71, 72) are under investigation to enhance understanding of MDSC function and assess the capacity of drug-induced MDSC cell reduction to treat cancer and enhance anti-tumour immunotherapy.

1.3.3 Increasing T-cell receptor affinity

Cytotoxic CD8⁺T cells recognize tumour-associated antigens (TAAs) through their T cell receptors (TCRs) which bind to small antigenic epitopes presented by the major histocompatibility complex (MHC) class I molecules on the surface of antigen-presenting cells (APCs) or tumour cells (73). The efficiency of triggering

T cell activation depends on the affinity of TAAs (bound to MHC Class I or Class II molecules) for the TCR, with TCR-MHC interaction stabilising the interaction: stronger interactions lead to superior T cell activation and high responsiveness to tumour antigens (74) (Figure 1). Induction of effective anti-tumour T cell immunity can be increased by enhancing TCR affinity and consequent ability to bind and recognize TAAs presented by MHC molecules. On the other hand, to avoid hyper-activation and pathological inflammation, T cells have the ability to reduce their antigen recognition and responsiveness through molecular mechanisms including interaction of the TCR with associated inhibitory molecules (75): Increased levels and activity of these molecules results in low anti-tumour T cell immunity, as they play a key role in TCR-mediated signaling (75). One example of these molecules is CD5, which can regulate TCR signaling (76). CD5 function in anti-tumour immunity is the focus of my thesis: current understanding of CD5 in solid and non-solid tumours is discussed below.

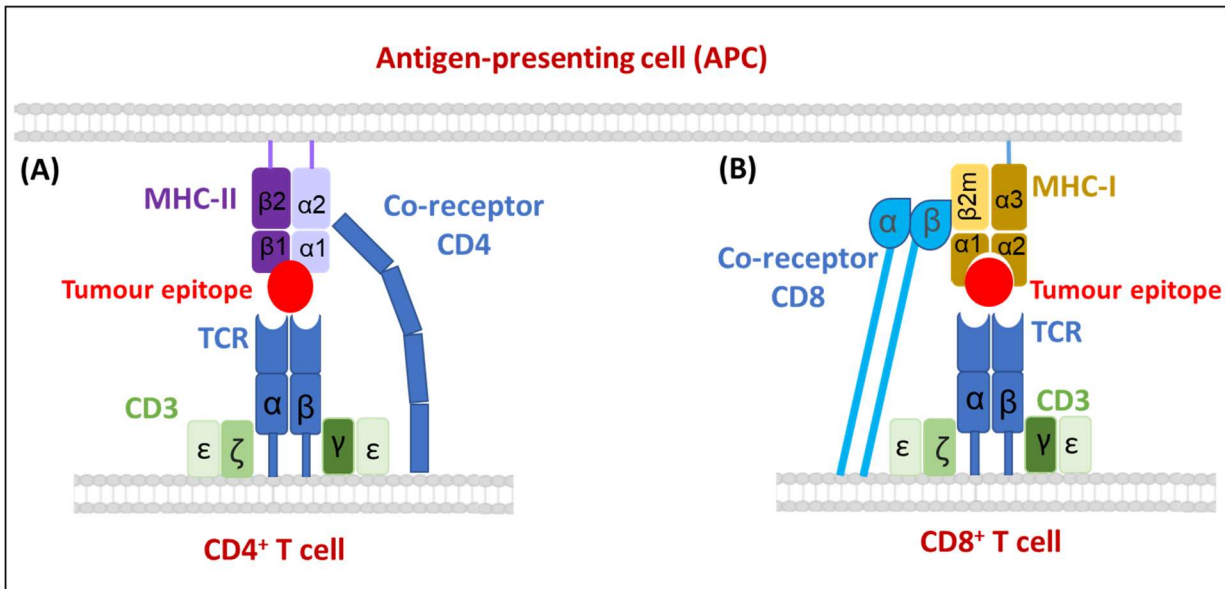


Figure 1. Antigen presenting cell interaction with CD4⁺T cell or CD8⁺ T cell.
(A) Structure of MHC-II binding to TCR on CD4⁺ T cells. **(B)** Structure of MHC-I binding to TCR on CD8⁺ T cells.

1.4 CD5

CD5 was first discovered in the late 1960s and was known as Lyt-1, Ly-A or Ly-1 (mouse), T1 or Leu-1 (human) (77) until its official name was assigned during the first International Workshop and Conference on Human Leukocyte Differentiation Antigens (HLDA) in the early 1980s (78). It was initially used as a cell surface marker for lymphocytes and to distinguish subpopulations of T and B cells (79, 80). It was not until the 1990s, after the first successful cloning of CD5 genes (81, 82) when more functional studies were performed to increase understanding of CD5 structure and function in T and B cells (83, 84) (Figure 2). CD5 is a 67 kDa type-I transmembrane glycoprotein and a member of the highly conserved group B scavenger receptor cysteine-rich (SRCR) protein superfamily (85). It contains three SRCR domains, protein modules of 90 -110 amino acids each (86). This superfamily includes more than 30 soluble or membrane-bound receptors including CD6, each with at least one SRCR extracellular domain in common, and some shown to regulate innate and adaptive responses (87) and to play a role in pathogen-associated molecular pattern (PAMP) recognition (88).

CD5 is associated with the antigen-specific receptor complex in both T and B cells (89-91) and is expressed on both $\alpha\beta$ and $\gamma\delta$ of T cells (92). It is detectable at early stage T cell development (the “double negative” when the TCR is present but CD8 and CD4 are not) and its expression increases during development from double negative to single positive ($CD4^+/CD8^-$ or $CD8^+/CD4^-$) stages (76). It has shown to fine-tune positive and negative selection and enhance the development of high affinity antigen binding (76, 89). The level of

CD5 has been linked to the strength of T cell receptor signaling (89). It has also been reported to be expressed on multiple subsets of immune cells such as monocytes (including macrophages (93)) and dendritic cells (94). It is increased in regulatory T and B cells (95, 96). Furthermore, T and B cells exposed to chronic stimulation are characterized by increased levels of CD5 (97, 98).

Ligands for CD5 were hard to identify due to the susceptibility of CD5 to denaturation and loss during experimental isolation. However, by using soluble CD5 purified at neutral pH to stabilize biological activity, CD5 has been shown to facilitate species-specific homophilic interactions and that CD5 is a ligand for itself (99). Further, the B cell surface protein CD72 in human or Lyb-2 in mice is a ligand for CD5 (100). Because CD5 is a member of scavenger receptors which play a role in PAMP recognition (101), CD5 can bind to exogenous ligands such as β -D-glucan (102) which is a polysaccharide found in the cell walls of yeast and mushrooms shown to promote immune response and is anti-tumorigenic (103, 104). Evaluation of the role of CD5 in the T-cell antigen receptor signaling pathway has been reviewed previously (105). Here, I review the potential therapeutic targeting of CD5 (both depleting or non-depleting approaches) in solid and non-solid tumours.

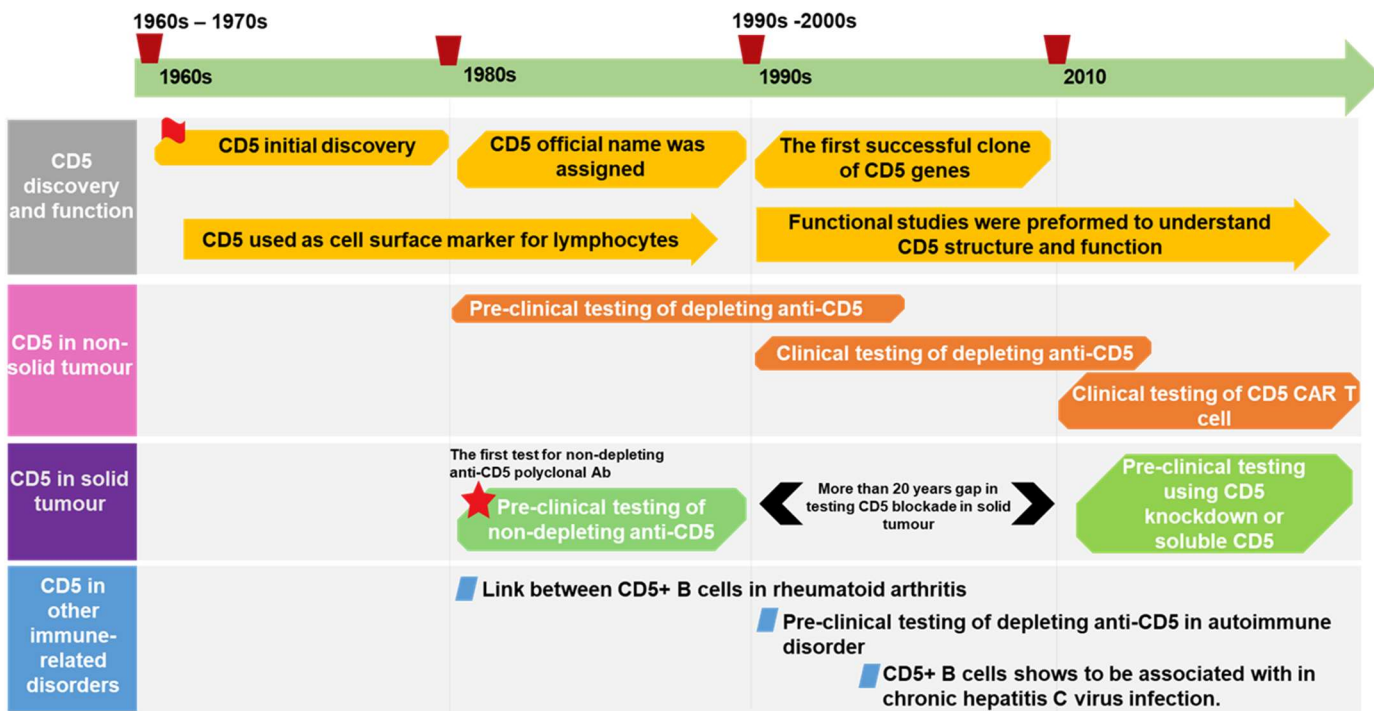


Figure 2. CD5 Timeline of its discovery and potential use as a therapeutic target in immune-related disorders.

1.5 CD5 in CD5⁺ non-solid tumours (T and B malignancies)

1.5.1 CD5 as diagnostic marker for T and B malignancies

Non-solid tumours (specifically, malignant lymphomas) are neoplasms that arise from B or T immune cells during various stages of development (106). Although the initial diagnosis of malignant lymphoma is made on the basis of cell morphology using histologic or cytologic tests (107), histologically similar malignant lymphomas are immunophenotypically and molecularly heterogeneous. Immunophenotyping of normal, non-transformed lymphocytes can be subdivided based on lineage markers expressed on their cell surfaces. For example, T cells express CD2, CD3, CD4, CD5, and CD8 while B cells express markers such as CD19, CD20 and CD22. In non-solid B and T cells tumours, lineage marker patterns may be similar (108). Hence, the majority of non-solid tumours are subdivided according to the pattern of marker expression.

CD5 is expressed on T cells and a subset of B cells (B1 cells)(109). CD5 has been used as characteristic surface marker of T and B cell malignancies. Nearly 70% of T-cell malignancies are CD5-positive, including T-cell acute lymphoblastic leukaemia (T-ALL) and T-lymphoma (110). CD5 has also been used as a useful marker to detect certain B-cell malignancies including B cell chronic lymphocytic leukemia (CLL), chronic non-Hodgkin B cell lymphoma (NHL), hairy cell leukemia, mantle cell lymphoma (MCL), and small cell leukemia/lymphoma (SLL)(111, 112). Approximately 6% of NHL are MCL (113) which are mostly CD5⁺, similar to their normal counterparts which are generated

from the mantle zone cells in lymphoid follicles. Furthermore, almost 10% of diffuse large B-cell lymphomas (DLBCLs) express CD5 (114). In contrast to CD5, other T-cell markers are rarely expressed in B-cell lymphomas, drawing attention to CD5 as a diagnostic marker for non-solid tumours.

1.5.2 CD5 as prognostic marker for T and B cell malignancies

CD5 on T and B malignancies vary in amount and is associated with clinical outcome and, sometimes, treatment resistance (115). High CD5 levels can be correlated with either a positive or negative prognosis, depending on the malignancy. Unlike T cell malignancy, CD5 is expressed by a smaller percentage of B cell malignancies. The level of CD5 varies among patients with B-CLL (116) and high CD5 is associated with better clinical outcome (117). CD5 acts as suppressor for B cell receptor (BCR) signaling (118) whereas, upon CD5 phosphorylation, Src homology-2 protein phosphatase-1 (SHP-1) is recruited to the cell membrane resulting in impaired BCR signaling. Because BCR signaling is critical for B-CLL tumour cell survival, increased CD5 is associated with better clinical outcomes and can be used as prognostic tool for B-CLL patients (119). In contrast, high CD5 is correlated with poor prognosis in patients with DLBCL (120), the most common subtype of NHL and highly invasive (121). 9.2% of DLBCL patients have tumours that express CD5, and this expression was not associated with age, sex, or clinical stages but rather with worse clinical outcomes (120). These lymphomas exhibit higher BCL-2 and increased recurrence in the central nervous system (122). In addition, CD5 has been proposed as an independent prognostic factor for DLBCL in elderly patients

(123). MCL is a disease consisting of several subtypes, most of which are CD5-positive as they are generated from mantle zone cells in lymphoid follicles (124). Mantle cell lymphoma (MCL) patients with a fraction of CD5⁺ cells less than 80% have decreased overall survival compared to those with a fraction higher than 80% (125). This indicates that favorable prognosis in high CD5⁺ in MCL is an indicator of a favourable prognosis.

1.5.3 CD5 as therapeutic target on T and B tumour cells

Several studies have validated therapeutic targeting of CD5 on T and B tumour cells as a therapeutic strategy. Anti-CD5 antibodies conjugated with various cytotoxic molecules (ADCs, or antibody-drug conjugates) have been used to treat non-solid CD5⁺ tumours. Anti-human CD5 monoclonal antibody (T101) conjugated to the chemotherapeutic drug doxorubicin (DOX) suppresses human T-cell leukaemia in athymic mice more effectively than T101 alone or DOX alone (126). Selective delivery of immunoconjugates consisting of monoclonal antibody T101 targeting CD5 linked to the potent toxin ricin A and labeled with radionuclides to the human T lymphoblastoid CEM and MOLT-4 tumour cell lines expressing CD5 suppressed tumour growth compared to control antibody that was minimally inhibitory (127). Killing of CD5⁺ non-solid tumours occurs after diffusion of the potent toxin ricin A through the cell membrane into the cytoplasm of the cell to inactivate ribosomes and inhibit protein synthesis to induce cell death (128). The use of radiolabeled antibody enhances tumour killing at a distance from the binding site and increases the killing directly by affecting DNA and plasma membrane and killing adjacent tumour cells relatively

insensitive to immunoconjugate within the heterogeneous population of transformed cells within the tumour (the “bystander” effect). In contrast, CAR-T cells exerted a better anti-tumour response against CD5⁺ tumour cells. In 2015, Mamonkin *et al.* transduced human T cells with chimeric antigen receptor targeting CD5 (CD5-CAR-T) to eliminate and kill malignant T cells lines as well as primary T-ALL blasts (129). CD5⁺ CAR-T cells were expanded *in vitro* and were able to target and eliminate CD5⁺ non-solid malignant T cells and to control disease progression in a xenograft mouse model (129). However, there was a lack of sustained effect as expansion of anti-CD5 CAR-T cells was transient due to cell self-killing (fratricide).

Anti-CD5 CAR-T cells have exerted better anti-tumour activity than anti-CD5 MAbs in directly targeting aggressive T-cell malignancies. However, because the majority of T cells express CD5, the treatment approach comes with some limitations due to fratricide in the engineered CAR-T cells expressing CD5 and the off-target toxicity from T-cell aplasia (130). T cell aplasia is a life-threatening condition resulting in immune system suppression, unlike CAR therapy in B cell malignancies which can be managed through intravenous infusion of immunoglobulins (131). This problem was resolved by switching to two other approaches: CD5-CRISPR-Cas9-edited T cells and CD5-negative NK cells (132). The first approach is use of genome-editing CRISPR-Cas9 to knock out CD5 in the human Jurkat T cell line. The CD5-edited CD5-CAR-modified Jurkat T cells exhibited reduced fratricide and self-activation which can result from interactions with self and neighboring CD5 antigens and enhanced

activation when co-cultured with target cells (132). The second approach involves use of CD5-negative NK cells engineered to express anti-CD5 CARs. An IL-2-dependent NK cell line (NK-92) expressing anti-CD5 CAR effectively targeted a CD5⁺ T cell leukemia cell line *in vivo* and *in vitro* (132). However, the lack of IL-2 led to low efficacy of the treatment which is essential to maintain NK-92 proliferation and function. The use of both approaches has potential to overcome fratricide in CAR-T cells and enhance the capacity of CAR-T cells to therapeutically target CD5 directly on non-solid immune cell malignancies. Therapeutic targeting of CD5 on non-tumour cells, on the other hand, has potential as a cancer immunotherapy and is described below.

1.6 CD5 blockade as a therapy to treat CD5⁻ solid tumours

The possible immunotherapeutic potential of anti-CD5 MAb in treatment of CD5⁻ solid tumours was first reported in the early 1980s (133). Passive administration of unconjugated/non-depleting anti-CD5 polyclonal antibody reduced tumour growth in mice harbouring non-solid tumour (EL-4 leukemia) and solid tumour (Lewis lung carcinoma) (133). This effect was abolished when mice were thymectomized, suggesting that the anti-tumour efficacy of anti-CD5 polyclonal antibody was not directly on the tumour cells but rather on CD5⁺ normal immune cells. Furthermore, genetic knockout of CD5 in mice enhances anti-tumour immunity and reduces homograft tumour growth without impairing mouse viability: syngeneic mouse B16 melanoma tumours have delayed growth in host CD5^{-/-} (knockout) mice (134). Transgenic mice with increased expression of soluble human CD5 (sCD5) displayed slower B16F10 tumour homograft

growth than control mice (135), a phenomenon associated with a reduced number of T_{regs} in lymph node and spleen and an increased number of natural killer cells in the spleen. Because soluble CD5 is capable of binding cell surface CD5 (99) the authors hypothesized that sCD5 acted to block CD5 from binding within the TCR/CD3 complex and impaired the capacity of cell surface CD5 to attenuate TCR signaling on T cells (135).

The correlation between CD5 level and anti-tumour immunity has been also reported in several human studies. TILs isolated from lung cancer patients have been reported to exhibit different anti-tumour activity based on CD5 expression, where CD5 levels were negatively correlated with anti-tumour activity (136). Increased tumour-mediated activation-induced death (AICD) has been reported in T cells with undetectable CD5 levels compared to CD5^{high} T cells, suggesting that CD5 could impair activation of anti-tumour T cells (137). This observation was associated with increased TIL activation as shown by increased activation markers such as CD25 and CD69 (134). However, the delay in tumour growth was transit due to increased AICD which can be reverted by blocking pathway that induced AICD such as Fas/FasL signaling pathway. Therefore, CD5 may negatively regulate T-cell responsiveness to tumour neoantigens and TAAs of low immunogenicity and if therapeutically reduced could lead to increased T cell recognition and killing of tumour cells expressing such antigenic molecules. As such, blocking CD5 signaling could lead to increased anti-tumour immunity and enhanced immune cell activation.

1.7 CD5 blockade-mediated immune cell activation

1.7.1 CD5 on T cells

CD5 has been shown to interact with TCR and impair its signaling pathway (89). Previous studies have shown that CD5 plays a role in signal transduction and participate in phosphorylation of intracellular substrates including protein kinase C (138, 139), and enhance intracellular Ca^{2+} concentration (140). CD5 has four tyrosine residues (Y378, Y429, Y411, and Y463) in its cytoplasmic domain. When tyrosine residues are phosphorylated they recruit several molecules and kinases away from the TCR complex, resulting in suppression of TCR signaling strength (141). Recruited and excluded molecules include casein kinase II (CK2), CBL, SHP-1, PI3K and zeta-chain-associated protein kinase 70 (ZAP70), which play roles in regulating the TCR signaling pathway (142). SHP1 inhibits Ca^{2+} and decrease activation of PLC gamma (142) while ZAP70 promotes Ca^{2+} signaling which activates ERK and recruits and activates other molecules such as PLC gamma (143).

The role of CD5 on the CD3/TCR signaling pathway has an impact on T cell function and survival. As previously reported, TILs with low CD5 levels have reduced survival: they undergo more AICD as a result of increased activation (137). Furthermore, T cells isolated from among TILs and peripheral blood lymphocytes from lung cancer patients have been reported to differ in their anti-tumour effect based on their CD5 levels: higher CD5 levels correlated with lower anti-tumour effect (136). Not only the presence or absence of CD5 but also its

level has an effect on T cell activation threshold (76). CD5 is detectable at very early stages of T cell development, and its level increases as T cells progress from hematopoietic precursors through to commitment to CD4⁺ or CD8⁺ expression (76). CD5 is upregulated by engagement of the antigen receptor in both immature and mature T cells. These observations support a previously study reporting that T cells with genetically-deleted CD5 are hyper-responsive to antigen stimulation, a potentially positive characteristic for anti-tumour immunity (141). Together, these data support the potential of blocking CD5 to increased anti-tumour immunity and enhanced immune cell activation.

1.7.2 CD5 on B cells

CD5 on B cells was first studied in B-CLL until it was shown that CD5 is also expressed in other subpopulations of B cells isolated from spleen, lymph nodes, and blood (144, 145). Further studies on B cell subpopulation revealed that B1 cells can be subdivided into two types based on CD5 level (CD5⁺ B1a cells and CD5⁻ B1b cells) (146). Similar to T cells, the physical correlation between CD5 and BCR suggests a potential functional role that can regulate B cell activation. It was reported that CD5 plays a negative role in BCR signaling: CD5-deficient B1 cells treated with anti-IgM antibodies show increased proliferation (147). Furthermore, CD5-deficient B1 cells showed decreased apoptosis and promoted NF κ B translocation to the nucleus (147). The link between CD5⁺ B cells and tumour immunity has been previously reported. CD5⁺ B cells lacking IL-6R α and isolated from within tumours was reported to bind IL-6 directly through CD5 (148). This interaction leads to activation of gp130 and Jak3 which results in

activation of STAT3 (148). Furthermore, CD5⁺ B cells but not CD5⁻ B cells promoted B16 melanoma growth, suggesting that CD5⁺ B cells might promote tumour progression (148). The same year, another study reported that IL-6-binding to CD5 in B cells promotes tumour growth via JAK-STAT signaling (149).

Most studies focus on CD5 as a marker for B malignancies and autoimmune diseases, which may not represent the normal physiological function of CD5 in untransformed B cells. Hence, the underlining mechanism through which CD5 regulates B cell survival and development is still not well understood. It has been shown that B cells expressing CD5 produced greater amounts of IL-10 compared to B cells lacking CD5 (150). Furthermore, BCR stimulation in B cells lacking CD5 are more susceptible to apoptosis compared to B cells expressing CD5 (151). These results suggest a regulatory role for CD5 in B cell survival. A further study investigated the role of CD5 as a regulator molecule for B cell survival using B-CLL cells as a model (152). In this study, Daudi B cells transfected with a CD5 expression vector had hyperphosphorylated tyrosine residues on CD5 suggesting that CD5 activation in B cells may enhance B cell survival in the context of B cell malignancy (152). The exact mechanism underling CD5-mediated B cell survival requires further investigation. Several studies reported potential involvement of specific signaling pathways: cross-linking of anti-CD5 in a subset of B-CLL cells induces PKC activation which resulted in induction of the Bcl-2 pro-survival family protein known as Mcl-1 (153). Another study supported a role for CD5 as an anti-apoptotic molecule in B-

CLL, and suggested that Lyn-mediated SHP-1 recruitment of CD5 could promote resistance to apoptosis (115).

In contrast to T cells, CD5 expressed in a subset of B cells exhibited a proapoptotic function. CD5-deficient B1 cell has been shown improved survival compared to wild type B1 cells, suggesting that CD5 may protect them from apoptosis. These data are difficult to interpret as CD5-deficient B1 cells represent a small fraction of all B cells and their function compared to B1a cells is not yet well understood (147). Furthermore, one study reported that CD5 on resting B cells could induce apoptosis, as opposed to T cells where it was not associated with apoptosis (154). In fact, treatment with anti-CD5 in B-CLL induced tumour apoptosis in some patients (155-157). These data suggest that CD5 function may have a dual function based on the type of cell; it may be proapoptotic in certain malignancies such as B-CLL but anti-apoptotic in normal B and T cells.

1.7.3 CD5 on dendritic cells

Dendritic cells (DCs) are a heterogeneous group of professional APCs comprised of conventional DCs (cDCs), including plasmacytoid DCs (pDCs) and CD11c myeloid DCs (mDCs)(158). The first study to report CD5-expressing DCs was published in 1992, showing that DCs isolated from human peripheral blood express CD5 (159). Later, human cDC2 isolated from lymph nodes, skin (Langerhans cells), and tonsils have been reported to be sub-dividable based on their CD5 level (160-162). Furthermore, in mice the majority of DC subtypes have been reported to express CD5 but to a lesser degree than T cells (159).

CD5 expression has not been reported to affect DC generation and maturation but rather to affect their function. The functional relevance of CD5 in DCs is not well understood: one published report indicated that CD5⁺ DCs isolated from blood represents a mature phenotype, however their function differ according to their CD5 expression (160). CD5^{high} DCs were able to induce proliferation of naïve T cells better than CD5^{low} DCs (160). Furthermore, CD5^{high} DCs induced increased generation of T_{regs} (*i.e.*, those expressing IL-10, IL-22, and IL-4) whereas CD5^{low} DCs were able to induce increased numbers of IFN γ -producing T cells (cytotoxic T cells)(160). It has been reported that CD5-deficient DCs produced higher levels of IL-12 compared to wild-type DCs when challenged with LPS (163). CD5-deficient DCs were able to induce anti-tumour immunity in mice (163). Furthermore, CD5-deficient DCs were capable of inducing higher activation of both CD4⁺ and CD8⁺ T cells as assessed by increased production of IL-2 and IFN- γ (163). These effects were abolished after restoring CD5 expression to the CD5-deficient DCs, suggesting that CD5 can reduce production of pro-inflammatory cytokines in DCs: that reduction can decrease their ability to activate T cells and induce strong adaptive immunity. The functional relevance of CD5 in DC, therefore, requires further investigation.

1.8 CD5 in clinical trials

Several phase I clinical trials have been conducting using an anti-CD5 monoclonal antibody. The first was to test the therapeutic effectiveness of a monoclonal mouse antibody (T101) directed against CD5 on human patients suffering from two types of leukemia: eight patients in total, four with cutaneous

T-cell lymphoma (CTCL) and four with chronic lymphocytic leukemia (164) and reported relative specificity and efficacy of monoclonal antibody therapy but the clinical benefit was limited. Another study reported testing of T101 in 13 patients [eight with CTCL and five with various other T cell malignancies] and reported complete remission in one patient with convoluted T cell lymphoma and partial remission in one patient with CTCL (165). These studies reported that higher doses of the antibody were associated with higher toxicity to the patients and the treatment outcome was limited due to antigen modulation as well as development of human anti-mouse antibodies resulting in neutralization of treatment. Further studies validated immunoconjugated T101 to enhance treatment outcome. A phase I clinical trial tested the use of radioimmunoconjugate 90Y-T101 in ten patients [eight with cutaneous T-cell lymphoma and two with chronic lymphocytic leukemia](166). All patients with cutaneous T-cell lymphoma developed human an anti-mouse response after finishing one cycle of the infusion and therefore did not receive any further infusion. Five of the patients, two with chronic lymphocytic leukemia and three with cutaneous T-cell lymphoma, showed partial responses and one patient with cutaneous T-cell lymphoma is in complete remission after 6 years (166). The use of monoclonal anti-CD5 immunoconjugate with ricin A chain toxin shows some limitation in patients due to development of human anti-mouse antibodies (167). Some durable limiting toxicities to kidney and liver due to non-specific uptake of the toxin conjugate were observed but were not fatal (167). These clinical trials show the need for further research into passive therapy and suggest that the use

of CAR-T cells may be of more clinical value. Work by Mamonkin *et al.* (129), showed the efficacy of CD5⁺ CAR T cells in targeting CD5⁺ malignant T cells and led to initiation of a phase I clinical trial (NCT03081910) in 2017 with an estimated primary completion date of 2021. This clinical trial has been recently published a promising result and demonstrate that CD5 CAR T cells are safe when administrated in five patients with relapsed or refractory (r/r) non-Hodgkin T cell lymphoma (T-NHL) and induce clinical responses without inducing complete T-cell aplasia (168). Furthermore, genetic variants of CD5 may affect tumour development and could be used as prognostic markers in patients with certain tumours. Two studies have shown the effect of two *CD5* single nucleotide polymorphisms SNPs (rs2241002 and rs2229177) in solid and non-solid tumours (169, 170). A study has investigated two cohort of patients with melanoma and revealed increased survival in patients with ancestral haplotype (169). Another study has reported chronic lymphocytic leukemia patients carrying ancestral P224-A471 haplotype showed increased progression-free survival (170)(Table 1).

Table 1: Clinical trials for CD5 in solid and non-solid tumours

Cancer type	Treatment approach	Phase	Year initiated	Publication
<ul style="list-style-type: none"> Chronic lymphocytic leukemia Cutaneous T-cell lymphoma 	Monoclonal antibody to human T-cell (T101)	Phase I	1984	(164)
<ul style="list-style-type: none"> Cutaneous T cell lymphomas (CTCL) Various other T cell malignancies 	Monoclonal antibody to human T-cell (T101)	Phase I	1986	(165)
<ul style="list-style-type: none"> Cutaneous T-cell lymphoma Chronic lymphocytic leukemia 	Radioimmunoconjugate 90Y-T101	Phase I	1998	(166)
<ul style="list-style-type: none"> Cutaneous T-cell lymphoma 	H65-RTA immunoconjugate	Phase I	1991	(167)
<ul style="list-style-type: none"> Melanoma 	CD5 single nucleotide polymorphisms SNPs rs2241002 and rs2229177	Prognostic study	2016	(169)
<ul style="list-style-type: none"> Chronic lymphocytic leukaemia 	CD5 single nucleotide polymorphisms SNPs rs2241002 and rs2229177	Prognostic study	2017	(170)
<ul style="list-style-type: none"> T-cell Acute Lymphoma T-non-Hodgkin Lymphoma T-cell Acute Leukemia 	CD5-CAR T cells	Phase I	2017	[NCT03081910]

1.9 Mouse models to study cancer immunotherapy drugs

The validation of immunotherapeutic drugs efficacy requires the availability of reliable mouse models that represent the complexity of immune cells interaction within the tumour. Several mouse models have been developed to test different aspect of cancer. Herein, I briefly describe the most widely-used mouse models employed to assess cancer immunotherapy.

1.9.1 Humanized tumour models

Human xenograft models are among the oldest models used to examine the efficacy of cytotoxic drugs to treat cancer by inoculating immunocompromised mice such as athymic nude or severe combined immunodeficiency (SCID) animals with human tumour cell lines (171). SCID mice are deficient in a DNA-dependent protein kinase required for T- and B-cell development therefore result in lack in normal thymic development and deficient in T-cell function. Although this model lacks T-cell function, innate immune cells such as neutrophils and dendritic cells remain present and limit the engraftment of human hematopoietic tumour cell lines. Therefore, the use of this model is limited in testing cancer immunotherapy drugs.

1.9.2 Syngeneic tumour models

These models are among the first preclinical models used to test anti-cancer agents. Fully immunocompetent mice (inbred strains such as C57BL/6 and BALB/c) have been used to validate immuno-oncology agents without the need for adoptive transfer of immune cells into host mice. In these models, mouse tumour cell lines are expanded *in vitro* and then injected into the host to

grow tumours either subcutaneously or orthotopically (*i.e.*, at the organ site specific to the tumour type). The use of syngeneic tumour cell lines is relatively easy compared to other models as it can be rapidly expanded prior to implantation into the host and are relatively reproducible in different research contexts. Another logistical advantage of using this model is the flexibility in selection of model tumour cell lines based on criteria such as poor or good immunogenicity, and relative ease of genetic manipulation of tumourigenic cell lines prior to implantation (as opposed genetic manipulation of mice to create tumour-prone animal lines) to induce expression of antigens of interest to examine antigen-specific anti-tumour responses.

1.9.2.1 The murine 4T1 breast tumour cell line

The 4T1 syngeneic mouse tumour cell was first isolated as subpopulation 410.4 from a mammary tumour arising in BALB/cfC3H mice (172). This is a poorly immunogenic tumour model that can metastasize to multiple distant sites such as lymph nodes, liver, lung and brain (173). It can grow progressively causing lethal disease even after tumour excision (174). There is several characteristics that makes 4T1 tumour a suitable experimental animal model to validate targeting CD5 in T cells. First, it recapitulates the triple-negative breast cancer in human as it metastasizes to the draining lymph nodes and other organs (174). Second, it can be easily transplanted into mammary gland as an orthotopic tumour. Also, 4T1 tumour cell expresses poorly immunogenic tumour antigens which makes it a suitable model to examine the ability of CD8⁺ T cells to recognize tumour antigen after CD5 blockade.

1.10 Hypothesis and objectives:

Hypothesis: Functional blockade of CD5 on the surface of primary mouse cytotoxic T cells will enhance recognition of poorly immunogenic tumour antigens and promote T cell activation (Figure 3).

Objectives:

- **Objective 1.** Characterize CD5 levels in T cell subsets and under different activation conditions (quiescence and induced activation).
- **Objective 2.** Explore the association of CD5 with T cell activation and exhaustion.
- **Objective 3.** Target CD5 receptor using CD5-blocking antibodies *ex vivo*, in primary CD8⁺ T cells isolated from mouse spleen, and assess CD8⁺ T cell activation and function.
- **Objective 4.** Evaluate the anti-tumour efficacy of anti-CD5 as single treatment *in vivo* and evaluate T cell function and activation in T cell subsets derived from spleen, lymph nodes, and tumours.
- **Objective 5:** Evaluate the anti-tumour efficacy of anti-CD5 antibody in combination with immune checkpoint inhibitors *in vivo*.

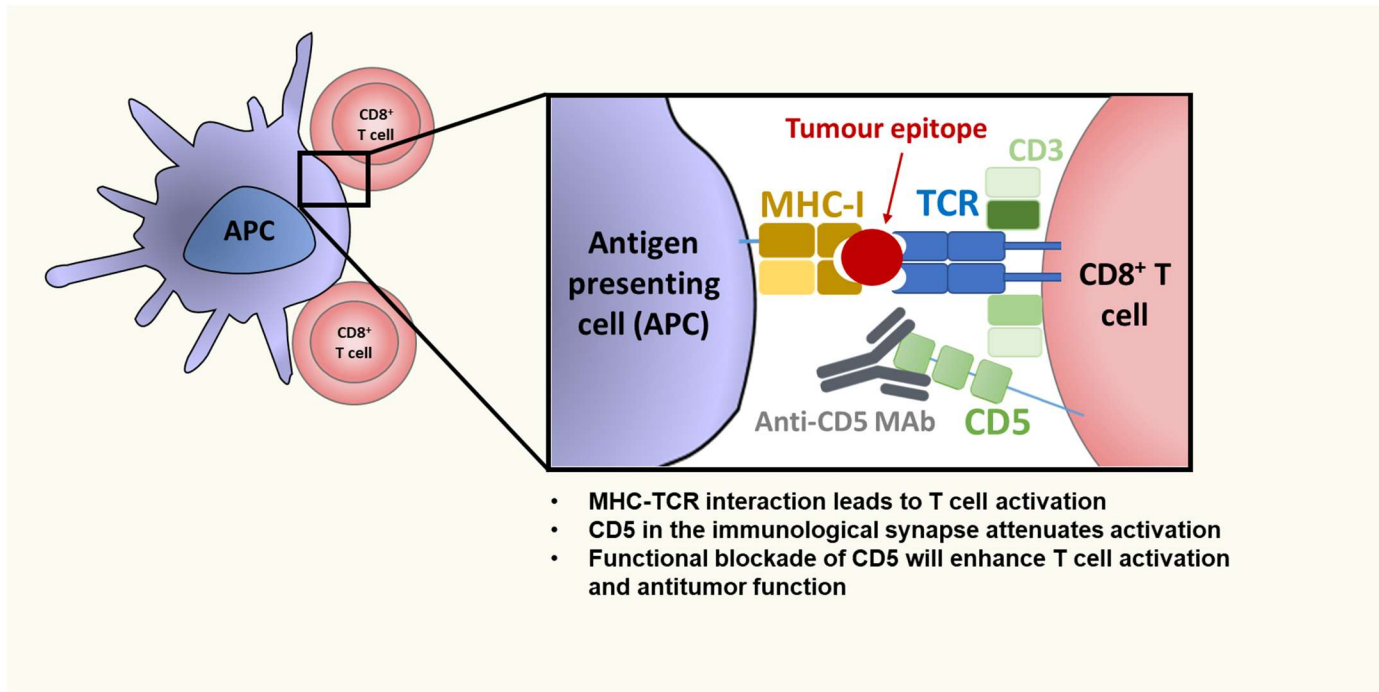


Figure 3: CD5 blocking and its possible effect during immunological synapse. In the immunological synapse in normal T lymphocytes, CD5 physically interacts with TCR, limiting TCR function and TAA recognition. When CD5 is targeted using anti-CD5 MAb, TCR affinity is increased which leads to enhanced T cell activation. This figure was generated by me and was selected for the “In This Issue” section in the *European Journal of Immunology* (vol. 50, no. 5). It can be accessed at <https://onlinelibrary.wiley.com/doi/10.1002/eji.202070053>

Chapter 2

1 Materials and methods

2.1 Table 2: Key resources

REAGENT or RESOURCE	SOURCE	CLONE	IDENTIFIER
<i>Antibodies (For in vivo administration)</i>			
Anti-CD5 Mouse IgG2a, Fc Silent™, Kappa	Absolute Antibody	YTS 121.5.2	Ab00208-2.3
<i>InVivoMAb</i> anti-mouse PD-1 (CD279), IgG2a	Bio X cell	RMP1- 14	BE0146
Anti-Fluorescein Mouse IgG2a, Fc Silent™, Kappa (isotype control)	Absolute Antibody	4-4-20	Ab00102-2.3
<i>Antibodies (For in vitro administration)</i>			
Purified NA/LE Hamster Anti-Mouse CD3e	BD Biosciences	145- 2C11	553057
Ultra-LEAF™ Purified anti-mouse CD3e	BioLegend	145- 2C11	100340

Purified NA/LE Hamster Anti-Mouse CD28	BD Biosciences	37.51	553294
Ultra-LEAF™ Purified anti-mouse CD28	BioLegend	37.51	102116
Purified anti-mouse CD5	BioLegend	53-7.3	100602
Purified Rat IgG2a, κ Isotype Ctrl Antibody	Biolegend	RTK275 8	400502
Purified Rat IgG2a, κ Isotype Ctrl Antibody	BD Biosciences	R35-95	553927
<i>Antibodies (For flow cytometry)</i>			
Brilliant Violet 711™ anti-mouse CD3	BioLegend	17A2	100241
Alexa Fluor® 700 anti-mouse CD4	BioLegend	GK1.5	100430
PE/Cyanine7 anti-mouse CD5	BioLegend	53-7.3	100622
FITC Rat Anti-Mouse CD5	BD Biosciences	53-7.3	553020
PerCP/Cyanine5.5 anti-mouse	BioLegend	53-6.7	100734

CD8a			
PE-Cy™7 Rat Anti-Mouse CD8a	BD Biosciences	53-6.7	552877
Brilliant Violet 605™ anti-mouse CD25	BioLegend	PC61	102036
PE Hamster Anti-Mouse CD69	BD Biosciences	H1.2F3	561932
PE anti-mouse CD95 (Fas) Antibody	BioLegend	SA367H 8	152608
Brilliant Violet 421™ anti-mouse CD107a (LAMP-1)	BioLegend	1D4B	121618
PE anti-mouse CD137	BioLegend	17B5	106106
PE Hamster Anti-Mouse CD152 (CTLA-4)	BD Biosciences	UC10- 4F10-11	561718
PE anti-mouse CD152 (CTLA-4)	BioLegend	UC10- 4B9	106306
PE anti-mouse CD178 (FasL)	BioLegend	MFL3	106606
PE/Dazzle™ 594 anti-mouse	BioLegend	29F.1A1	135228

CD279 (PD-1)		2	
PE/Dazzle™ 594 anti-mouse/human CD11b	BioLegend	M1/70	101256
APC anti-mouse Ly-6G/Ly-6C (Gr-1)	BioLegend	RB6-8C5	108412
PE anti-mouse IFN-γ	BioLegend	XMG1.2	505808
Purified anti-mouse CD16/32 (For Fc block)	BioLegend	93	101302
Alexa Fluor® 488 anti-mouse/rat/human FOXP3	BioLegend	150D	320012
<i>Isotype antibodies (for flow cytometry)</i>			
FITC Rat IgG2a, κ Isotype Control Antibody	BD Biosciences	R35-95	553929
PE Armenian Hamster IgG Isotype Control Antibody	BioLegend	HTK888	400908
PE-Cy™7 Rat IgG2a, κ Isotype Control Antibody	BD Biosciences	R35-95	552784
PE-CF594 Hamster IgG2, λ1	BD	Ha4/8	562522

Isotype Control	Biosciences		
FITC Mouse IgM, κ Isotype Control	BD Biosciences	G155-228	553474
<i>Antibodies (For western blot)</i>			
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody	Cell Signal	Polyclonal	9101
p44/42 MAPK (Erk1/2) Antibody	Cell Signal	Polyclonal	9102
Anti-actin Antibody	Sigma-Aldrich	MM2/193	SAB4200248
Anti-mouse horse radish peroxidase antibody conjugate	Cell Signaling		7076S
Anti-rabbit horse radish peroxidase antibody conjugate	Cell Signaling		7074S
<i>Recombinant Proteins and Media for Cell Culture</i>			
Roswell Park Memorial Institute (RPMI)-1640 Medium	Sigma-Aldrich	R8758-500ML	
Dulbecco's Modified Eagle's	Sigma-	D5796	

Medium (DMEM)	Aldrich	
Fetal Bovine Serum (FBS)	Invitrogen	12483020
Recombinant Human IL-2	Peprotech	200-02
<i>Other reagents</i>		
Propidium iodide	BioLegend	421301
FITC Annexin V	BioLegend	640906
Annexin V Binding Buffer	BioLegend	422201
Zombie NIR™ Fixable Viability Kit	BioLegend	423106
MojoSort™ Magnet	BioLegend	480019
MojoSort™ Buffer (5X)	BioLegend	480017
MojoSort™ Mouse CD8 T Cell Isolation Kit	BioLegend	480008
Fixation Buffer	BioLegend	420801
Brefeldin A Solution (1,000X)	BioLegend	420601
Intracellular Staining Permeabilization Wash Buffer (10X)	BioLegend	421002

Female BALB/c mice	The Jackson Laboratories	000651
4T1 mouse breast tumour cells	The American Type Culture Collection	(ATCC® CRL-2539™)
Bradford Assay Kit	abcam	(ab102535)
Collagenase A	Sigma-Aldrich	C2674
CFSE Cell Division Tracker Kit	BioLegend	423801
Western Lightning Plus-ECL, Enhanced Chemiluminescence Substrate	Perkin Elmer, Inc Products	NEL103E001EA
<i>Apparatus</i>		
Incucyte® SX5 Live-Cell Analysis System	Essen BioScience	n/a
Cytomics FC 500	Beckman Coulter, Inc	n/a

BD™ LSR II Flow Cytometer	BD Biosciences	n/a
Beckman Coulter Z1 Particle Counter	Beckman Coulter, Inc	n/a
Vibra Cell™ ultrasonic processor	Sonics & Materials Inc., Danbury, CT	n/a
Semi-dry transfer apparatus	Bio-Rad	n/a
Software		
Prism, version 8	Graphpad	https://www.graphpad.com/
Flowjo, version 10	FlowJo, LLC	https://www.flowjo.com/

2.2 Experimental model and subject details

2.2.1 Mice

Female BALB/c mice were purchased from The Jackson Laboratories (Jackson Laboratories, Bar Harbor, ME). All animals were between 8 and 12 weeks of age and housed in the Animal Care and Veterinary Services Facility at the Victoria Research Building, Lawson Health Research Institute, according to

guidelines of the Canadian Council for Animal Care and under the supervision of the Animal Use Subcommittee of the University of Western Ontario.

2.2.2 Cell lines

4T1 mouse breast mouse tumour cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (FBS)(Invitrogen). All cells were kept at 37°C in 5% CO₂.

2.2.3 Syngeneic mouse tumour model

4T1 tumour cells were counted by Coulter counter and resuspended into sterile PBS at a concentration of 5×10^5 cells/ml. One hundred microliters of cells (5×10^4) were injected subcutaneously into the right flanks of 2-month-old female BALB/c mice and allowed to grow until 21 days before animals were euthanized by CO₂ inhalation for immune cell assessment. For tumour growth assessment, the tumour growth was measured by caliper and the largest length and width dimensions were used to calculate tumour volumes and mice were euthanized once tumour size reach endpoint at 1500 mm³.

2.2.4 *In vivo* drug treatment

Four different treatment protocols were used to study the effect of CD5 blockade on tumour suppression *in vivo*.

First treatment design

This experiment was design to test the impact of anti-CD5 MAb to reduce tumour growth as a single agent and in combination with anti-PD-1 MAb. Mice were injected subcutaneously with 4T1 tumour cells and as soon as tumours reached 50 mm³ (day 7), mice were divided into 4 different groups and were administered one of the following treatments on days 7, 10, 13, and 16: isotype control MAb alone, anti-CD5 MAb alone, anti-PD-1 MAb alone, or a combination of both anti-CD5 + anti-PD-1 MAbs. Mice received their treatments by i.p injection of 200 µg of each agent/mouse (Figure 4).

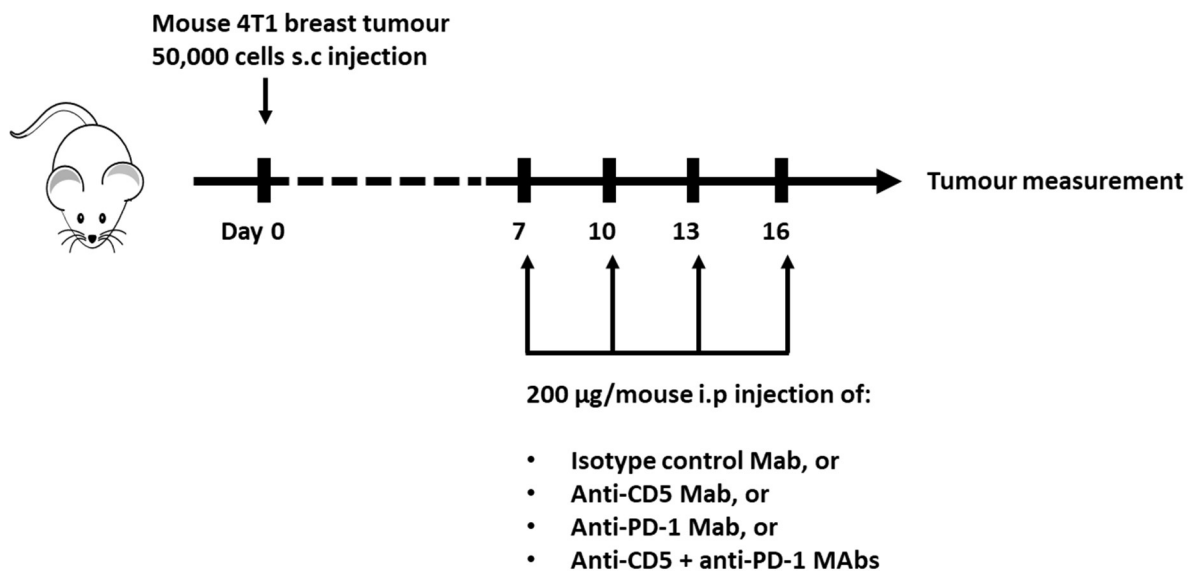


Figure 4. Scheme of the first *in vivo* treatment design

Second treatment design

This experiment was designed to assess the less dosage of anti-CD5 MAb in reducing tumour growth as a single agent or in combination with anti-PD-1 MAb. Mice were injected subcutaneously with 4T1 tumour and cells as soon as tumours reached 50 mm³ (day 7), mice were divided into 4 different groups and received one of the following treatments: isotype control MAb alone (200 µg) (day 7), anti-CD5 MAb alone (200 µg) (day 7), anti-PD-1 MAb alone (200 µg) (days 10, 13, and 16), or a combination of anti-CD5 MAb (day 7) + anti-PD-1 MAb (days 10, 13, and 16) (Figure 5).

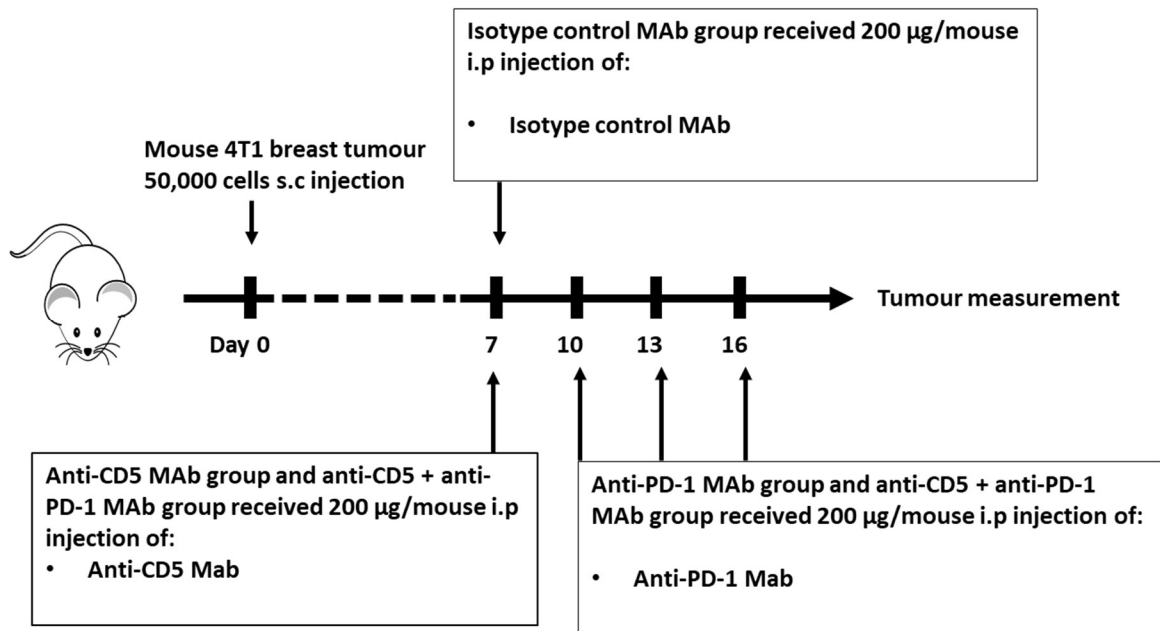


Figure 5. Scheme of the second *in vivo* treatment design

Third treatment design

This experiment is design to assess whether anti-CD5 can enhance efficacy of chemotherapy gemcitabine to suppress tumour growth. Mice were injected subcutaneously with 4T1 tumour cells and as soon as tumours reached 50 mm³ (day 7), mice were divided into 4 different groups and received one of the following treatments: PBS (day 7 and 10), gemcitabine alone (day 7), anti-CD5 MAb alone (day 10), or a combination of gemcitabine (day 7) + anti-CD5 MAb (day 10). Mice received only one treatment of 100 µg anti-CD5 MAb/mouse one treatment of 60 mg/kg gemcitabine (Figure 6).

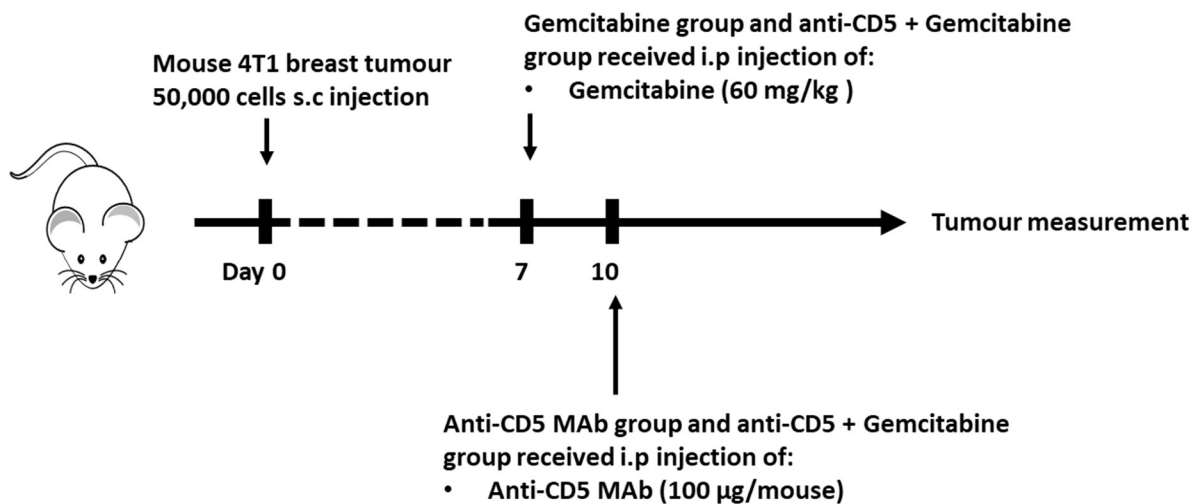


Figure 6. Scheme of the third *in vivo* treatment design

Fourth experiment design

This experiment is design to assess whether anti-CD5 can enhance efficacy of chemotherapy 5-FU to suppress tumour growth. Mice were injected subcutaneously with 4T1 tumour cells and as soon as tumours reached 50 mm³ (day 7), mice were divided into 4 different groups and received one of the following treatments: PBS (day 7, 10 and 14), anti-CD5 MAb alone (days 7, 10, and 14), 5-FU alone (day 7), or both anti-CD5 MAb (days 7, 10, and 14) + 5-FU (day 7). The 5-FU dose was 50 mg/kg and the anti-CD5 MAb doses were 50 µg/mouse each (Figure 7).

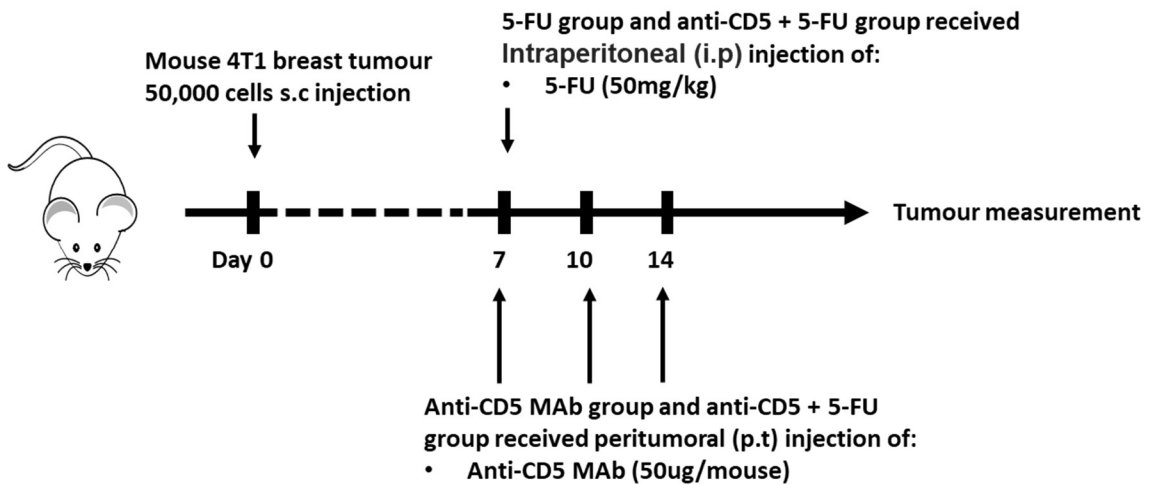


Figure 7. Scheme of the fourth *in vivo* treatment design

Fifth experiment design

This experiment is designed to assess whether anti-CD5 MAb has the capacity to suppress tumour growth when administered at lower dosage, and in treatment of a smaller number of injected tumour cells, than described in the "Fourth *in vivo* treatment design". Mice were injected subcutaneously with 5000 4T1 tumour cells and, as soon as tumours reached 50 mm³ (day 7), mice were divided into two different groups and received one of the following treatments: isotype control on day 0 and every three to four days of a total of 11 injections and anti-CD5 MAb alone (on day 0 and every three to four days of a total of 11 injections). The isotype control and the anti-CD5 MAb doses were 25 µg/mouse each (Figure 8).

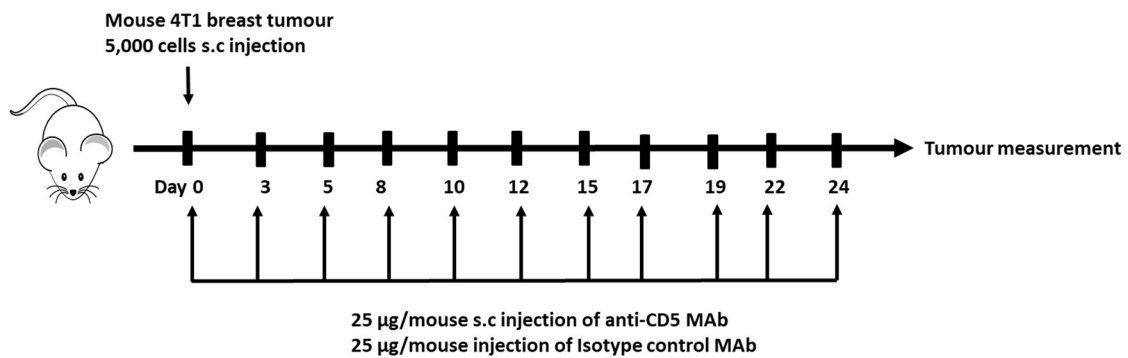


Figure 8. Scheme of the fifth *in vivo* treatment design.

2.3 Methods

2.3.1 Splenocyte and lymphocyte preparation

Single cell suspensions of lymphocytes were obtained from mice by pressing spleens or lymph nodes through a 70 µm Falcon Cell Strainer (VWR, Mississauga, ON) into RPMI 1640 medium (GIBCO). Cells were then centrifuged (300xg, 10 mins, 4°C), and erythrocytes were lysed using Ammonium-Chloride-Potassium (ACK) red cell lysis buffer. The resulting live (trypan blue-negative) splenocytes and lymphocytes were counted manually (microscope slide) and stained directly or cultured for further assessment.

2.3.2 Tumour-infiltrating lymphocyte (TIL) preparation

TILs were obtained from freshly-resected tumours, which were isolated immediately after euthanization of mice. Tumours were cut into 2-3 mm³ fragments and each tumour was placed into individual wells of a 6 well plates and incubated in 2 ml of an enzyme digest mix consisting of RPMI1640 complete media containing 15% fetal bovine serum (FBS)(Invitrogen) and 10 mg/ml collagenase A (Sigma-Aldrich Canada, Oakville, ONT) and incubated for 2 hours at room temperature under continuous rotation.

2.3.3 4T1 mouse breast tumour lysate preparation

4T1 tumour lysate was prepared by detaching confluent cultures of 4T1 tumour cells with 0.01% EDTA for 10 min, washing the cells twice with PBS, resuspending cells in serum-free medium (5X10⁶ cells/ml), and lysis by 5 freeze/thaw cycles (-80 to 37°C) accomplished by repetitive 5 min on dry ice followed by 5 min in a water bath at 37°C. To quantify lysate loading, lysate

protein concentrations were then determined using a Bradford assay following the manufacturer's recommended protocol.

2.3.4 Western immunoblotting

Splenocytes were stimulated for 30 min with 2 µg/ml anti-CD3 MAb (Clone: 145-2C11. BD Biosciences) or 2 µg/ml anti-CD3 + 5 µg/ml anti-CD5 MAbs (Clone: 53-7.3. BioLegend) to induce activation of T lymphocytes. After stimulation, cells were washed twice with cold PBS and lysed with RIPA lysis buffer (10 mM Tris pH 7.2, 2mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton 100), supplemented with protease inhibitors, including 10 µg/ml aprotinin, 10 µg/ml leupeptin, 100 µM sodium orthovanadate, 100 µM phenylmethylsulfonyl fluoride, and sonicated at 4°C using a Vibra Cell™ ultrasonic processor (Sonics & Materials Inc., Danbury, CT) to disrupt membranes. Lysates were centrifuged at 13,000 × g for 15 min at 4°C. Protein concentration was measured using the Bradford assay. For SDS PAGE, 30 µg protein per lane was electrophoresed on a 7% polyacrylamide gel and proteins were then transferred to a nitrocellulose membrane using a semi-dry transfer apparatus (Bio-Rad) and blocked with 5% skim milk in TBST for 1 hour. Membranes were incubated overnight with the primary antibody at 4°C. After washing three times in TBST, membranes were incubated with the appropriate secondary antibody-horse radish peroxidase conjugate for 1 hour at 21° C and proteins were then detected by using enhanced chemiluminescence reagents (Western Lightning Plus – ECL [Perkin Elmer, Inc Products]). Primary antibodies were used to detect anti-phospho-ERK, (Cell Signaling Technology/New England

Biolabs, Whitby, ON) and anti-actin (Sigma Aldrich Canada, Oakville, ON). Anti-mouse or anti-rabbit horse radish peroxidase antibody conjugates were purchased from Cell Signaling.

2.3.5 *In vitro* activation

Single cell suspensions of splenocytes were generated from naïve mice using the protocol described in section 2.3.1 and in accord with MIATA guidelines. On day 0, cells (2×10^5) were treated with or without purified anti-mouse anti-CD5 MAb at 5 µg/ml (Clone: 53-7.3. BioLegend) for 15 min before seeding into U-bottom 96-well plates pre-coated with 5 µg/ml anti-CD3 antibody, in RPMI media containing 10% FBS, IL-2 (50 IU/ml)(PeproTech Canada Inc), and soluble anti-CD28 antibody (Clone: 37.51. BD Biosciences, 2 µg/ml). Media was replaced every 48 hours with a fresh RPMI media containing 10% FBS, IL-2 (50 IU/ml) were kept at 37°C in 5% CO₂ for the period of experiment.

2.3.6 *Ex vivo* activation

Single cell suspensions of splenocytes were generated from 4T1-harboured mice using the protocol described in section 2.3.1 and in accord with MIATA guidelines. Cells (2×10^5) were reactivated by addition of 4T1 tumour cell lysate (200 µg/well) in a 96-well plate treated with or without purified anti-mouse anti-CD5 MAb at 5 µg/ml (Clone: 53-7.3. BioLegend), in RPMI media containing 10% FBS and IL-2 (50 IU/ml)(PeproTech Canada Inc). Cultured splenocytes, activated and unactivated, were kept at 37°C in 5% CO₂ and the media refreshed every 48 hours.

2.3.7 T cell apoptosis

On day 7 of the *in vitro* and *ex vivo* activation, apoptotic and dead cell fractions were determined using a FITC-labeled Annexin-V and propidium iodide (PI) kit (Biolegend, San Diego, CA) according to the manufacturer's protocol. Briefly, cells were stained with anti-mouse PE-Cy7-labeled anti-CD8 MAb at 4°C for 30 min, washed twice with cold FACS buffer, and resuspended in Annexin V staining buffer (Biolegend, San Diego, CA). FITC-Annexin V (1 µg/ml) and PI (10 µg/ml) were added to the cell suspension and incubated at 21°C for 15 min in the dark. Annexin V staining buffer (400 µl) was added and cells were analyzed using a Cytomics FC 500 (Beckman Coulter, Inc).

2.3.8 T cell proliferation

For CFSE labelling, isolated splenocytes were stained with CFSE (1 µg/ml; Biolegend, San Diego, CA) for 15 min at 37°C and washed twice with FBS-containing buffer to stop the reaction. Cells were then cultured as described in section 2.3.5 and 2.3.6 for 7 days at 37°C in 5% CO₂, followed by flow cytometry using a Cytomics FC 500 flow cytometer (Beckman Coulter, Inc). Data were analyzed using Flowjo software (BD Bioscience).

2.3.9 Flow cytometry

To assess the levels of surface protein expression, the antibodies described in Table 2 (Key Resources) were used for flow cytometry. Flow cytometry was performed using a Cytomics FC 500 (Beckman Coulter, Inc.) and

BD™ LSR II Flow Cytometer and data analyzed using Flowjo software (BD Bioscience). To assess the level of the indicated markers, spleens, lymph nodes and TILs were collected from either tumour-naïve mice or tumour-bearing mice 21 days after tumour cell injection. Cells were prepared as previously described, and 2×10^5 cells were treated with purified anti-mouse CD16/32 antibody (Clone 93)(Biolegend, San Diego, CA) for 15 min at 21°C in the dark to block CD16/CD32 interactions with the Fc domain of immunoglobulins. Cells were then stained with appropriate antibodies for 25 mins on ice in the dark, washed twice with FACS staining buffer, suspended in 0.5 ml FACS staining buffer, and analyzed by flow cytometry. The European Journal of Immunology Guidelines for the use of flow cytometry and cell sorting in immunological studies (175) were followed.

2.3.10 Intracellular cytokine staining (ICS)

To measure IFN γ in CD8⁺ T cells, ICS was restricted to detection of IFN γ (a cytokine produced by CD8⁺ cells upon activation (176)). Splenocytes from either tumour-naïve or 4T1 tumour-bearing BALB/c mice were isolated and single cell suspensions prepared as described. Splenocytes from tumour-naïve mice (2×10^5) were treated as described above. For splenocytes obtained from tumour-bearing mice, 4T1 tumour lysate was added to re-stimulate the cells, with or without addition of function-blocking anti-CD5 MAb. Cells were incubated at 37°C overnight. Brefeldin A (10 μ g/ml) was added to retain secretion of IFN γ in the Golgi apparatus. After 3 hours cells were stained with anti-mouse FITC-CD8a (clone 53-6.7) MAb (Biolegend, San Diego, CA)(1 μ g/ml in 50 μ l FACS buffer) on

ice and incubated in the dark for 30 min. The samples were washed twice and fixed in 2% paraformaldehyde (50 μ l). To detect CD8⁺ T cell activation, samples were stained for 30 min with PE-conjugated anti-mouse IFN γ (1 μ g/ml in intracellular staining permeabilization wash buffer) (Biolegend, San Diego, CA). Samples were then washed and harvested in FACS buffer for flow cytometry. Flow cytometric data were analyzed using Flowjo software (BD Bioscience).

2.3.11 *Ex vivo* cytotoxic T lymphocyte (CTL) assay

4T1 cells were stained with CFSE cell tracking dye (green) and plated in glass-bottomed 96-well plates (5X10³ cells/well) in triplicate. After cells adhered to the plate for 2 hours, CD8⁺ T cells isolated from spleens of tumour-bearing mice using a MojoSort™ Mouse CD8 T Cell Isolation Kit (Biolegend, San Diego, CA) were treated with isotype control or anti-CD5 MAb and added into each well at a 1:1 ratio with a final well volume of 200 μ l. Propidium iodide was added to wells to detect dead cells. Images were taken every 2 hours, and the cytotoxic capacity of CD8⁺ T cells was measured using an IncuCyte Zoom live cell imaging system (Essen BioScience, Ann Arbor, MI) by counting yellow objects using metric phase object confluence (propidium iodide [red] binding to dead CFSE⁺ 4T1 cells [green]).

2.3.12 CD8⁺ T cell purification

MojoSort™ Mouse CD8 T Cell Isolation Kit (BioLegend, San Diego, CA) were used for magnet-based cell separation of CD8⁺ T cells. The protocol was followed based on the instructions provided by the kit manual. Briefly, non CD8⁺

T cells were depleted by incubating splenocytes with the biotin antibody cocktail followed by incubation with magnetic streptavidin nanobeads. The untouched CD8⁺ T cells were collected by decanting the liquid in a clean tube and the magnetically labeled fraction was retained using a magnetic separator.

2.3.13 Cell counting

4T1 tumour cells were grown for 3 days before harvesting for an experiment, washed with PBS, trypsinized and counted on a Beckman Coulter Z1 Particle Counter (Beckman, Mississauga, Ontario, Canada). Results were analyzed by calculating the average number and multiple by 80 as the following

$$\text{number of cells/mL} = \frac{A + B}{2} \times 80$$

Splenocytes and lymphocytes were isolation from mice and prepared for single cell suspension. The resulting cells were stained with trypan blue to distinguish dead cells from live cells and counted manually on microscope slides. Cell numbers were calculated using the following equation:

$$\text{number of cells/ml} = \frac{A + B}{2} \times 2 \times 10^4$$

2.3.14 Statistical analysis

Statistical differences were assessed using a Student's unpaired one-tailed *t*-test (GraphPad Prism 8.2.1) to test the relationship between independent variables. No comparisons were made between dependent variables (that is, groups defined by segregation on the basis of one variable before making comparisons on the basis of a second variable) thereby validating use of *t*-testing

rather than ANOVA analysis. One-tailed analysis was employed in preference to two-tailed to analysis because only the capacity of experimental treatments to alter response of cells or tumours in one direction (increased or decreased levels of relevant measures) was of interest. Unpaired rather than paired testing was conducted because test subjects (mice and cells) were independent of each other (that is, not the same test subjects prior to and after treatments). Data points indicate means of n values \pm standard deviation (SD). Differences between data sets where $p \leq 0.05$ were considered to be significant. Asterisks represent statistical significance.

Chapter 3

CD8⁺ T tumour-infiltrating lymphocytes with downregulated cell surface expression of CD5 exhibited an increased level of activation and exhaustion

3.1 Preamble

CD5, a member of the scavenger receptor cysteine-rich superfamily, is a marker for T cells and a subset of B cells (B1a). CD5 associates with T-cell and B-cell receptors and increased CD5 is an indication of B cell activation. In tumour-infiltrating lymphocytes (TILs) isolated from lung cancer patients, CD5 levels were negatively correlated with anti-tumour activity and tumour-mediated activation-induced T cell death, suggesting that CD5 could impair activation of anti-tumour T cells. I determined CD5 levels in T cell subsets in different organs in mice bearing syngeneic 4T1 breast tumour homografts and assessed the relationship between CD5 and increased CD69 and PD-1 (markers of T cell activation and exhaustion) by flow cytometry. I report that T cell CD5 levels were higher in CD4⁺ T cells than in CD8⁺ T cells in 4T1 tumour-bearing mice, and that high CD5 levels on CD4⁺ T cells were maintained in peripheral organs (spleen and lymph nodes). However, both CD4⁺ and CD8⁺ T cells recruited to tumours had reduced CD5 compared to CD4⁺ and CD8⁺ T cells in peripheral organs. In addition, CD5^{high}CD4⁺ T cells and CD5^{high}CD8⁺ T cells from peripheral organs exhibited higher levels of activation and associated exhaustion compared to CD5^{low}CD4⁺ T cell and CD5^{low}CD8⁺ T cell from the same organs. Interestingly,

CD8⁺ T cells among TILs and downregulated CD5 were activated to a higher level, with concomitantly increased exhaustion markers, than CD8⁺CD5⁺ TILs. Thus, differential CD5 levels among T cells in tumours and lymphoid organs can be associated with different levels of T cell activation and exhaustion, suggesting that CD5 may be a therapeutic target for immunotherapeutic activation in cancer therapy.

3.2 Results

3.2.1 Differential CD5 expression among organs and T cell subsets

CD5 is expressed on the majority of T cells (76). However, differences in the levels of expression among T cell subsets isolated from different peripheral organs are not well-described. To determine the level of CD5 among T cell subsets and within peripheral organs and tumour cells, mice subcutaneously implanted with syngeneic triple-negative 4T1 breast tumours were euthanized on day 21 after implantation and spleen, lymph nodes, and tumour tissue were harvested and processed to generate single cell suspensions containing immune cells. The recovered cells were stained for CD5 and surface markers of T cell subsets and analyzed by flow cytometry. The results show that CD4⁺ T cells in spleen and lymph nodes had higher levels of surface CD5 than CD8⁺ T cells in those tissues (Figure 9A). There was no significant difference between CD5 levels in CD4⁺ T cells from spleen and lymph nodes (Figure 9B), and no significant difference in CD5 levels between CD8⁺ T cells from spleen and lymph nodes (Figure 9C). Interestingly, both CD4⁺ and CD8⁺ T cells infiltrated into tumours (TILs) had lower CD5 surface levels than CD4⁺ and CD8⁺ T cells from

lymph nodes and spleen (Figures 9B, 9C). Taken together, these data indicate that CD5 levels are significantly higher in CD4⁺ T cells compared to CD8⁺ T cells. Furthermore, a fraction of both CD4⁺ T cell and CD8⁺ T cells recruited to the tumour have lower CD5 levels than T cells in lymph and spleen, either because CD5 is downregulated by the tumour or T cells with lower CD5 are preferentially infiltrated into tumours.

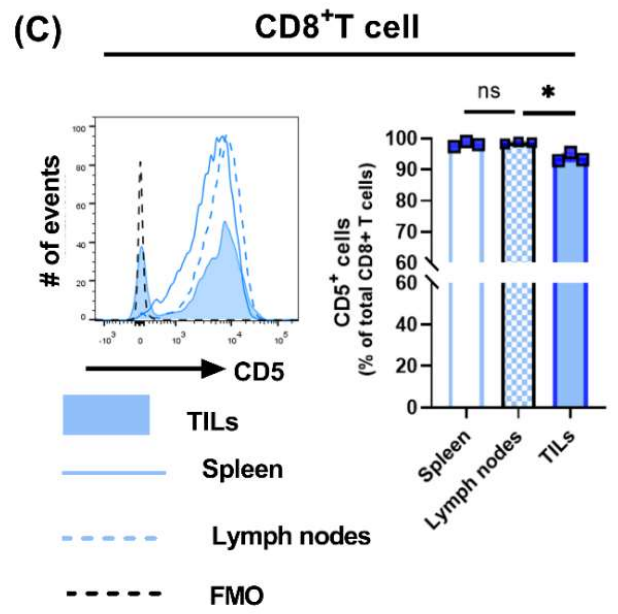
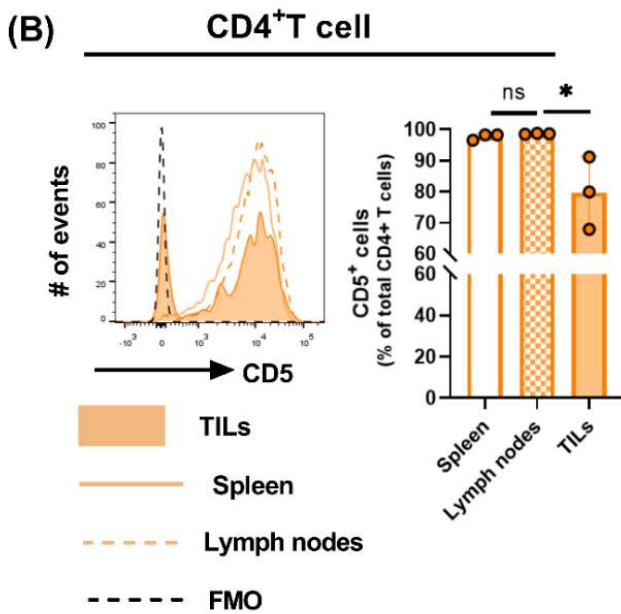
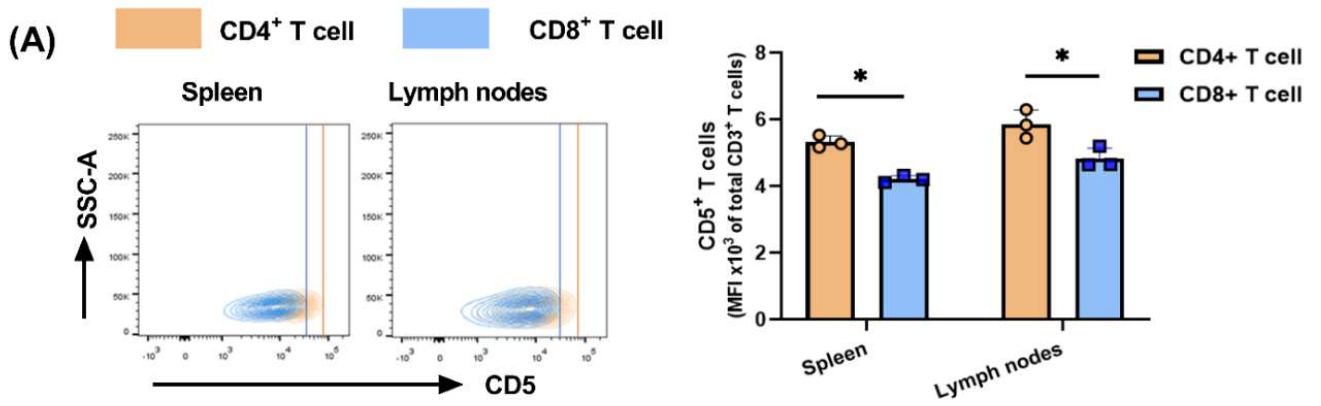


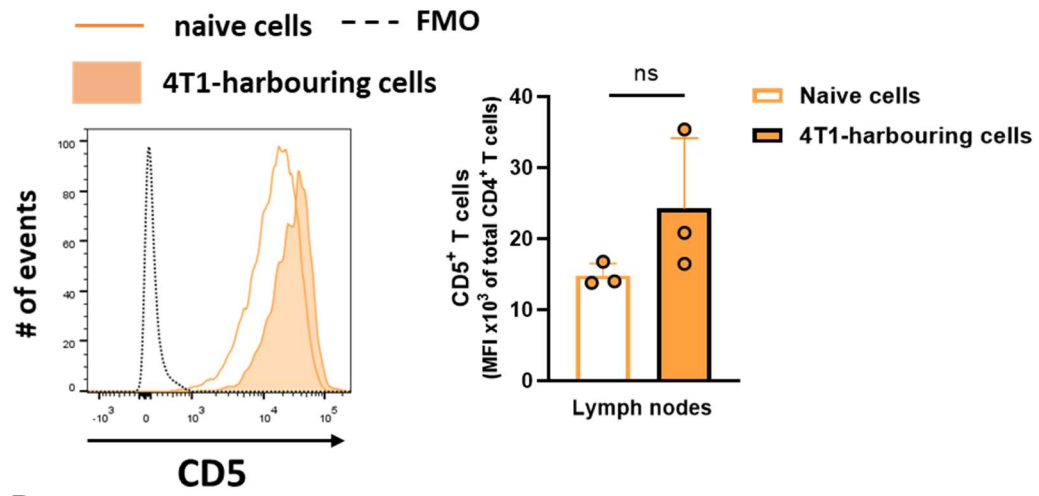
Figure 9: CD5 levels in T cell subsets and lymphoid organs. T cells isolated from 4T1-harboring BALB/c mice were stained with fluorescence-conjugated anti-CD3, anti-CD4, anti-CD8, and anti-CD5 MAbs. **(A)** CD5 levels on CD8⁺ T cells and CD4⁺ T cells in lymph nodes and spleen. **(B)** The fraction of CD5⁺CD4⁺ T cell isolated from the lymph nodes, spleen and TILs. **(C)** The fraction of CD5⁺CD8⁺ T cells isolated from the lymph nodes, spleen, and TILs. Data are shown as means \pm SD of 3 mice per group and from one representative experiment of three independent experiments. FMO: Fluorescence Minus One Control. TILs: tumour-infiltrating lymphocytes. MFI: mean fluorescence intensity. NS: not significant. * $p < 0.05$ (Student's unpaired one-tailed t -test).

3.2.2 CD5 level on CD4⁺ and CD8⁺ T cells is induced upon TCR/CD3

stimulation To determine if CD5 levels on T cells are affected by the presence of tumour homografts, T cells were isolated from lymph nodes of naïve mice and 4T1-harboring mice on day 21 after tumour cell implantation. Cells were stained with anti-CD5 MAb in addition to antibodies targeting CD3, CD4, and CD8. The levels of CD5 on the surfaces of naïve T cell and T cell from 4T1 tumour homograft-harboring mice showed different patterns of expression. CD5 levels on both CD4⁺ T cells (Figure 10A) and CD8⁺ T cells (Figure 10B) were significantly higher in lymph nodes of mice harbouring 4T1 tumours than in tumour-naïve mice. Furthermore, as previously reported by us, CD5 levels on CD8⁺ T cell splenocytes were significantly increased after TCR/CD3 stimulation by *ex vivo* treatment with anti-CD3/anti-CD28 MAbs compared to non-stimulated CD8⁺ T splenocytes (Figure 10C)(177). Together, these results reveal that the presence of tumour homografts in mice leads to elevated CD5 on T cells in lymph nodes, similar to the increase in CD5 seen after *ex vivo* stimulation of the TCR/CD3 complex on CD8⁺ T cells.

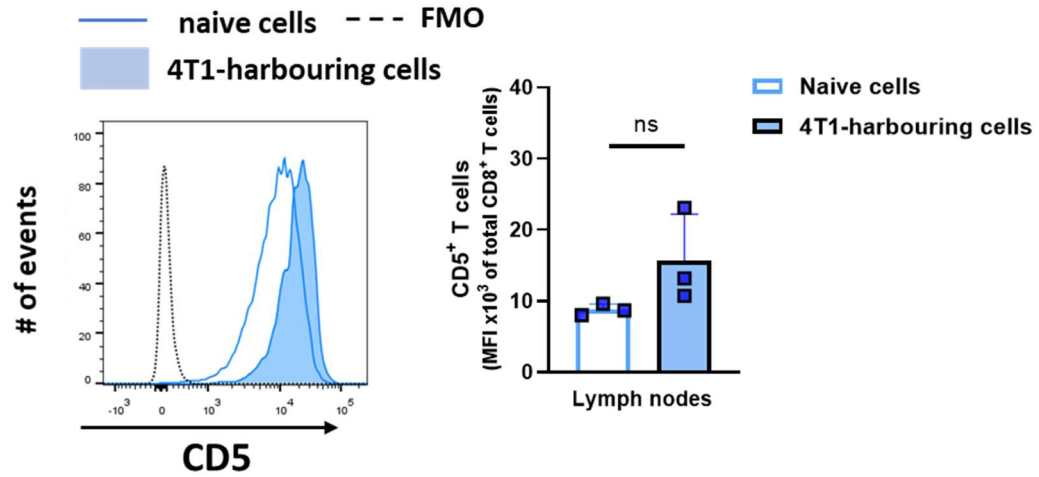
A.

CD4+ T cell



B.

CD8+ T cell



C.

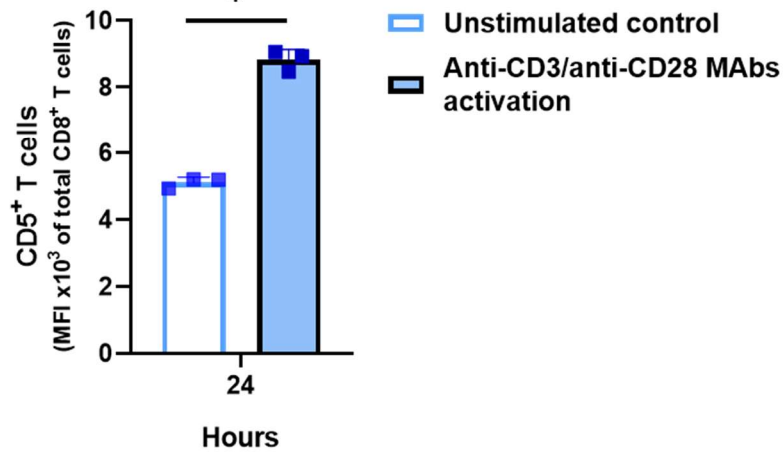


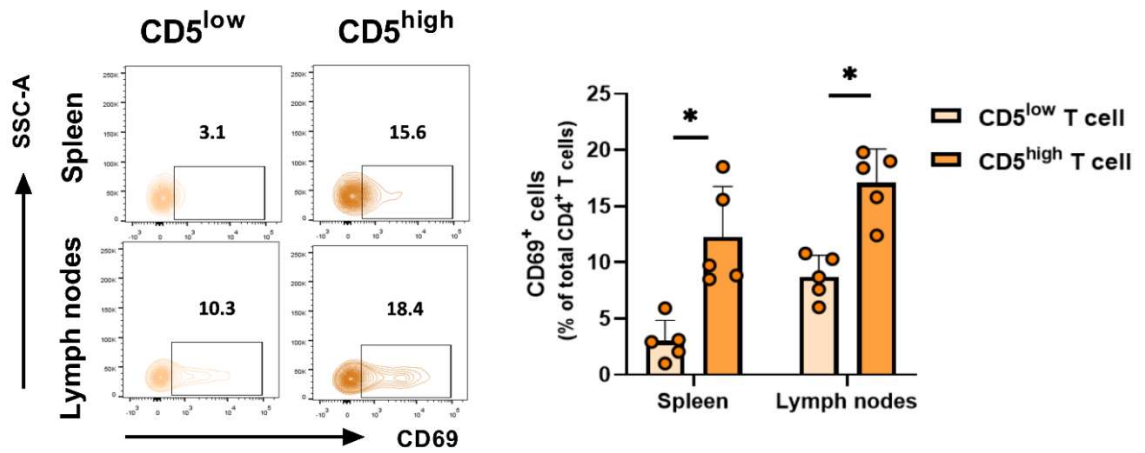
Figure 10: CD5 expression by CD4⁺ and CD8⁺ T cells is increased upon activation. Lymphocytes isolated from naïve mice or 4T1 tumour-harboring-BALB/c mice were stained with MAbs targeting CD4, CD8, and CD5. **(A)** The level of CD5 on CD4⁺ T cells isolated from lymph nodes of naïve mice and 4T1 tumour-harboring mice. **(B)** The level of CD5 expression on CD8⁺ T cell isolated from lymph nodes of naïve mice and 4T1 tumour-harboring mice. **(C)** Splenocytes isolated from naïve BALB/c mice were treated with anti-CD3/anti-CD28 MAb on day 0 and cells were quantified by flow cytometry after 24 h for CD5 level on CD8⁺ T cells. Data are shown as means ± SD of 3 mice per group and from one representative experiment of three independent experiments. FMO: Fluorescence Minus One Control. MFI: mean fluorescence intensity. * $p < 0.05$ (Student's unpaired one-tailed t -test)

3.2.3 CD5^{high} T cells in spleen and lymph nodes exhibit increased activation

The increased level of CD5 on T cells upon TCR/CD3 stimulation suggests that CD5 level may be directly increased by that activation. To address how CD5 levels may be associated with T cell activation, a gating strategy was applied to determine the activation level of CD5^{high} T cells and CD5^{low} T cells based on the level of the T cell activation marker CD69 (178)(gating strategy described in Figure 11C). Mice were challenged by subcutaneous injection of 4T1 tumour cells 21 days prior to euthanasia, at which time lymph nodes and spleens were collected. T cells isolated from those organs were stained with anti-CD69 and anti-CD5 MAbs in addition to antibodies against T cell markers (anti-CD3/anti-CD8/anti-CD4 MAbs). The results show that the fraction of CD69⁺CD5^{high}CD4⁺ T cells in spleen and lymph nodes was significantly higher than the fraction of CD69⁺CD5^{low}CD4⁺ T cells (Figure 11A). Similarly, the fraction of CD69⁺CD5^{high}CD8⁺ T cells was significantly higher than the fraction of CD69⁺CD5^{low}CD8⁺ T cells in spleen and lymph nodes (Figure 11B). Collectively, these data suggest a correlation between CD5 level and T cell activation in peripheral organs.

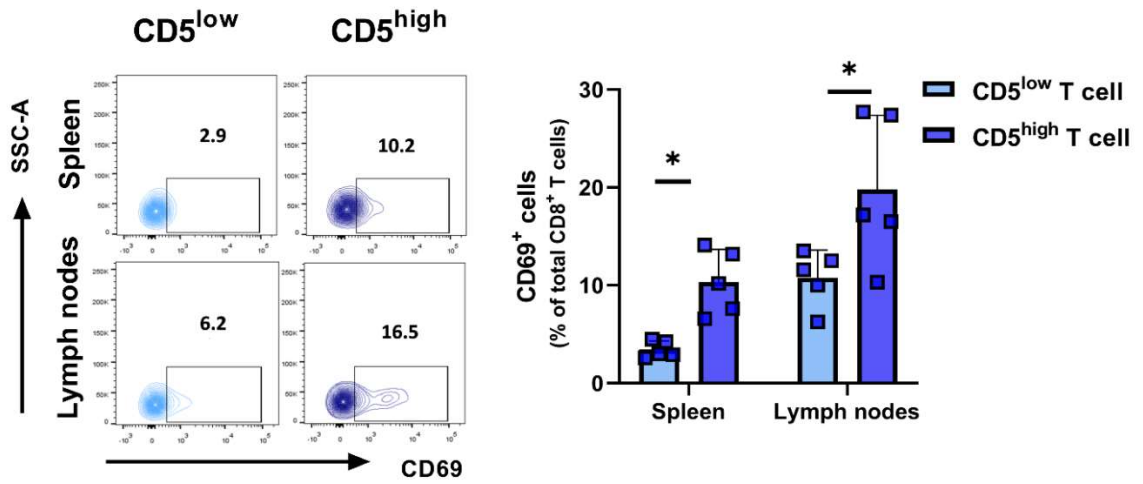
(A)

CD4⁺T cell



(B)

CD8⁺T cell



(c)

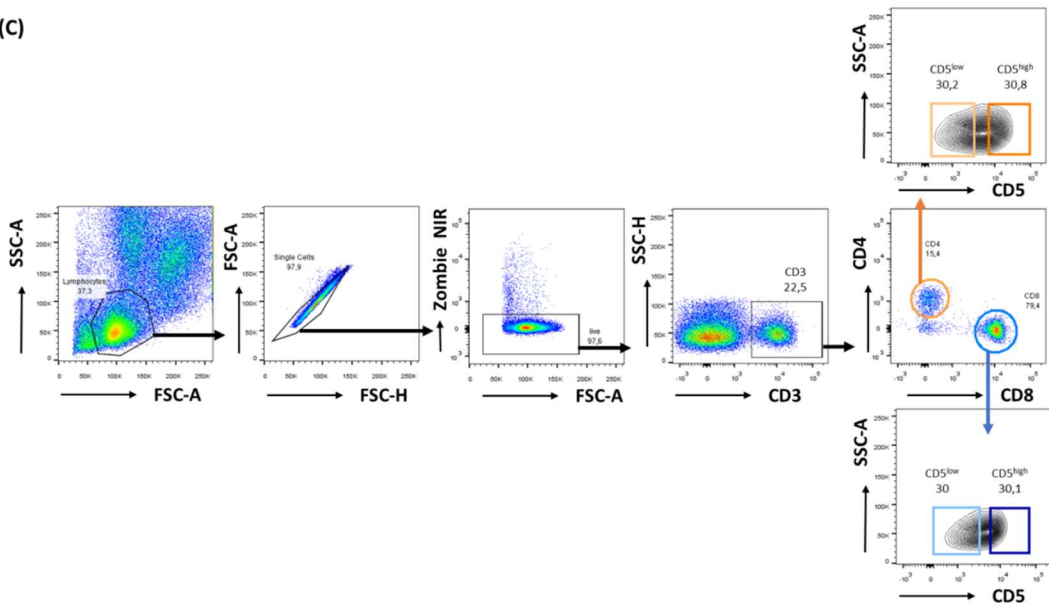


Figure 11: The correlation of CD5 with CD69 on CD4⁺ and CD8⁺ T cells.

Lymphocytes isolated from 4T1-harboring BALB/c mice were stained with fluorescence-conjugated anti-CD3, anti-CD4, anti-CD8 and anti-CD5 MAbs. **(A)** The fraction of CD69⁺CD5^{high}CD4⁺ T cell and CD69⁺CD5^{low}CD4⁺ T cell in spleen and lymph nodes. **(B)** The fraction of CD69⁺CD5^{high}CD8⁺ T cell and CD69⁺CD5^{low}CD8⁺ T cell in spleen and lymph nodes. **(C)** Gating strategy. Data are shown as means \pm SD of 5 mice per group and from one representative experiment of three independent experiments. * $p < 0.05$ (Student's unpaired one-tailed *t*-test).

3.2.4 CD5^{-/low} T cells display increased activation in the tumour

T cells recruited to the tumour have reduced CD5 levels compared to T cells in lymph nodes and spleens. To determine the level of activation of CD5⁺ and CD5^{-/low} tumour-infiltrating T cells, the level of the activation marker CD69 in both CD4⁺ and CD8⁺ T cell subpopulations in T cells isolated from 4T1 tumours excised from mice at 21 days following subcutaneous implantation of tumour cells was determined. The data show that the fraction of CD69⁺CD5^{-/low} CD4⁺ T cells from tumours was significantly higher than the fraction of CD69⁺CD5⁺CD4⁺ T cells (Figure 12A). Similarly, the fraction of CD69⁺CD5^{-/low}CD8⁺ T cells was significantly higher than the fraction of CD69⁺CD5⁺CD8⁺ T cells (Figure 12B). This suggests that the fraction of CD4⁺ and CD8⁺ TILs with reduced CD5 levels in the tumour have higher levels of activation compared to CD5⁺CD4⁺ and CD5⁺CD8⁺ TILs, respectively.

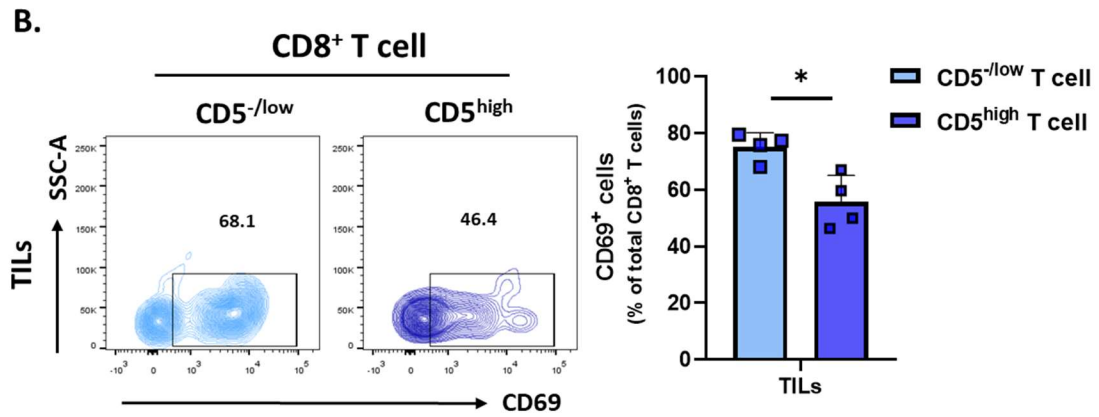
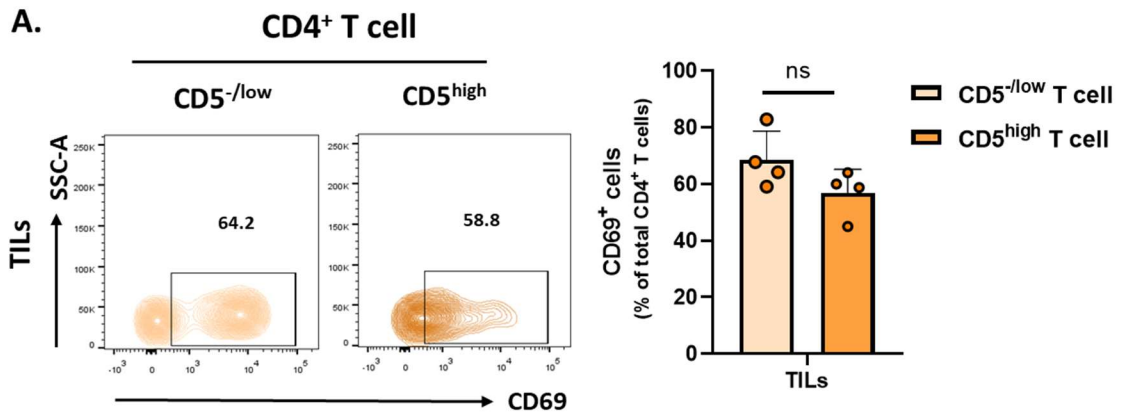


Figure 12. The fraction of CD5^{high} and CD5^{-low} tumour-infiltrating lymphocytes (TILs) expressing CD69. TILs isolated from 4T1-harboring-BALB/c mice were stained with fluorescence-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD5 and anti-CD69 MAbs. **(A)** The fraction of CD69⁺CD5^{high}CD4⁺ T cell and CD69⁺CD5^{-low}CD4⁺ T cell in TILs. **(B)** The fraction of CD69⁺CD5^{high}CD8⁺ T cell and CD69⁺CD5^{-low}CD8⁺ T cell in TILs. Data are shown as means \pm SD of 4 mice per group and from one representative experiment of three independent experiments. * $p < 0.05$ (Student's unpaired one-tailed *t*-test).

3.2.5 CD5^{high} T cells in spleen and lymph nodes exhibit increased exhaustion

Upon activation, T cells express high levels of CD5. T cells can also up-regulate exhaustion/activation markers such as PD-1 upon activation (179). To determine whether CD5^{high}T cells exhibit a higher exhaustion phenotype compared to CD5^{low} T cells, T cells from spleen and lymph nodes of 4T1 tumour-harboring mice were isolated on day 21 following subcutaneous implantation of the tumour cells. Recovered cells from spleen and lymph nodes were then stained with anti-PD-1 and anti-CD5 MAbs in addition to T cell panel markers (anti-CD3/anti-CD8/anti-CD4 MAbs) and analyzed by flow cytometry. The results show that the fraction of PD-1⁺CD5^{high}CD4⁺ T cell was substantially higher than the fraction of CD69⁺CD5^{low}CD4⁺ T cells in spleens and lymph nodes (Figure 13A). Similarly, the fraction of PD-1⁺CD5^{high}CD8⁺ T cells was significantly higher than the fraction of PD-1⁺CD5^{low}CD8⁺ T cells in spleen and lymph nodes (Figure 13B). Collectively, these data suggest that elevated CD5 levels on T cells is associated with T cell exhaustion.

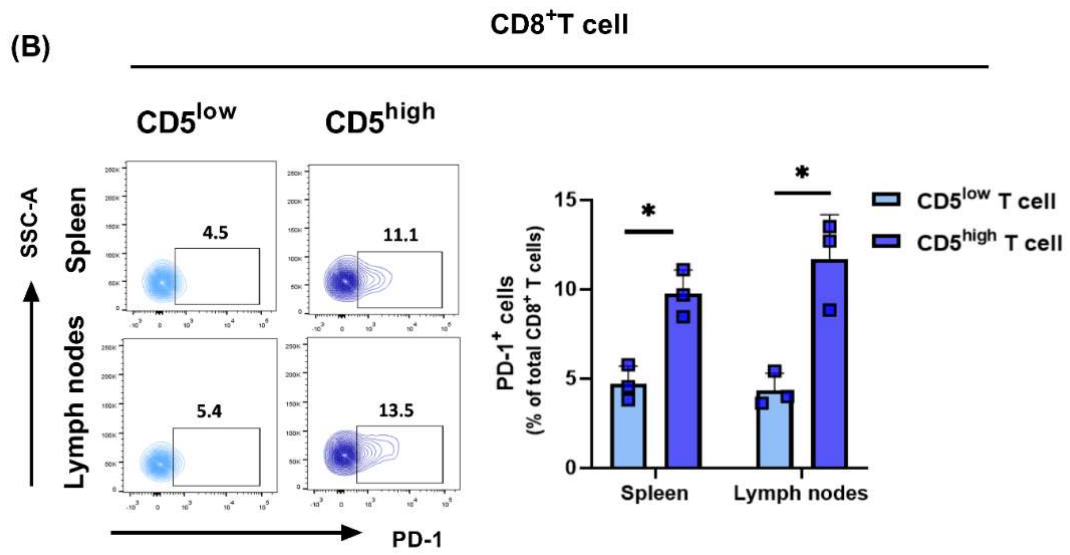
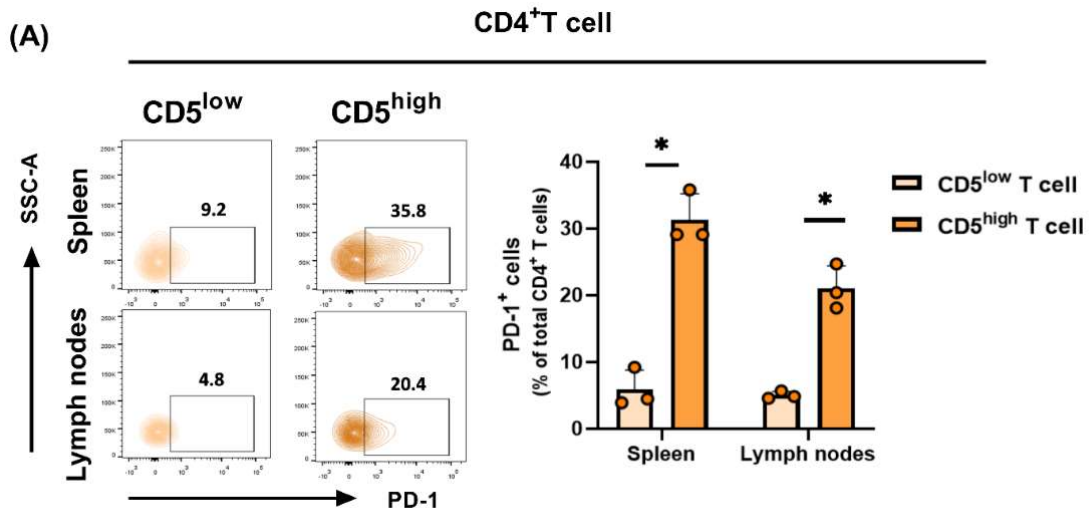


Figure 13: The correlation of CD5 level with PD-1 level in CD4⁺ and CD8⁺ T cells from spleen and lymph nodes of tumour-bearing mice. Lymphocytes isolated from 4T1-harboured-BALB/c mice were stained with fluorescence-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD5 and anti-PD-1 MAbs. **(A)** The fraction of PD-1⁺CD5^{high}CD4⁺ T cell and PD-1⁺CD5^{low}CD4⁺ T cell in spleen and lymph nodes. **(B)** The fraction of PD-1⁺CD5^{high}CD8⁺ T cell and PD-1⁺CD5^{low}CD8⁺ T cell in spleen and lymph nodes. Data are shown as means \pm SD of 3 mice per group and from one representative experiment from three independent experiments. * $p < 0.05$ (Student's unpaired one-tailed t -test).

3.2.6 CD5^{-/low}CD8⁺T cells display increased exhaustion in the tumour

Downregulation of CD5 level was associated with an increased fraction of activated CD4⁺ and CD8⁺ T cells in the tumour. To determine the exhaustion level in CD5^{-/low}CD4⁺ vs CD5⁺CD4⁺ TILs and in CD5^{-/low}CD8⁺ vs CD5⁺CD8⁺ TILs, TILs were stained with anti-PD-1 MAb and with antibodies to detect CD5, CD4, CD8 and CD3 and analyzed by flow cytometry. The results show a significantly elevated fraction of PD-1⁺CD5^{-/low}CD8⁺ TILs compared to PD-1⁺CD5⁺CD8⁺ TILs (Figure 14B). Interestingly, this was the opposite of what was observed in CD4⁺ TILs, where the fraction of PD-1⁺CD5⁻CD4⁺ TILs was significantly lower than PD-1⁺CD5⁺CD4⁺ TILs (Figure 14A). These data suggest that, the fraction of CD5^{-/low} CD8⁺ TILs become exhausted in the tumour compared to CD5⁺CD8⁺ TIL but not in the CD5^{-/low} CD4⁺ TILs.

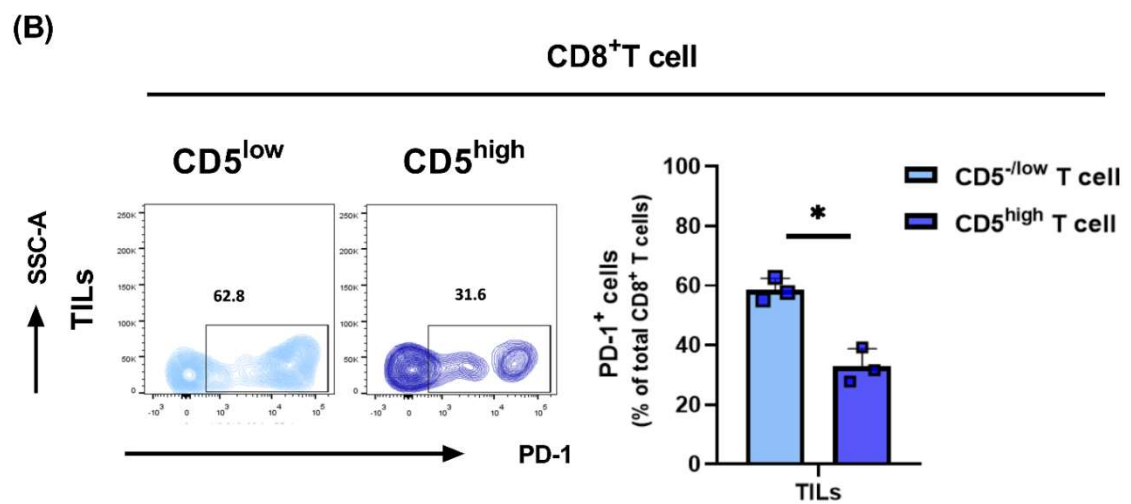
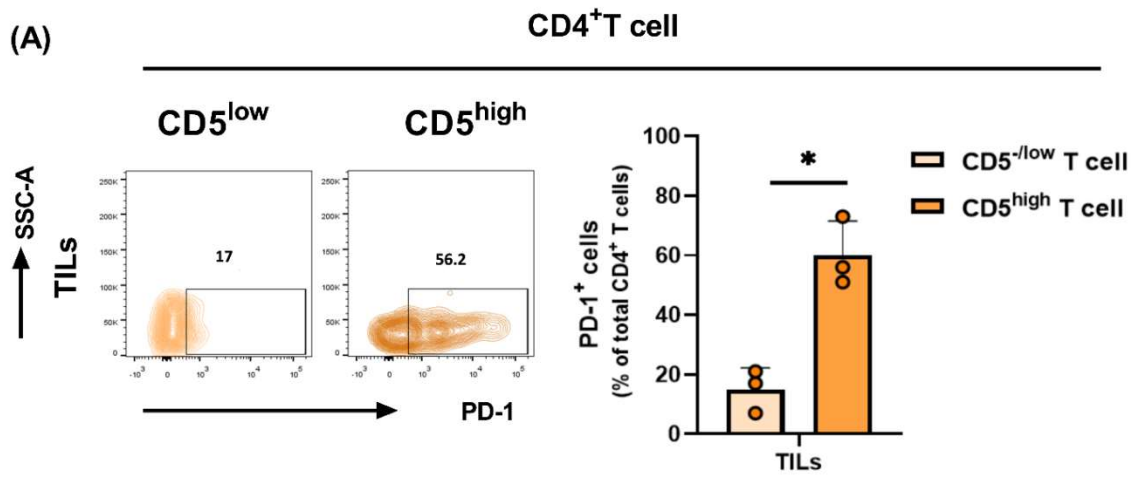


Figure 14. CD5^{-/low}CD8⁺ T cells from tumour-bearing mice exhibit an exhaustion phenotype compared to CD5⁺CD8⁺ T cells. TILs isolated from 4T1-harboring-BALB/c mice were stained with fluorescence-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD5 and anti-PD-1 MAbs. **(A)** The fraction of PD-1⁺CD5^{high}CD4⁺ T cell and PD-1⁺CD5^{-/low}CD4⁺ T cell in TILs. **(B)** The fraction of PD-1⁺CD5^{high}CD8⁺ T cell and PD-1⁺CD5^{-/low}CD8⁺ T cell in TILs. Data are shown as means \pm SD of 3 mice per group and from one representative experiment of three independent experiments. * $p < 0.05$ (Student's Unpaired one-tailed *t*-test).

Chapter 4

CD5 blockade enhances *ex vivo* CD8⁺ T cell activation and tumour cell cytotoxicity

4.1 Preamble:

CD5 is a therapeutically-targetable tumour antigen expressed on leukemic T and B cells. However, the potential therapeutic effect of blocking CD5 function to increase T cell anti-tumour activity against tumours other than leukemia (including solid tumours) has not been explored. CD5 knockout mice show increased anti-tumour immunity and reduced homograft tumour growth: reducing CD5 on CTLs may be therapeutically beneficial to enhance the anti-tumour response. Here I show that *ex vivo* administration of a blocking anti-CD5 monoclonal antibody (MAb) to primary mouse CTLs isolated from spleens of both naive mice and mice bearing mouse 4T1 breast tumour homografts enhances their capacity to respond to activation by treatment with anti-CD3/anti-CD28 MAbs or 4T1 tumour cell lysates. Furthermore, *ex vivo* CD5 blocking MAb treatment reduced CD5 signaling (erk activation) and increased markers of spleen T cell activation including proliferation, CD69 levels, interferon- γ production, apoptosis, and Fas receptor and Fas ligand levels. Finally, CD5 blocking MAb treatment enhanced the capacity of primary mouse spleen CD8⁺ T cells to kill 4T1 mouse tumour cells in an *ex vivo* assay. These data support the potential of blockade of CD5 function to enhance T cell-mediated anti-tumour immunity.

4.2 Results

4.2.1 CD5 blockade enhances CD8⁺ T cell receptor signaling

Increased phosphorylation of Erk is a downstream effect of activating TCR signaling pathway (180). Erk2 regulates CD8⁺ T cell proliferation and survival (181). A previous report showed elevated Erk phosphorylation in the absence of CD5-CK2 signaling (182). To determine if using anti-CD5 MAb could lead to impaired CD5 signaling pathway and increased Erk activation. I treated naïve splenocytes with anti-CD3 MAb alone or anti-CD3 and anti-CD5 MAbs and showed increased phosphorylation of Erk1/2 in cells receiving anti-CD3 and anti-CD5 MAbs using western blot (Figure 15A and B). Furthermore, treatment with anti-CD5 MAb alone did not induce Erk phosphorylation (Figure 15C).

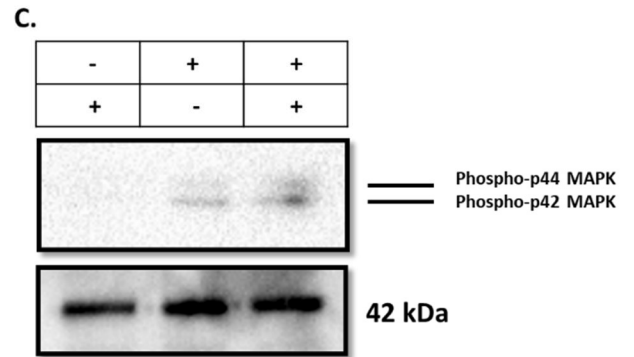
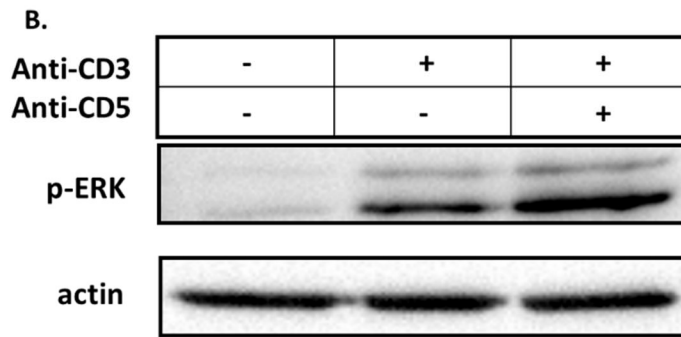
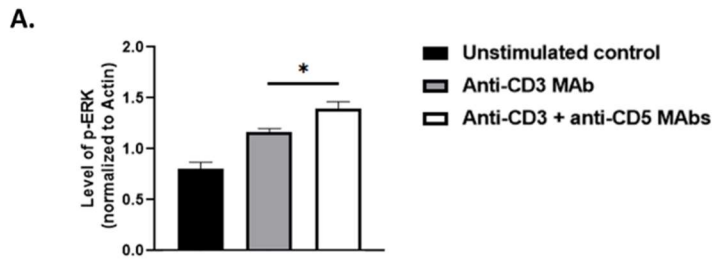


Figure 15. Increased ERK phosphorylation in anti-CD3/anti-CD5 MAb-treated splenocytes. Splenocytes isolated from naive BALB/c mice were treated with anti-CD3 MAb or with anti-CD3 and anti-CD5 MAbs for 30 min and then cells were lysed and phosphorylation of Erk was detected by western blot. **(A)** The level of ERK phosphorylation was normalized to loading control actin. **(B)** Membrane immunoblotted for p-ERK and actin from cells treated with nothing, anti-CD3 and anti-CD3/anti-CD5. **(C)** Membrane immunoblotted for p-ERK and actin from cells treated with anti-CD5, anti-CD3 and anti-CD3/anti-CD5. Data are mean \pm SD ($n = 3$ mice), one representative experiment of three. $*p < 0.05$ (Student's unpaired one-tailed t -test).

4.2.2 CD5 blockade enhances CD8⁺ T cell activation and proliferation

CD5^{-/-} T cells exhibit increased levels of T cell activation markers (CD25, CD69) after treatment with anti-CD3 MAb (183). To determine whether blocking CD5 function using an anti-CD5 MAb, in combination with activation by anti-CD3 MAb/anti-CD28 MAb, would enhance CD8⁺ T cell activation, I treated primary splenocytes isolated from tumour-naïve mice with anti-CD3/anti-CD28 MAbs and anti-CD5 MAb for 24 hours *in vitro* and then assessed the level of the activation marker CD69 on CD8⁺ T cells among those splenocytes. Cells treated with anti-CD3/anti-CD28 MAbs in addition to anti-CD5 MAb showed an increased fraction of CD69⁺CD8⁺ T cells compared to cells received anti-CD3/anti-CD28 MAbs and isotype control MAb (82% vs 78% respectively, $p < 0.05$)(Figure 16A; for the gating strategy Figure 16B). To further assess CD8⁺ T cell activation upon treatment with anti-CD5 MAb, proliferation of CD8⁺ T cells was examined. Splenocytes from either tumour-naïve mice or mouse 4T1 tumour-bearing mice were stained with CFSE cell tracking dye and treated with anti-CD3/anti-CD28 MAbs or 4T1 tumour lysate, in addition to anti-CD5 MAb or isotype control MAb. CD8⁺ T cells treated with anti-CD5 MAb exhibited increased proliferation compared to those treated with isotype control MAb up to 5 divisions with almost 20% cells in each division with anti-CD3/anti-CD28 MAbs and up to 5 divisions with 4T1 tumour lysate with almost 20% cells in division 3 and less than 10% cells in division 4 and 5. The increase percentage of cells in each divisions was significantly different in cells with anti-CD5 in division 3, 4 and 5 with $p < 0.05$ (Figure 17A,B).

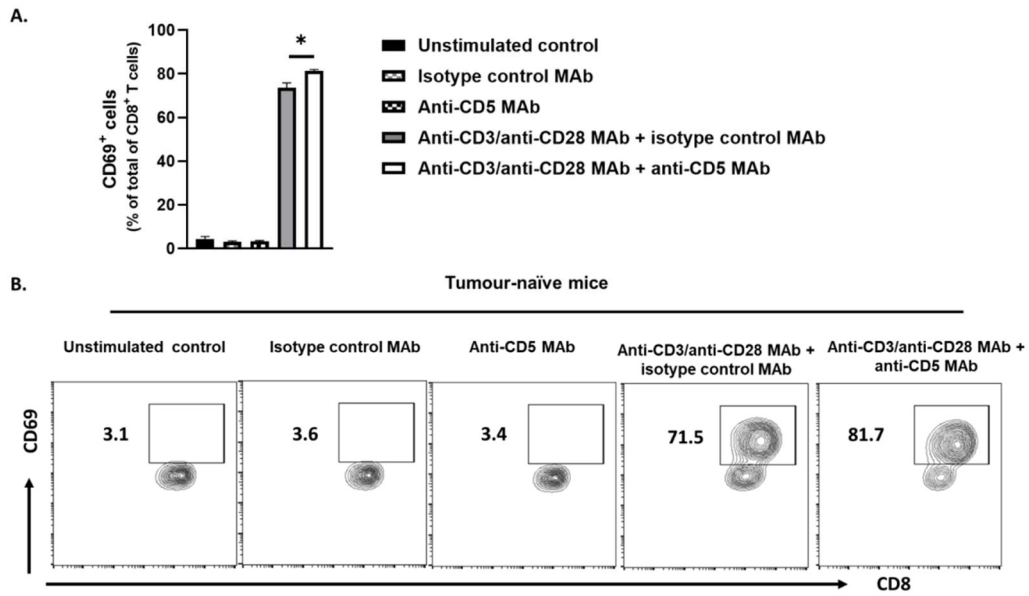


Figure 16. CD8⁺ T cell activation after treatment with anti-CD5 MAb blockade. (A) Quantification of fraction of CD69⁺CD8⁺ T cells. (B) Gating strategy for splenocytes isolated from naive BALB/c mice were treated with anti-CD3/anti-CD28 MAb with or without anti-CD5 MAb (day 0), CD69⁺CD8⁺ cells were quantified by flow cytometry 24 h later using PE anti-mouse CD69. Data are mean \pm SD ($n = 3$ mice), one representative experiment of two. * $p < 0.05$ (Student's unpaired one-tailed t -test).

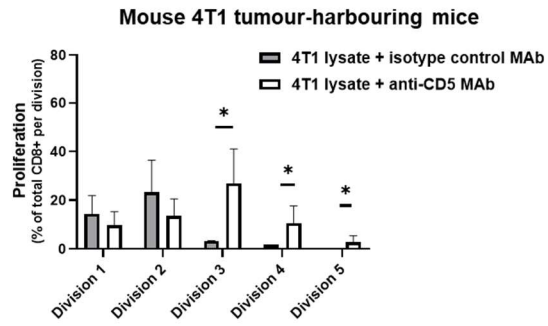
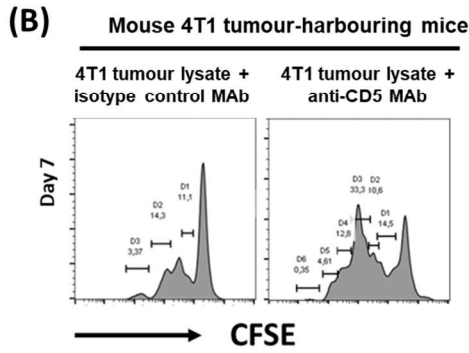
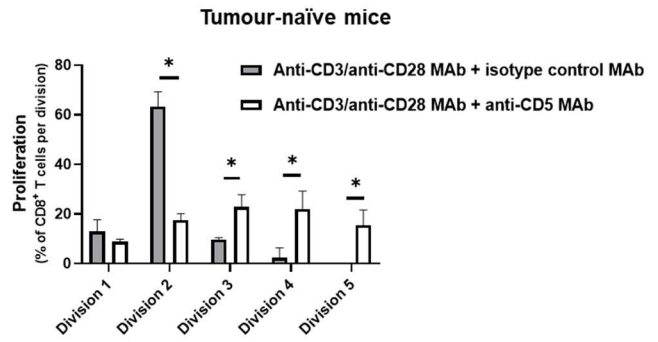
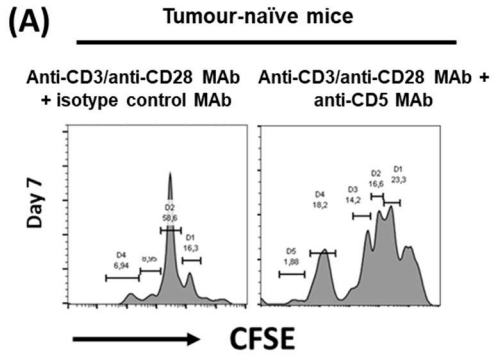


Figure 17. CD8⁺ T cell proliferation after treatment with anti-CD5 MAb

blockade. (A) Splenocytes isolated from naive BALB/c mice were stained with cell tracking dye (CFSE) then activated with anti-CD3/anti-CD28 MAb, with or without anti-CD5 MAb (day 0). **(B)** Splenocytes from mouse 4T1 breast tumour harbouring BALB/c mice were stained with cell tracking dye (CFSE) and then were treated with 4T1 lysate with or without anti-CD5 MAb (day 0). Cells were incubated for 7 days and media were replaced every 48 h. CFSE dye levels were quantified by flow cytometry: increased dilution of dye indicated increased CD8⁺ T cell proliferation (A and B). Data are mean \pm SD ($n = 3$ mice), one representative experiment of two. * $p < 0.05$ (Student's unpaired one-tailed t -test).

4.2.3 Increased fraction of CD8⁺ IFN γ ⁺ T cells after treatment with anti-CD5 MAb

IFN γ is an effector cytokine produced by activated CD8⁺ T cells (176). To determine whether treatment with anti-CD5 MAb enhances production of IFN γ in CD8⁺ T cells, splenocytes from either naïve mice or mouse 4T1 tumour-bearing mice were stimulated for 24 h *in vitro* with anti-CD3/anti-CD28 MAbs or 4T1 tumour lysate, respectively, in addition to anti-CD5 MAb or isotype control MAb. The fraction of cells positive for IFN γ after treatment with both anti-CD3/anti-CD28 MAbs and anti-CD5 MAb was greater than the fraction after treatment with anti-CD3/anti-CD28 MAbs and isotype control MAb, 18% vs 21% respectively $p < 0.05$ (Figure 18A). The MFI was higher in cells treated with both anti-CD3/anti-CD28 MAbs and anti-CD5 MAb treatment 3×10^3 vs 5×10^3 respectively $p < 0.05$ indicating that not only was fraction of IFN γ ⁺ cells increased, but the level of IFN γ in those cells was also higher (Figure 18A and B). The fraction of CD8⁺ T cells from 4T1 tumour-bearing mice treated *ex vivo* with tumour lysate in addition to anti-CD5 MAb also had an increased fraction of IFN γ ⁺ T cells from 0.6% to 1.5%, $p < 0.05$ (Figure 19A and B). Furthermore, to assess if blocking CD5 has a direct affect on CD8⁺ T cells, I purified CD8⁺ T cells from the whole splenocytes and then treat the cells with anti-CD3/anti-CD28 MAbs with or without anti-CD5 MAb. After 24 hours, the cells treated with addition of anti-CD5 MAb has greater production of IFN γ compared to the cells not received anti-CD5 MAb (Figure 20).

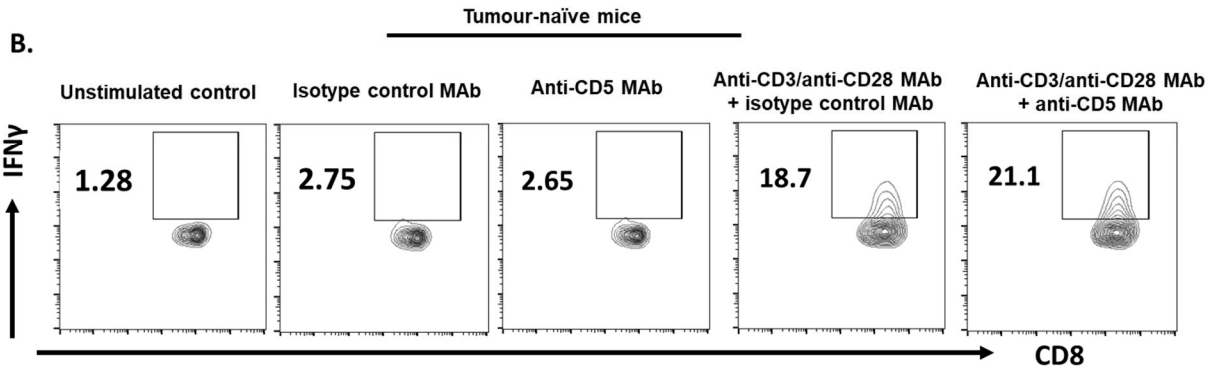
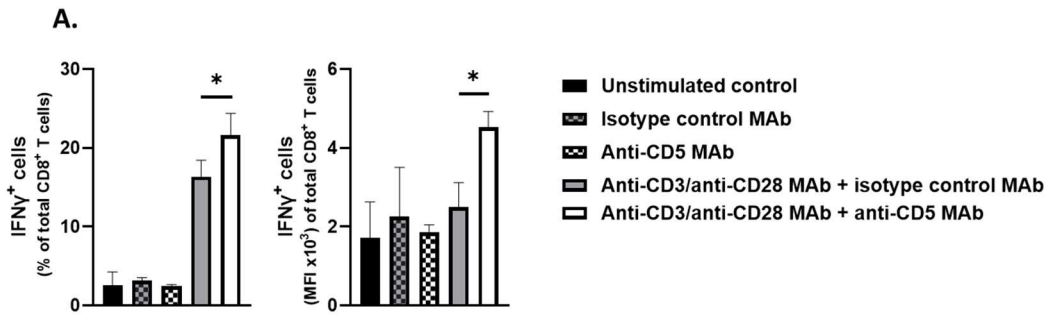


Figure 18. Increased fraction of CD8⁺ IFN- γ ⁺ T cell after treatment with anti-CD5 MAb. Splenocytes isolated from naive BALB/c mice were treated with anti-CD3/anti-CD28 MAb with or without anti-CD5 MAb (day 0). **(A)** Quantification of the fraction of CD8⁺ IFN- γ ⁺ T cell and the MFI of IFN- γ . **(B)** Gating strategy. Data are mean \pm SD ($n = 3$ mice), one representative experiment of three, $*p < 0.05$, (Student's unpaired one-tailed t -test), MFI: mean fluorescence intensity.

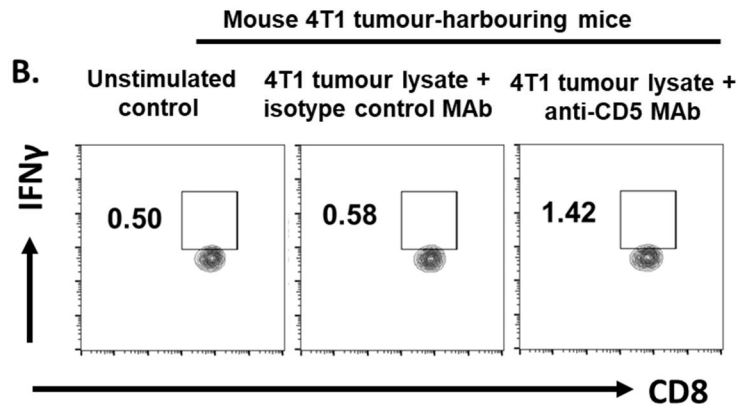
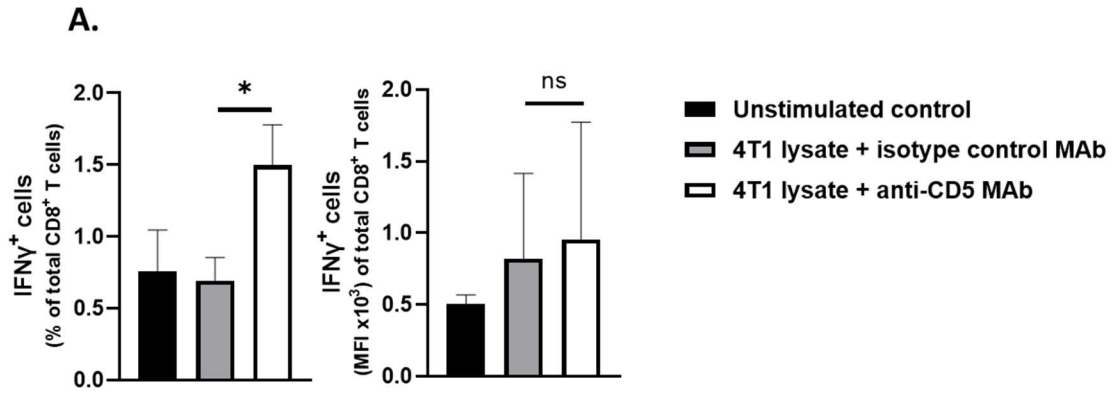


Figure 19. Increased fraction of CD8⁺IFN- γ ⁺ T cells after treatment with anti-CD5 MAb *ex vivo*. Splenocytes from mouse 4T1 breast tumour-harboring BALB/c mice were treated with 4T1 lysate with or without anti-CD5 MAb (day 0). **(A)** Quantification of the fraction of CD8⁺ IFN- γ ⁺ T cell and the MFI of IFN- γ . **(B)** Gating strategy. Data are mean \pm SD ($n = 3$ mice), one representative experiment of three. * $p < 0.05$, ns, not significant (Student's unpaired one-tailed t -test).

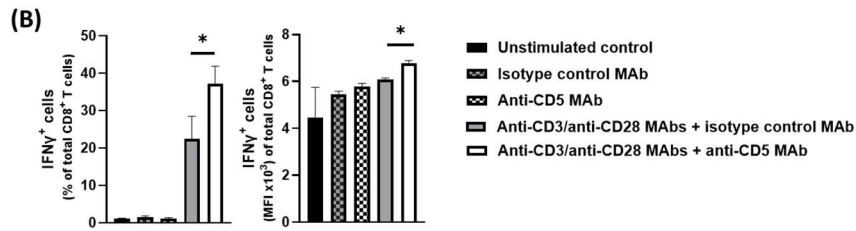
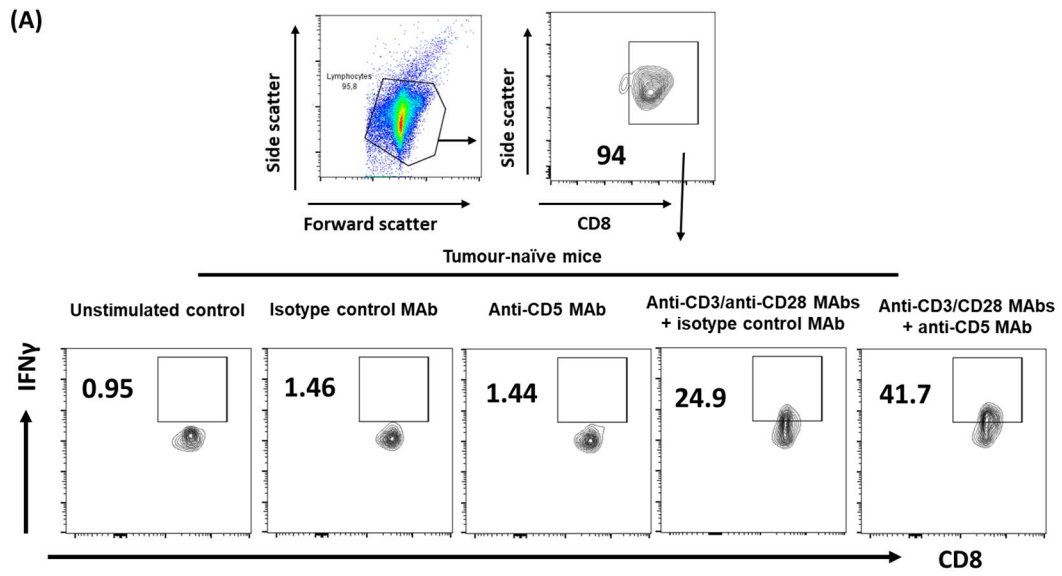


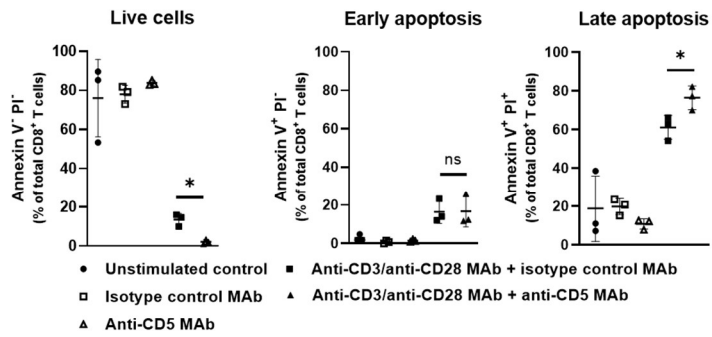
Figure 20: IFN γ in purified CD8⁺ T cells following activation, with or without anti-CD5 MAb blockade. (A) Gating strategy for purified CD8⁺ T cells after activation with or without anti-CD5 MAb (B) Quantified data. CD8⁺ T cells positive for IFN γ ⁺ were quantified by flow cytometry 24 hours later. Gating was based on the size of splenocytes, then gated on CD8⁺ cells and then on IFN γ and CD8. Data are mean \pm SD (n=3 mice), * p < 0.05 (Student's unpaired one-tailed t -test), one representative mouse of 3 is shown, MFI: mean fluorescence intensity .

4.2.4 CD5 blockade increases FasR- and FasL-dependent death of CD8⁺ T cells

CD5 may decrease TCR tumour antigen recognition, and CD5 blockade could enhance antigen recognition and intracellular TCR signaling. A consequence of repeated antigen stimulation of the CD3/TCR complex is activation-induced cell death (AICD)(20, 184). To assess whether anti-CD5 MAb could increase CD8⁺ T cell death after activation as a consequence of enhanced TCR sensitivity to antigen, splenocytes from tumour-naive mice were treated with anti-CD3/anti-CD28 MAbs, with or without anti-CD5 MAb, on day 0. Cells were cultured as previously described with media refreshed every 48 hours. On day 7 cells were stained with anti-CD8 MAb followed by staining with FITC-annexin V and PI (Figure 21A and B for gating strategy). The fraction of CD8⁺ T cells undergoing AICD (Annexin V⁺/PI⁺) was increased among those activated with anti-CD3/anti-CD28 MAbs and anti-CD5 MAb, compared to those treated with anti-CD3/anti-CD28 MAbs and isotype control MAb: 78% vs 60%, $p < 0.05$. For splenocytes from 4T1 tumour-bearing mice, treatment with anti-CD5 MAb enhanced AICD compared to treatment with isotype control MAb: 59% vs 40%, $p < 0.05$ (Figure 22A and B for gating strategy). AICD has been shown to depend on FasR/FasL interaction (185): consequently, I assessed the fraction of FasR⁺ CD8⁺ and FasL⁺ CD8⁺ T cells. Splenocytes from tumour-naïve mice and mouse 4T1 tumour-bearing mice were isolated and treated with anti-CD3/anti-CD28 MAbs or 4T1 lysate, respectively, with or without anti-CD5 MAb. Cells obtained from mice euthanized at 24, 48, 72, and 96 hours thereafter were stained with

fluorescent antibodies and analyzed by flow cytometry to detect FasR and FasL on CD8⁺ T cells. Splenocytes treated with anti-CD3/anti-CD28 MAbs in addition to anti-CD5 MAb had an elevated fraction of FasR⁺CD8⁺ T cells compared to the isotype control MAb-treated group at 48, 72, and 96 hours after activation 40% vs 30%, 50% vs 39% and 78% vs 50% respectively with $p < 0.05$ (Figure 23A and B for gating strategy). In addition, the level of FasR in FasR⁺ cells was increased after anti-CD5 MAb treatment compared to isotype control MAb-treated group at 48 hours 1.3×10^3 vs 1.2×10^3 and at 96 hours 1.6×10^3 vs 1.3×10^3 respectively with $p < 0.05$ (Figure 23A and B for gating strategy). The fraction of FasL⁺CD8⁺ T cells was also increased in cells treated with anti-CD5 MAb at 72 hours 60% vs 30% and at 96 hours 80% vs 60% respectively with $p < 0.05$ (Figure 23A and B). Similarly, the fraction of FasR⁺CD8⁺ T cells among mouse 4T1 tumour-bearing splenocytes treated with anti-CD5 MAb was increased compared to cells treated with isotype control MAb, at 48 and 72 hours after activation 45% vs 35% and 40% vs 35% respectively with $p < 0.05$; the fraction of FasL⁺CD8⁺ T cells treated with anti-CD5 MAb was increased compared to cells treated with isotype control MAb at 96 hours 65% vs 60% respectively with $p < 0.05$ (Figure 24A and B for gating strategy); and the level of FasR on FasR⁺CD8⁺ T cells was higher at 96 hours after anti-CD5 treatment compared to isotype control MAb 3×10^3 vs 2×10^3 (Figure 24A and B gating strategy).

A. Tumour-naïve mice



B. Tumour-naïve mice

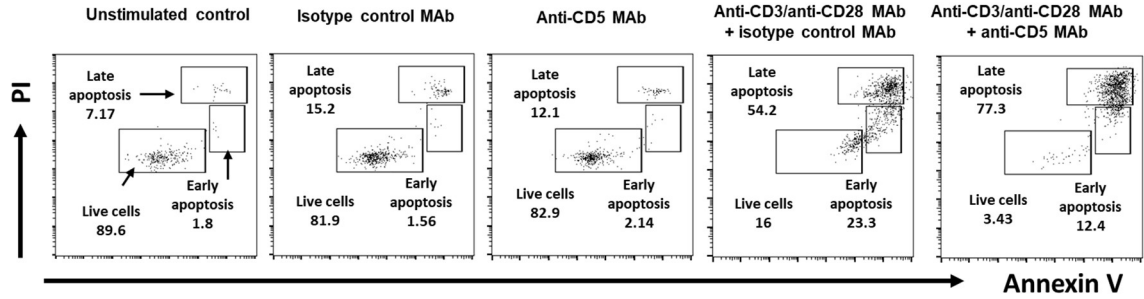
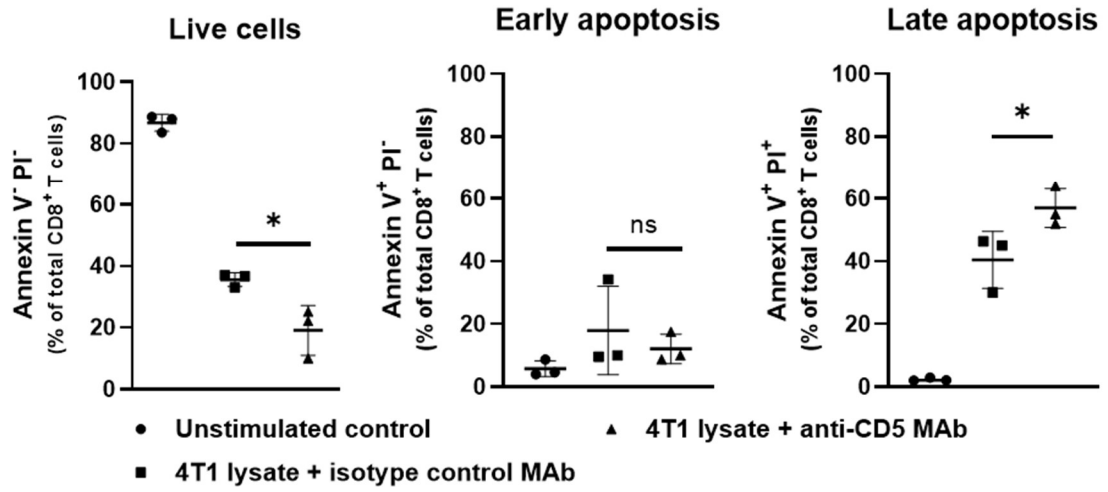


Figure 21. Increased CD8⁺ T cell apoptosis after activation with anti-CD3/anti-CD28 antibodies and treatment with anti-CD5 MAb. Splenocytes from naive, non-tumour bearing BALB/c mice were activated with anti-CD3/anti-CD28 MAb, with or without anti-CD5 MAb. Cell viability was assessed by flow cytometry on day 7 (cells positive for Annexin V only were deemed to be undergoing early apoptosis; for both Annexin V and PI labelled as late apoptosis). **(A)** Quantified data **(B)** Gating strategy. Data are mean \pm SD ($n = 3$ mice), one representative experiment of two, * $p < 0.05$, ns, not significant (Student's unpaired one-tailed t -test).

A. Mouse 4T1 tumour-harboring mice



Mouse 4T1 tumour-harboring mice

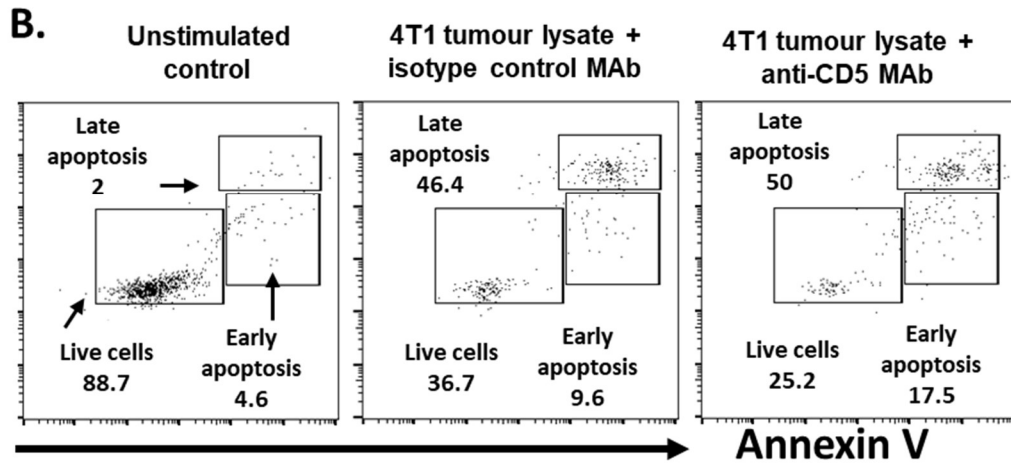


Figure 22. Increased CD8⁺ T cell apoptosis after activation with 4T1 tumour cell lysate and treatment with anti-CD5 MAb. Splenocytes from mice harbouring 4T1 breast tumours were treated with 4T1 lysate, with or without anti-CD5 MAb. Cell viability was assessed by flow cytometry on day 7 (cells positive for Annexin V only were deemed to be undergoing early apoptosis; for both Annexin V and PI labelled as late apoptosis). **(A)** Quantified data **(B)** Gating strategy. Data are mean \pm SD ($n = 3$ mice), one representative experiment of two, * $p < 0.05$, ns, not significant (Student's unpaired one-tailed t -test).

A. Tumour-naïve mice

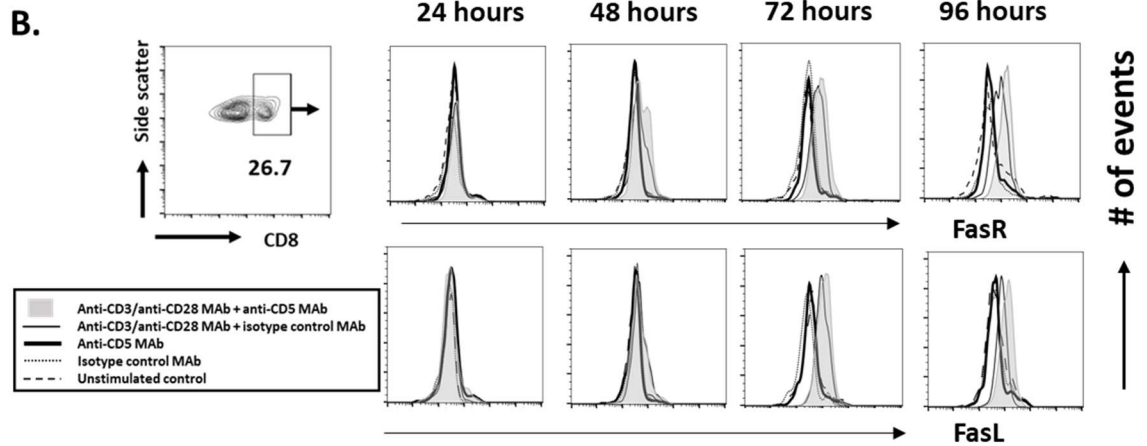
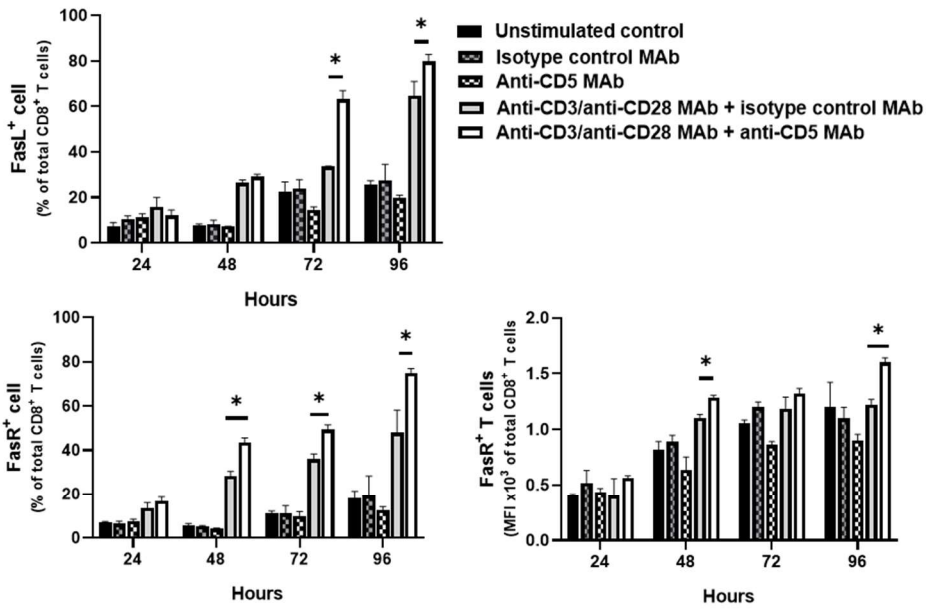
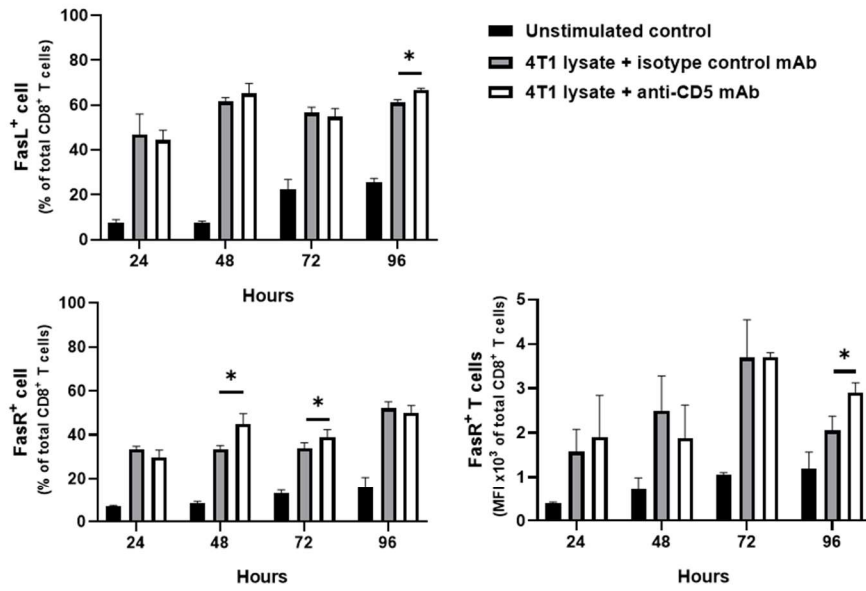


Figure 23. Increased fractions of FasR⁺CD8⁺ and FasL⁺CD8⁺ T cells after stimulation with anti-CD3/anti-CD28 MAbs and treatment with anti-CD5 MAb *ex vivo*. Splenocytes from naive, non-tumour bearing BALB/c mice were treated on day 0 with anti-CD3/anti-CD28 MAb with or without anti-CD5 MAb. CD8⁺ T cells were quantified by flow cytometry every 24 h for FasR and FasL positivity. **(A)** Quantified data. **(B)** Gating strategy. Data are mean \pm SD ($n = 3$ mice), one representative experiment of three, * $p < 0.05$ (Student's unpaired one-tailed t -test), MFI: mean fluorescence intensity.

A.

Mouse 4T1 tumour-harboring mice



B.

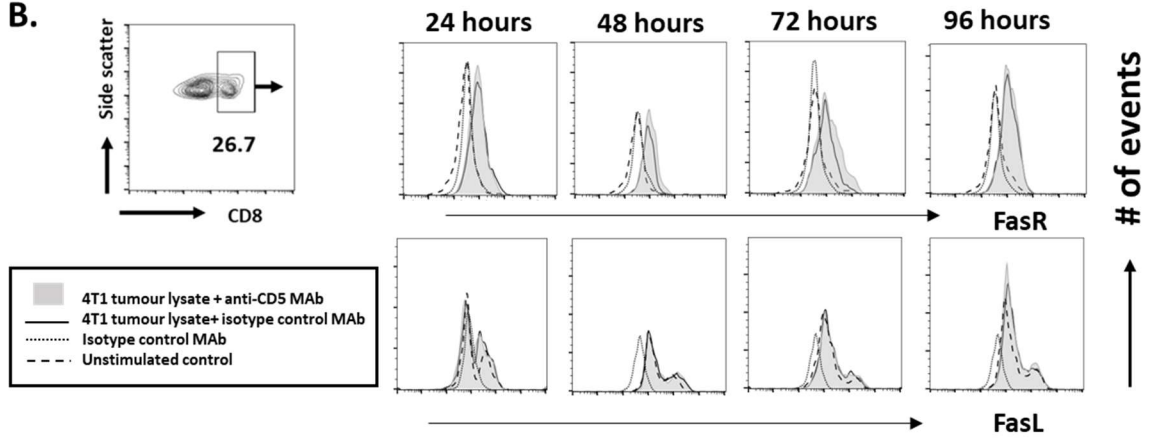


Figure 24. Increased fractions of FasR⁺CD8⁺ and FasL⁺CD8⁺ T cells after stimulation with 4T1 tumour cell lysate and treatment with anti-CD5 MAb *ex vivo*. Splenocytes from BALB/c mice harbouring 4T1 breast tumours were treated with 4T1 lysate, with or without anti-CD5 MAb (day 0). CD8⁺ T cells were quantified by flow cytometry every 24 h for FasR and FasL positivity. **(A)** Quantified data. **(B)** Gating strategy. Data are mean \pm SD ($n = 3$ mice), one representative experiment of three, * $p < 0.05$ (Student's unpaired one-tailed t -test), MFI: mean fluorescence intensity.

4.2.5 CD5 blockade enhances tumour killing by CD8⁺ T cells

To determine if treatment of CD8⁺ T cells with anti-CD5 MAb could enhance T cell-mediated cytotoxicity against tumour cells, I assessed *ex vivo* killing of mouse 4T1 tumour cells by primary CD8⁺ T cells isolated from mice and treated with blocking anti-CD5 MAb. Mice were challenged with 4T1 tumour cells for 21 days and CD8⁺ T cells were isolated and co-cultured with 4T1 cells *in vitro* in combination with *ex vivo* treatment with anti-CD5 MAb. Two-dimensional cell cultures containing T cells and 4T1 cells were maintained in an IncuCyte live cell analysis system to capture live images to detect the ability of CD8⁺ T cells to kill and lyse tumour cells. CD8⁺ T cells treated with anti-CD5 were more capable in killing tumour cells compared to cells that received isotype control or received nothing. At 6 and 10 hours post-treatment, there were more than 30 dead cells/mm³ with cells that received anti-CD5 blockade compared to less than 20 dead cells/mm³ with cells that received isotype control MAb with $p < 0.05$ (Figure 25).

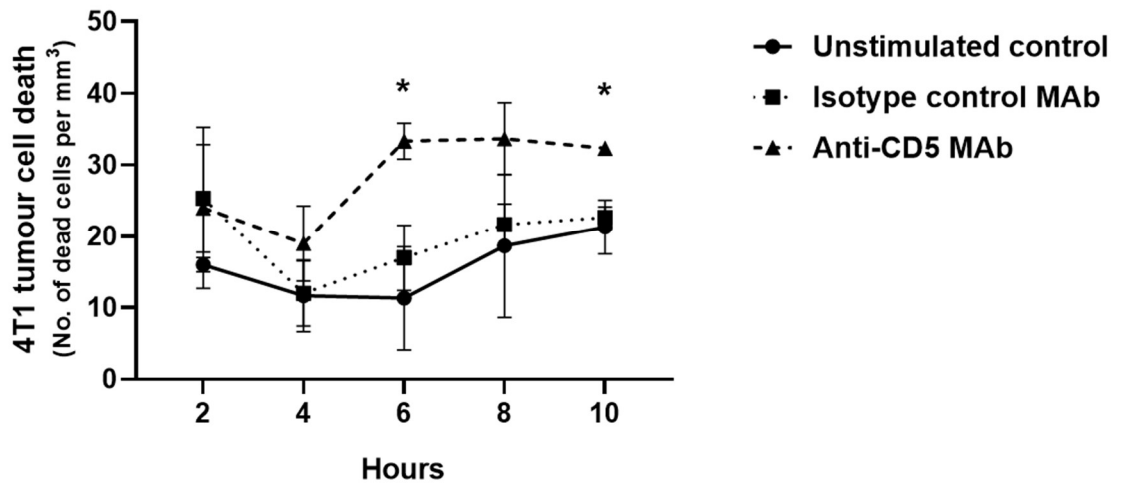


Figure 25. Anti-CD5 MAb enhances *ex vivo* CD8⁺ T cell-mediated killing of mouse 4T1 breast tumour cells. Cytotoxic CD8⁺ T cells were isolated from BALB/c mice bearing mouse 4T1 breast tumours, treated with the isotype control MAb or anti-CD5 MAb or control (nothing) and mixed with CFSE-stained 4T1 cells *in vitro* at a 1:1 ratio. PI was added to measure cell death and an IncuCyteR_S3 Live-Cell Analysis System was used to image the cells every 2 h. Yellow objects which represent CFSE stained 4T1 (green) binding to PI (red) were counted as a measure of 4T1 tumour cell death. Data are mean \pm SD ($n = 3$ mice), one representative experiment of two. $*p < 0.05$ (Student's unpaired one-tailed *t*-test).

Chapter 5

Administration of anti-CD5 MAb *in vivo* enhances CD8⁺ T cell activation and function in a poorly immunogenic 4T1 breast tumour model

5.1 Preamble

CD5 is a characteristic surface tumour marker for T and B cell malignancies. The therapeutic potential of depletion of non-solid CD5⁺ tumour cells has been explored using anti-CD5 depleting antibodies and CD5 CAR T cells. In solid CD5⁻ tumour cells, CD5 knockout mice exhibit increased anti-tumour immunity (134). This suggests that blocking CD5 function may have a therapeutic effect by enhancing cytotoxic T lymphocyte activity. The effect of administering an anti-CD5 antibody to block CD5 function and enhance T cell activation and function *in vivo* has not been explored. Here I injected immunocompetent mice with poorly immunogenic, triple-negative mouse 4T1 breast tumour cells and tested whether administration of anti-CD5 MAb *in vivo* could enhance normal T cell activation and effector function. The data show that administration of anti-CD5 MAb increased the ration of CD8⁺ T/CD4⁺ T cells in draining lymph nodes and within tumours. In addition, I observed significantly increased activation and effector function of T cells isolated from spleen, draining lymph nodes, and tumours. Furthermore, the fraction of exhausted T cells was significantly enhanced after administration of effector-blocking anti-CD5 MAb. These data suggest a potential use of anti-CD5 MAb as an immune blockade to

enhance immune activation in response to poorly immunogenic antigens, either alone or in combination with other drugs including immunotherapeutic drugs.

5.2 Results

5.2.1 Anti-CD5 MAb treatment increases the fraction of CD8⁺ T cells relative to CD4⁺ T cells in tumours and draining lymph nodes

Increased CD8⁺ T cells relative to CD4⁺ T cells in tumours correlate with better progression-free survival in breast cancer patients (186). I determined whether the administration of an anti-CD5 MAb *in vivo* affected the ratio of CD8⁺/CD4⁺ T cell ratio in spleen, draining lymph nodes, and within tumours. As assessed by flow cytometry, there was a higher fraction of CD8⁺ T cells and a lower fraction of CD4⁺ T cells among all CD3⁺ cells (*i.e.*, T lymphocytes) in draining lymph nodes and among tumour-infiltrating lymphocytes (TILs) after treatment with anti-CD5 MAb (Figure 26A). However, treatment with anti-CD5 MAb did not induce a similar increase the fraction of CD8⁺ T cells and decrease in the fraction of CD4⁺ T cells in spleens (Figure 26A). These results were further confirmed in analysis of CD8⁺/CD4⁺ T cell ratios (Figure 26B). As expected, there was a significant increase in the ratio of CD8⁺/CD4⁺ T cells in draining lymph nodes and TILs, but not in spleens of mice treated with anti-CD5 MAb compared to those treated with isotype control MAb (Figure 26B). These data suggested that treatment with anti-CD5 MAb enhances CD8⁺ T cell fractions within tumours and draining lymph nodes.

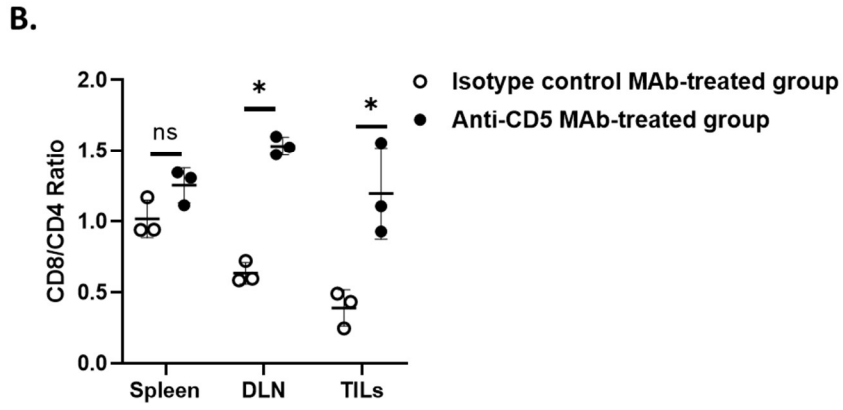
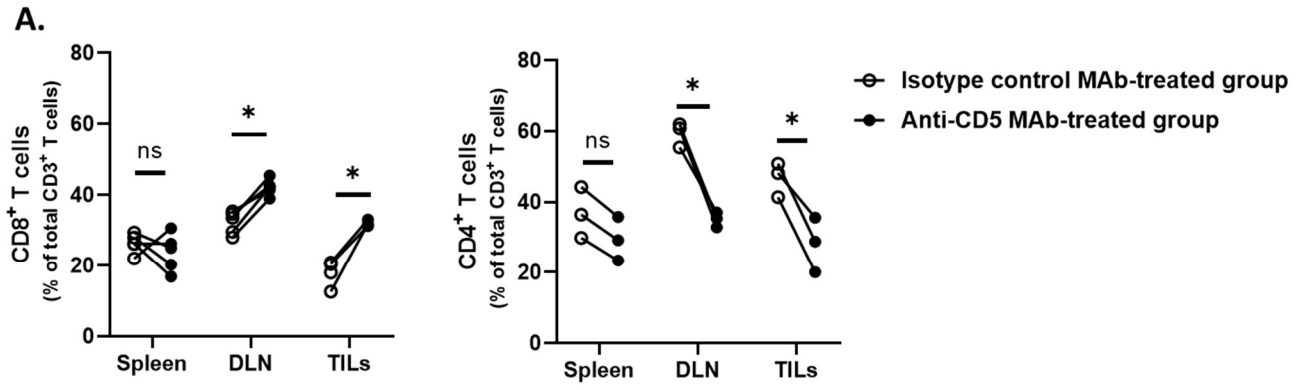


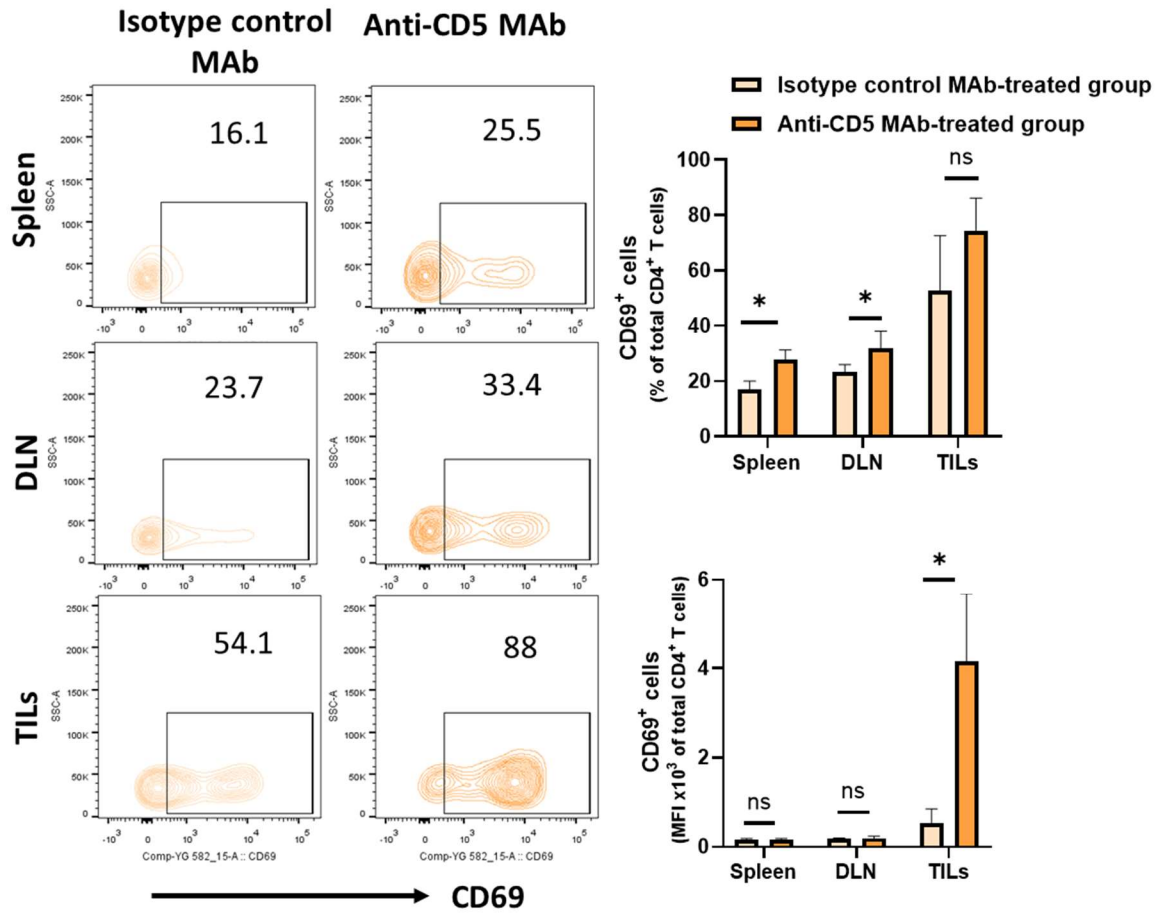
Figure 26. Fraction of CD8⁺ T and CD4⁺ T cells after treatment with anti-CD5 MAb *in vivo*. (A) CD8⁺ T and CD4⁺ T cells from spleen, draining lymph node, and TILs isolated from 4T1 tumour-bearing BALB/c mice treated with anti-CD5 MAb or isotype control MAb. (B) CD8⁺/CD4⁺ T cell ratios calculated from spleen, draining lymph node, and TILs. Data are mean ± SD ($n = 3$ mice), one representative experiment of three, ns, not significant, * $p < 0.05$ (Student's unpaired one-tailed t -test).

5.2.2 Increased CD8⁺ T cell activation after treatment with anti-CD5 MAb *in vivo*

In a published study I show that splenocytes stimulated *ex vivo* with anti-CD3/anti-CD28 MAbs or 4T1 tumour lysate and treated with anti-CD5 MAb had an increased fraction of CD8⁺CD69⁺ T cells compared to cells stimulated with anti-CD3/anti-CD28 or 4T1 tumour lysate and isotype control (177). To investigate whether *in vivo* administration of anti-CD5 MAb enhanced T cell activation, I excised spleens, draining lymph nodes, and 4T1 tumours from mice and assessed the levels of CD69 (a marker of T cell activation) on T cells in those organs. The results show an increase in the fraction of CD69⁺CD4⁺ T cells in spleens and draining lymph nodes in anti-CD5 MAb-treated mice (Figure 27A). Furthermore, I assessed the levels of CD69 on CD4⁺ T cells and I found the mean fluorescence intensity of CD69 was higher in CD4⁺ TILs in anti-CD5 MAb-treated mice (Figure 27A). Furthermore, I found increased fraction of CD69⁺CD8⁺ T cells in spleen and draining lymph nodes of mice treated with anti-CD5 MAb compared to isotype control MAb-treated mice (Figure 27B). Furthermore, similar to CD4⁺ TILs, CD8⁺ TILs isolated from anti-CD5 MAb-treated mice had a higher level of CD69 compared to isotype control MAb-treated mice (Figure 27B). These data indicate that treatment with anti-CD5 MAb enhanced T cell activation *in vivo*.

CD4⁺ T cell

A.



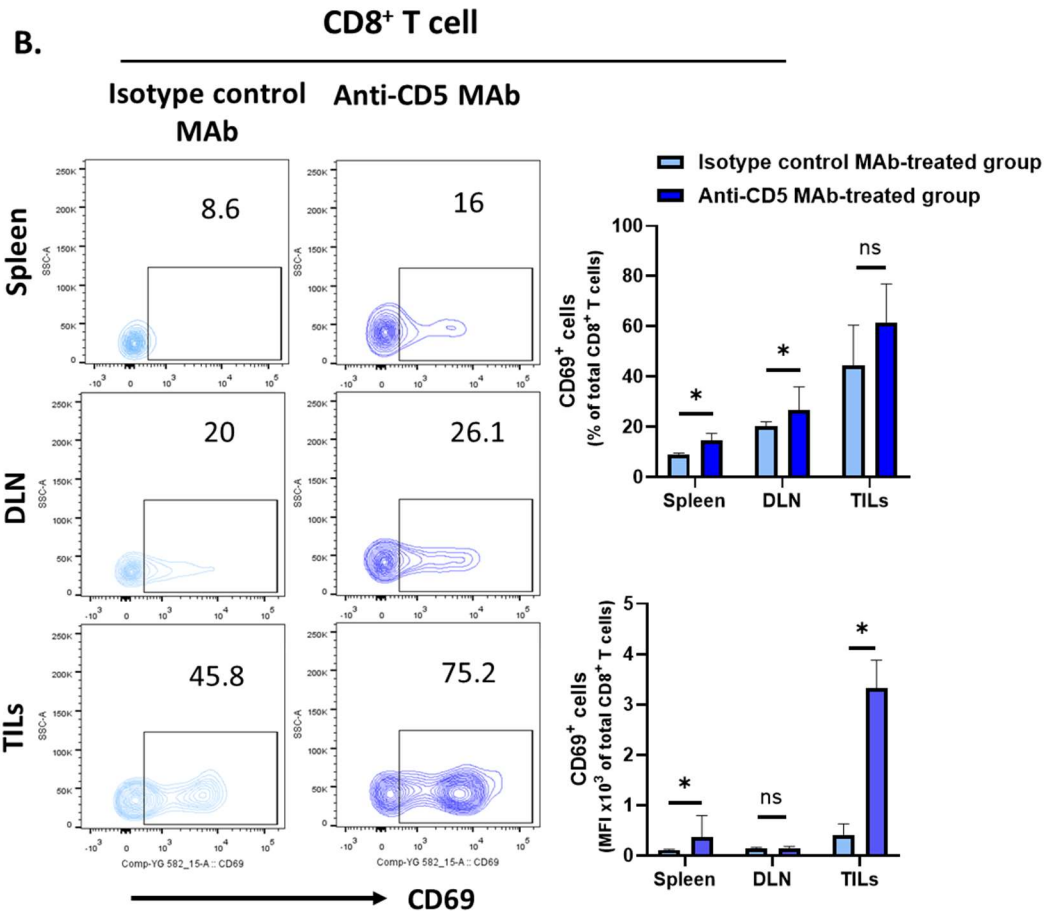


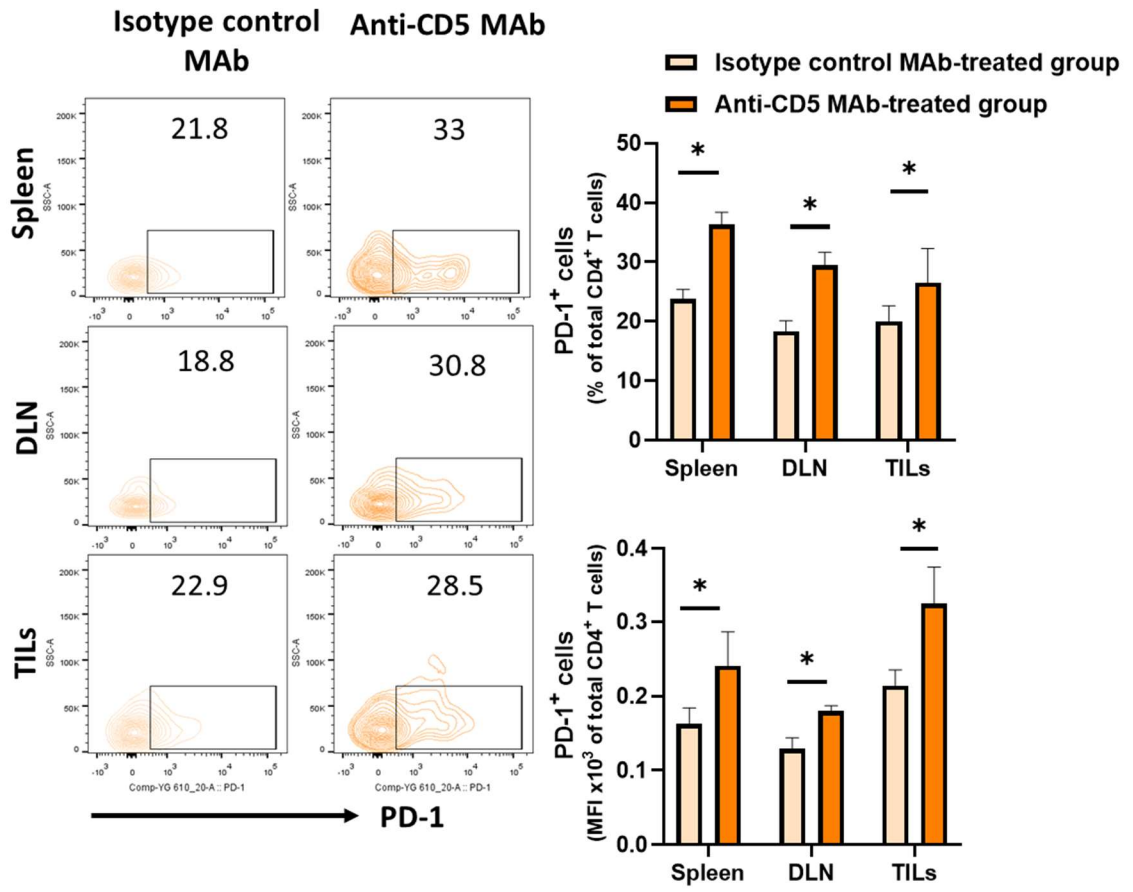
Figure 27. Fraction of CD8⁺CD69⁺ T and CD4⁺CD69⁺ T cells after treatment with anti-CD5 MAb *in vivo*. (A) The fraction of CD4⁺CD69⁺ T and MFI of CD69 on CD4⁺ T cells isolated from spleens, draining lymph nodes, and TILs excised from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb or isotype control MAb. (B) The fraction of CD8⁺CD69⁺ T cells and MFI of CD69 on CD8⁺ T cells isolated from spleens, draining lymph nodes, and TILs isolated from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb or isotype control MAb. Data are mean ± SD ($n = 3$ mice), one representative experiment of three, ns, not significant, * $p < 0.05$ (Student's unpaired one-tailed t -test), MFI: mean fluorescence intensity.

5.2.3 Increased CD8⁺ T cell exhaustion after treatment with anti-CD5 MAb *in vivo*

PD-1 is a co-inhibitory receptor that is highly expressed on exhausted T cell (187). It can also indicate chronic stimulation of T cells and is an indication of activation (179). The expression of the T cell exhaustion marker PD-1 was assessed on T cells following treatment with anti-CD5 MAb *in vivo*. The data show that mice treated with anti-CD5 MAb had a higher fraction of PD-1⁺CD4⁺ T cells in spleen, draining lymph nodes, and within 4T1 tumours compared to mice treated with isotype control MAb (Figure 28A). The MFI of PD-1 was also higher in anti-CD5 MAb-treated CD4⁺ T cells isolated from spleens, draining lymph nodes, and tumours (Figure 28A). Furthermore, treatment with anti-CD5 MAb increased the fraction of PD-1⁺CD8⁺T cells and MFI of PD-1 in spleens and draining lymph nodes (Figure 28B). No significant difference in the fraction of PD-1⁺CD8⁺T cells or PD-1 level was observed in CD8⁺ TILs (Figure 28B) after treatment with anti-CD5 MAb.

CD4⁺ T cell

A.



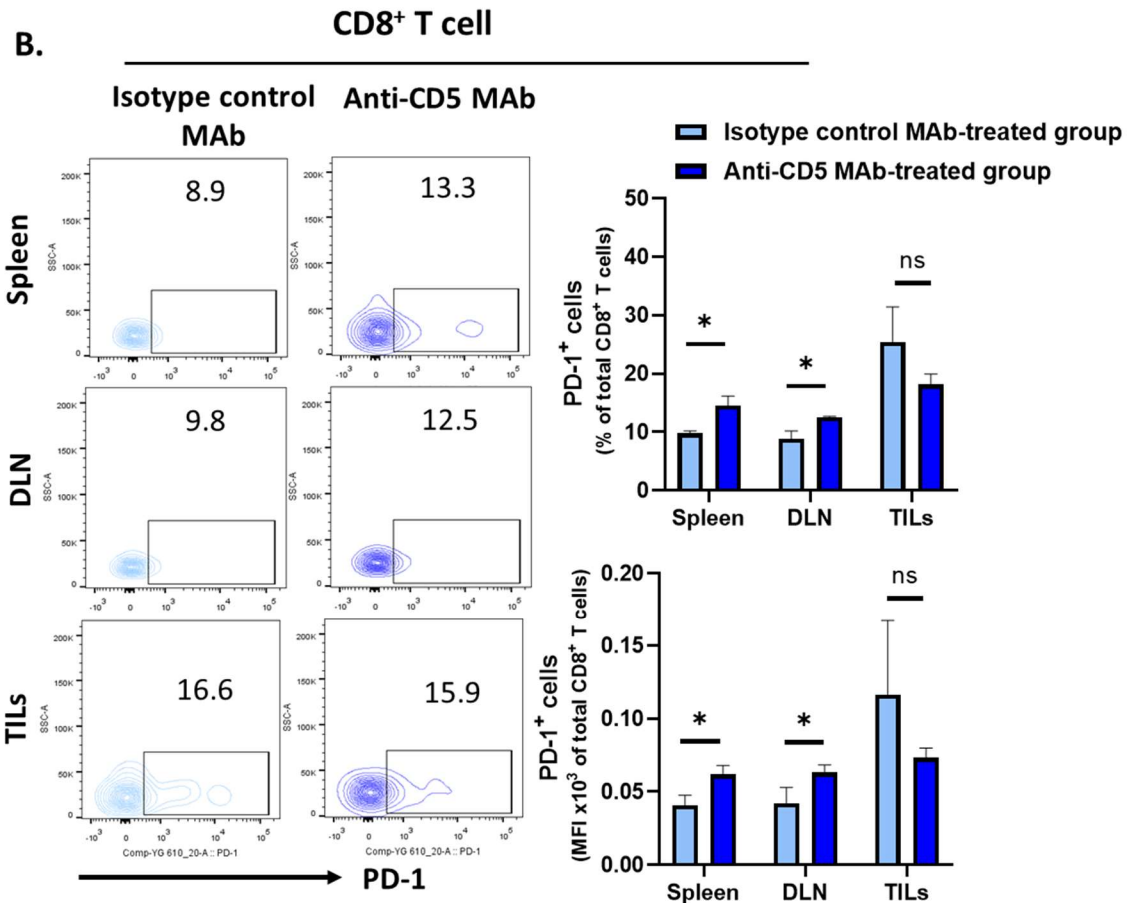


Figure 28. The fraction of CD8⁺PD-1⁺ T and CD4⁺PD-1⁺ T cells after treatment with anti-CD5 MAb *in vivo*. **(A)** The fraction of CD4⁺PD-1⁺ T cells and the MFI (mean fluorescence intensity) of PD-1 on CD4⁺ T cells isolated from spleens, draining lymph nodes, and TILs isolated from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb or isotype control MAb. **(B)** The fraction of CD8⁺PD-1⁺ T cells and MFI of PD-1 on CD8⁺ T cells isolated from spleens, draining lymph nodes, and TILs isolated from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb or isotype control MAb. Data are mean ± SD ($n = 3$ mice), one representative experiment of three, * $p < 0.05$ (Student's unpaired one-tailed t -test), MFI: mean fluorescence intensity.

5.2.4 Increased activation-induced cell death and level of Fas receptor on CD8⁺ T cells after anti-CD5 MAb treatment

Increased T cell activation after CD5 blockade can lead to activation-induced cell death (AICD). To assess whether anti-CD5 MAb treatment resulted in increased T cell AICD, cells isolated from draining lymph nodes and TILs were stained with FITC-annexin V and PI and anti-Fas receptor MAb. The fraction of CD8⁺ T cells undergoing AICD (Annexin V⁺/PI⁺) and early apoptosis (Annexin V⁺/PI⁻) was increased among CD8⁺ T cells in draining lymph nodes and tumours from anti-CD5-treated mice (Figure 29). Because Fas receptor/Fas ligand interaction is important for AICD (185), I further determined the level of Fas receptor on T cells. There was an increased level of Fas receptor on CD4⁺ T cell in draining lymph nodes (Figure 30A). Furthermore, treatment with anti-CD5 MAb also induced an increase in the level of Fas on CD8⁺ T cells from draining lymph nodes and tumours, but not spleens, from anti-CD5-treated mice (Figure 30B).

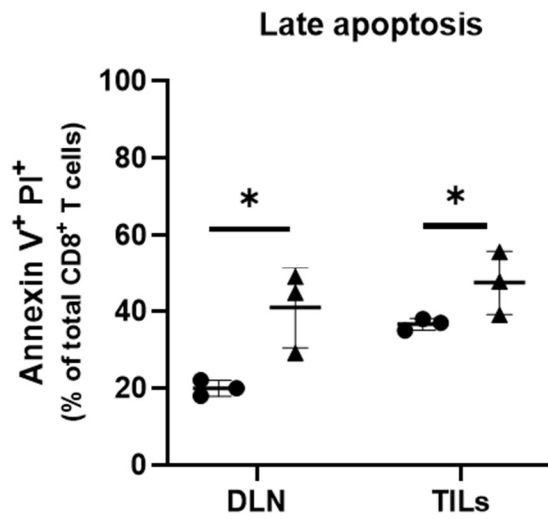
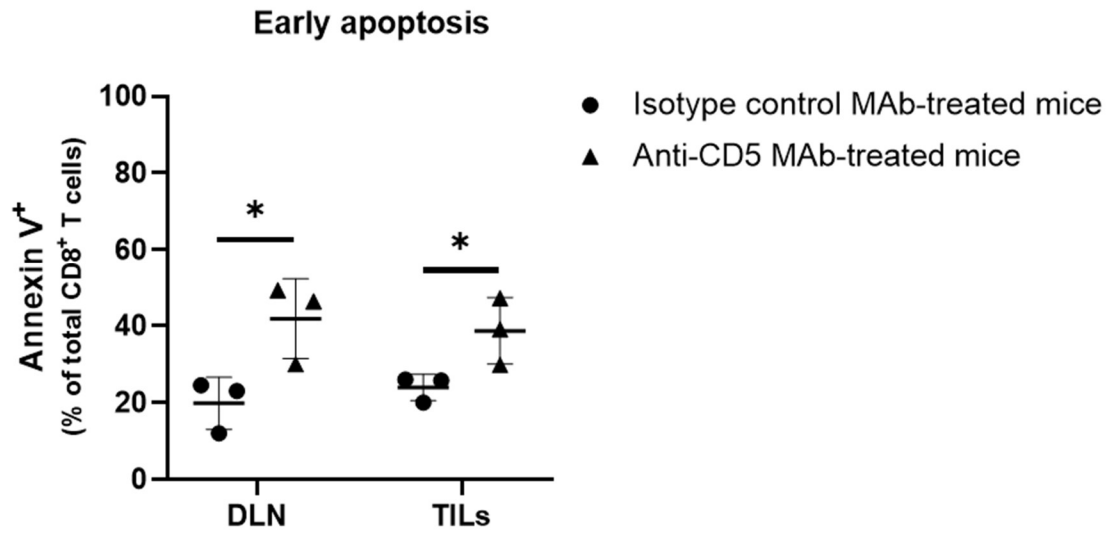
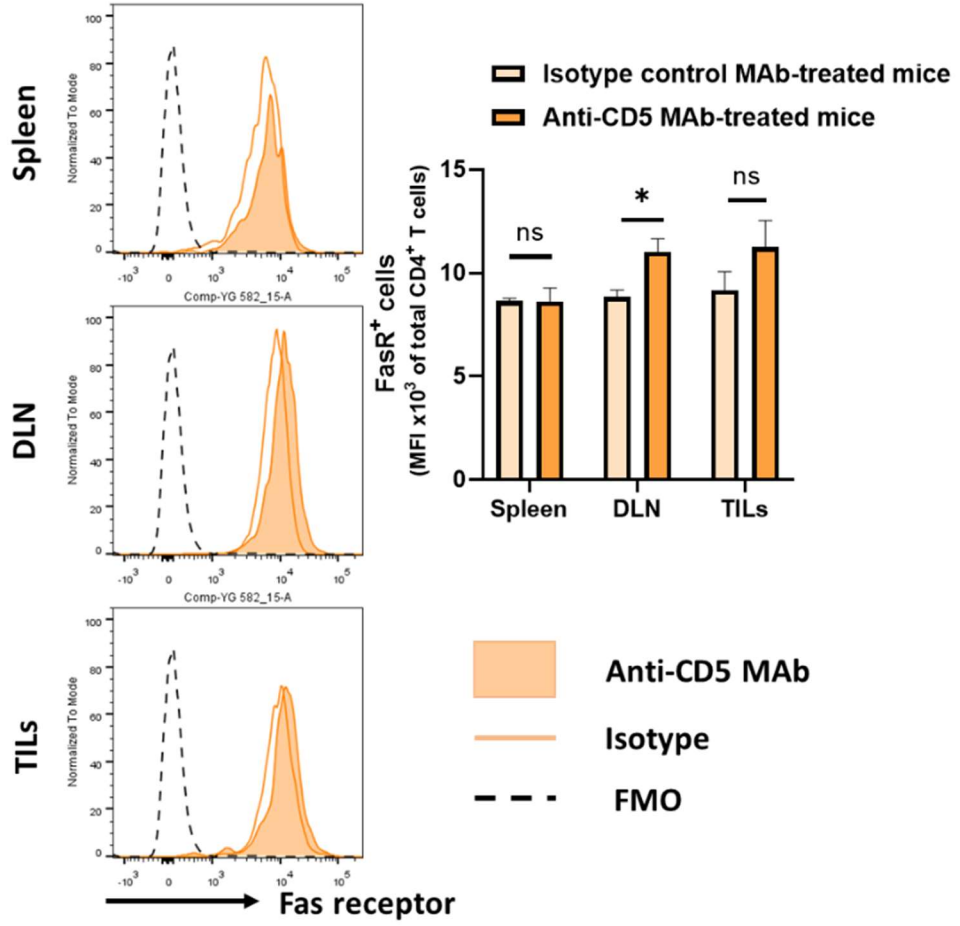


Figure 29. Increased early apoptosis of CD8⁺ T cells apoptosis (upper graph) and late apoptosis (low graph) after treatment with anti-CD5 MAb.

Draining lymph nodes (DLN) and TILs from tumour-bearing BALB/c mice that were treated with anti-CD5 MAb, stained for Annexin V and/or PI, and analyzed by flow cytometry. Data are mean \pm SD ($n = 3$ mice), one representative experiment of two. * $p < 0.05$, (Student's unpaired one-tailed t -test).

A.

CD4⁺ T cell



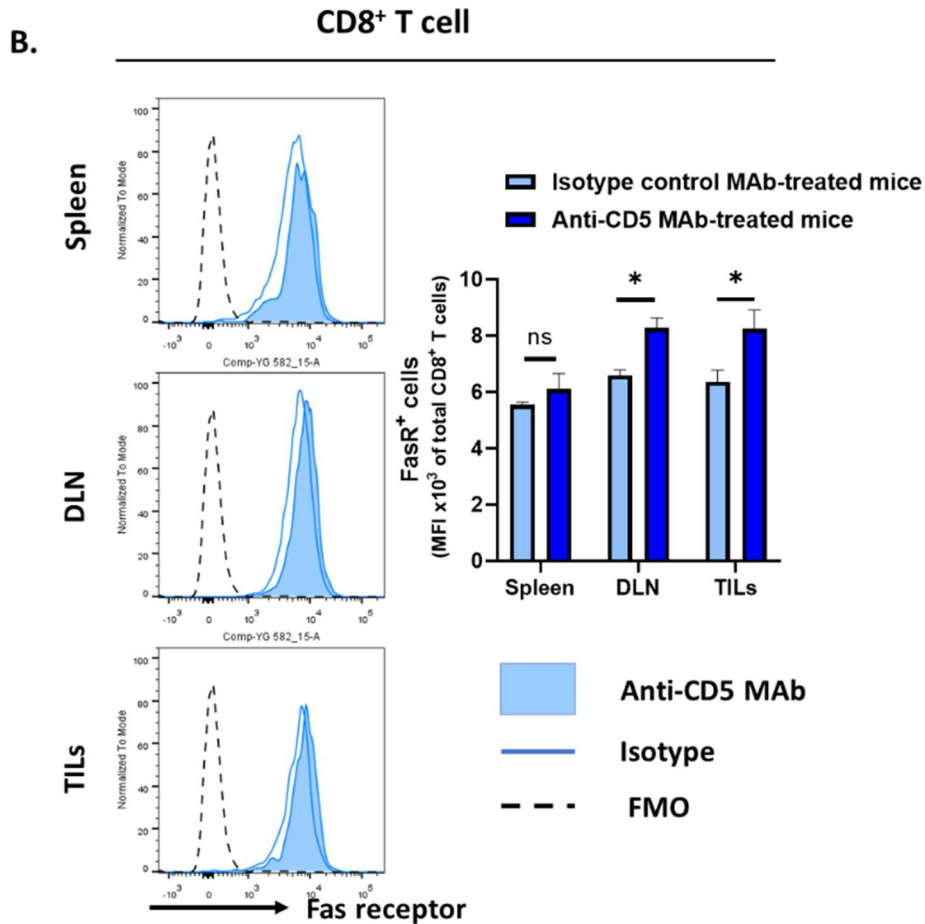


Figure 30. The level of Fas receptor on CD4⁺ T cell and CD8⁺ T cells after treatment with anti-CD5 MAb *in vivo*. (A) The MFI of Fas receptor on CD4⁺ T cells isolated from spleens, draining lymph nodes, and tumours (TILs) excised from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb or isotype control MAb. (B) The MFI of Fas receptor on CD8⁺ T cells isolated from spleens, draining lymph nodes, and tumours (TILs) isolated from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb or isotype control MAb. Data are mean \pm SD ($n = 3$ mice), one representative experiment of three, ns, not significant, $*p < 0.05$ (Student's unpaired one-tailed *t*-test), FMO = Fluorescence Minus One Control, MFI: mean fluorescence intensity.

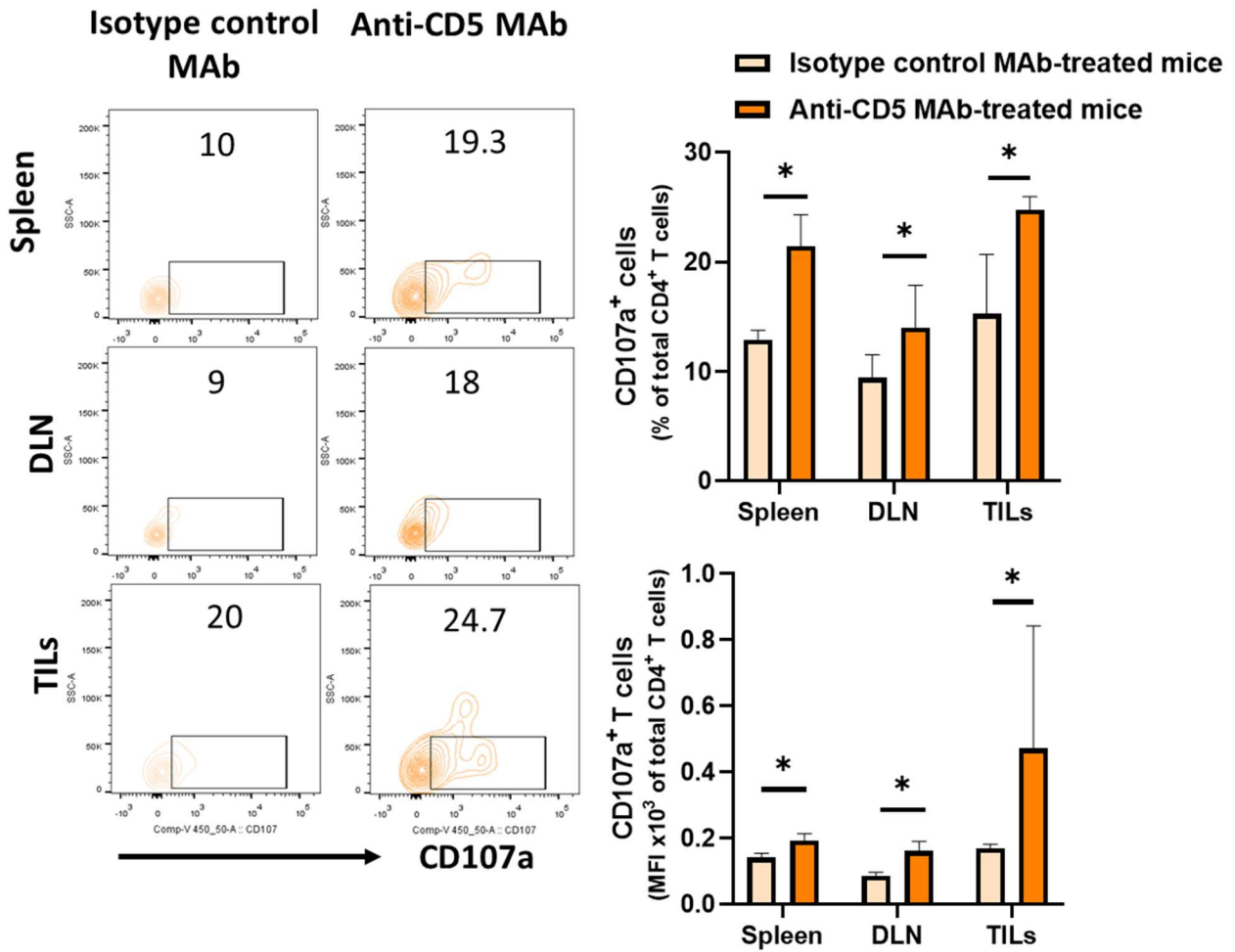
5.2.5 Enhanced T cell tumour reactivity and degranulation after treatment with anti-CD5 MAb *in vivo*

I further determined cytotoxic T lymphocyte (CTL) effector function after treatment with anti-CD5 MAb *in vivo*. CTL effector function was assessed by flow cytometric measurement of a surrogate marker for CTL degranulation: the level of CD107a (188). The fraction of CD107a⁺CD4⁺ T cells and the mean fluorescence intensity of CD107a were significantly higher in spleens, draining lymph nodes, and tumours (TILs) from anti-CD5 MAb-treated mice (Figure 31A). Furthermore, the fraction of CD107a⁺CD8⁺ T cells among all CD8⁺ T cells in both spleens and draining lymph nodes (DLN) from mice treated with anti-CD5 MAb was significantly increased compared to mice treated with isotype control MAb (Figure 31B). The MFI was also significantly higher in spleens, DLN, and TILs in anti-CD5 MAb-treated mice (Figure 31B). In addition, antigen-specific activation of T cells was assessed using a surrogate marker for antigen-specific T cell activation: CD137, a member of the TNFR family with costimulatory function (189). Anti-CD5 MAb-treated mice had an increased fraction of CD137⁺CD4⁺ T cells in spleens and draining lymph nodes but not among TILs (Figure 32A). The MFI of CD137 was up-regulated in CD4⁺ TILs after treatment with anti-CD5 MAb (Figure 32A). Moreover, there was a significant increase in the fraction of CD137⁺CD8⁺ T cells in spleens and among TILs (Figure 32B). The numerical increase in the fraction of CD137⁺CD8⁺ T cells in DLN was not significant (Figure 32B). The MFI of CD137 was significantly higher in CD8⁺ T cells in spleens, DLN, and among TILs from anti-CD5 MAb-treated mice (Figure 32B). Together these

data suggest antigen-specific and effector functions of CD8⁺ T cells and CD4⁺ T cells are enhanced after treatment with anti-CD5 MAb.

CD4⁺ T cell

A.



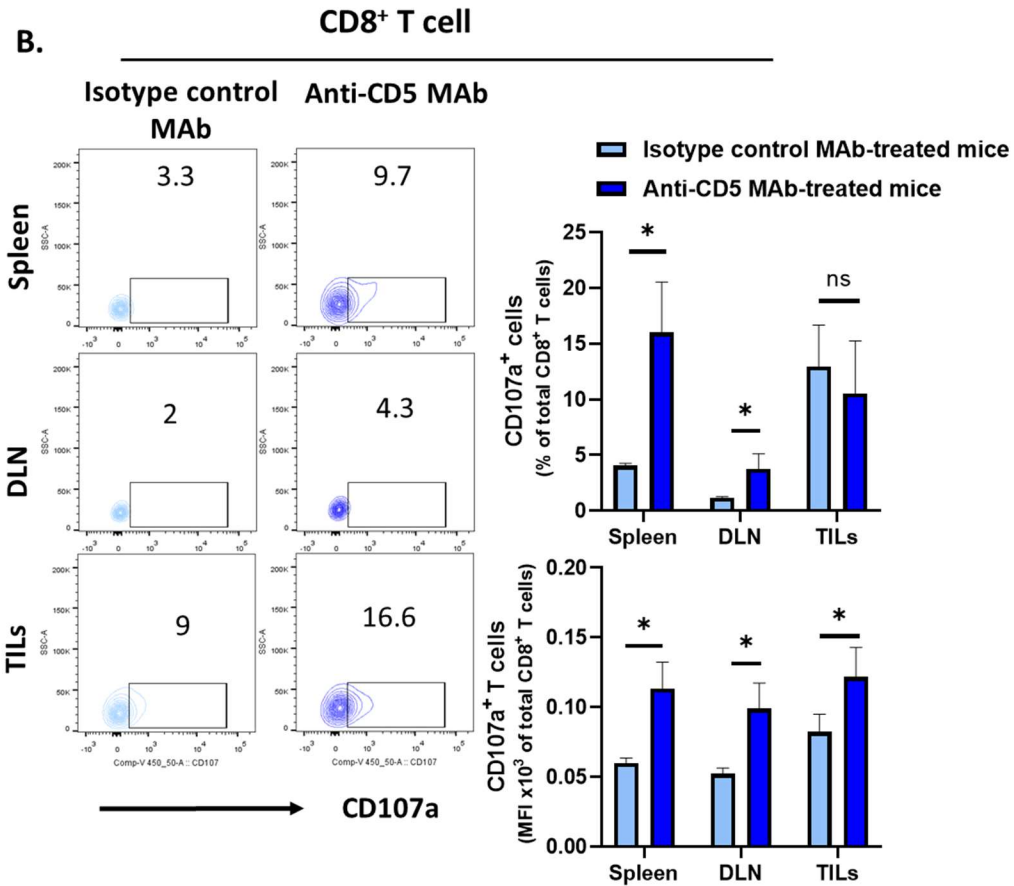
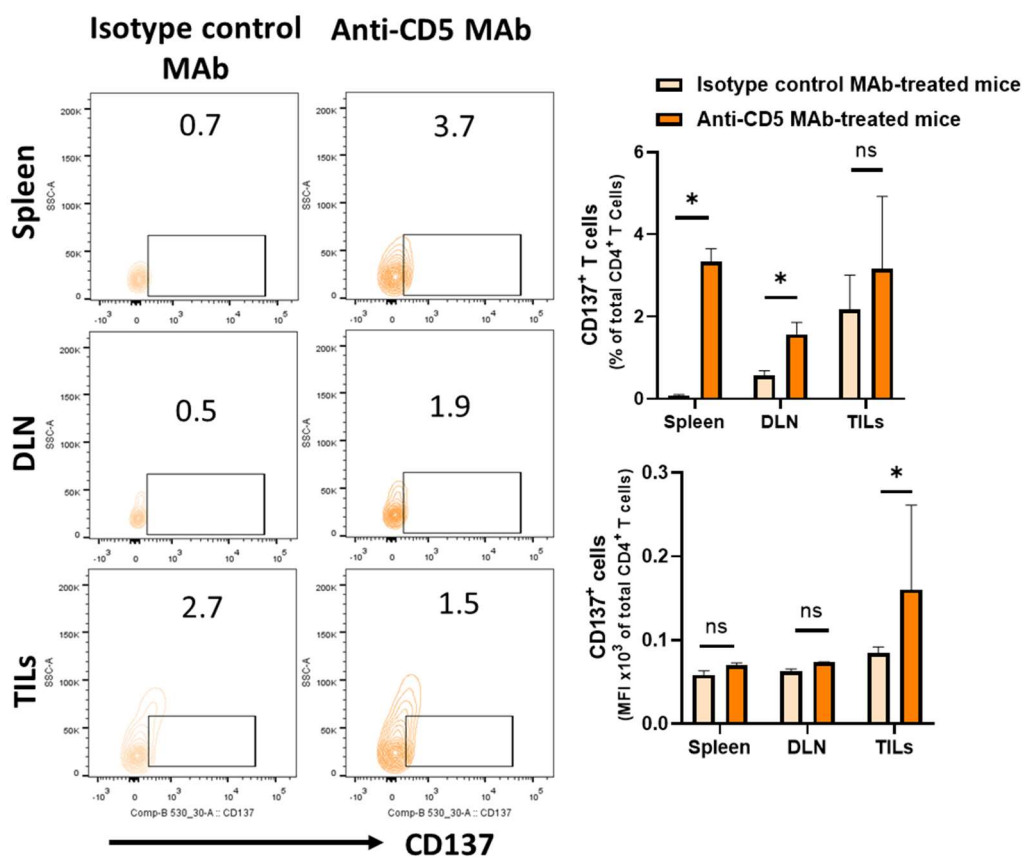


Figure 31. The fraction of CD8⁺CD107a⁺ T and CD4⁺CD107a⁺ T cells after treatment with anti-CD5 MAb *in vivo*. (A) The fraction of CD4⁺CD107a⁺ T cells and MFI of CD107a on CD4⁺ T cells isolated from spleens, draining lymph nodes, and TILs from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb or isotype control MAb. (B) The fraction of CD8⁺CD107a⁺ T cells and MFI of CD107a on CD8⁺ T cells isolated from spleens, draining lymph nodes, and TILs isolated from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb or isotype control MAb. Data are mean \pm SD ($n = 3$ mice), one representative experiment of three, ns, not significant, $*p < 0.05$ (Student's unpaired one-tailed *t*-test), MFI: mean fluorescence intensity.

CD4⁺ T cell

A.



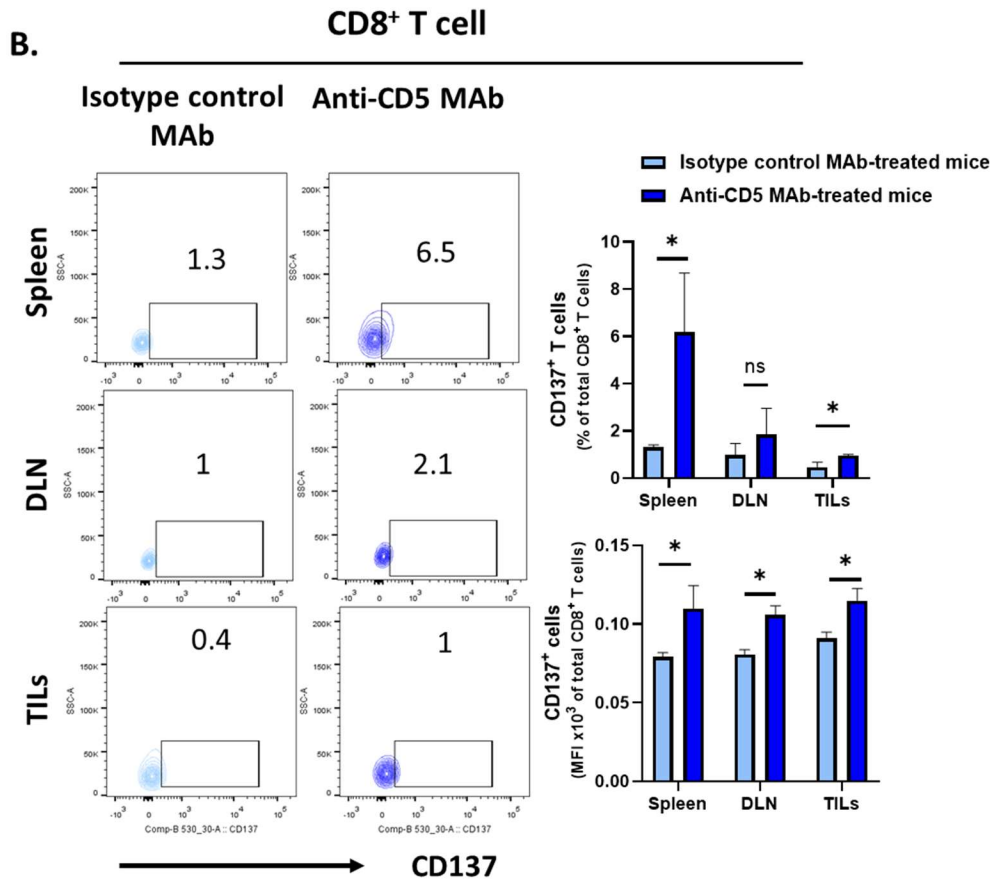


Figure 32. The fraction of CD8⁺CD137⁺ T and CD4⁺CD137⁺ T cells after treatment with anti-CD5 MAb *in vivo*. (A) The fraction of CD4⁺CD137⁺ T cells and MFI of CD137 on CD4⁺ T cells isolated from spleen, draining lymph nodes, and tumours (TILs) from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb or isotype control MAb. (B) The fraction of CD8⁺CD137⁺ T cells and MFI of CD137 on CD8⁺ T cells isolated from spleens, draining lymph nodes, and tumours (TILs) from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb or isotype control MAb. Data are mean ± SD (*n* = 3 mice), one representative experiment of three, ns, not significant, **p* < 0.05 (Student's unpaired one-tailed *t*-test), MFI: mean fluorescence intensity.

5.2.6 No overt toxicity is associated with anti-CD5 MAb treatment *in vivo*

To determine whether *in vivo* administration of anti-CD5 MAb has any overt toxicity, I measured body weight for all groups of mice from the first day of antibody injection and monitored the weight over the course of the treatment. No toxicity by this relatively crude measure (decreased body weight compared to mice treated with isotype control MAb) was observed as indicated by the body weight when mice received single treatments or when they received a combination of anti-CD5 MAb + anti-PD-1 MAb (Figure 33).

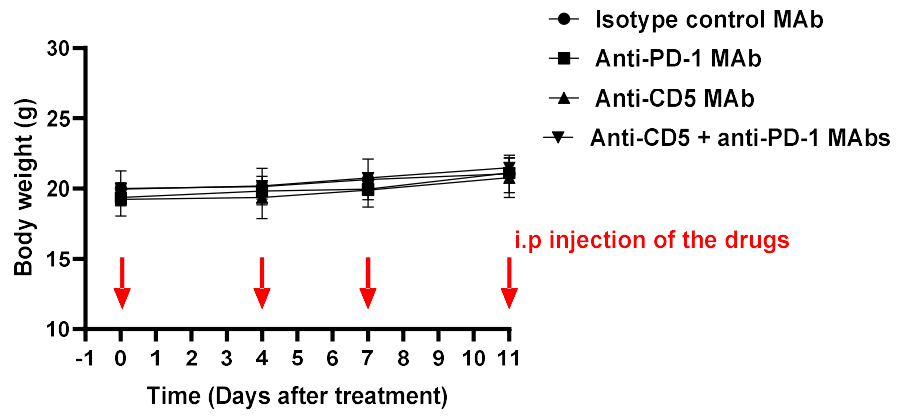


Figure 33. Body weight measurements. BALB/c mice bearing 4T1 tumours were treated with the indicated therapeutic modalities. Their body weights were measured and recorded regularly after treatments. Means and SDs of data are shown, ($n = 6$ mice).

5.2.7 Mice treated with anti-CD5 MAb had significant accumulation of T_{reg} cells in tumour-draining lymph nodes

Accumulation of myeloid-derived suppressor cells (MDSC) has been shown to suppress immunotherapy (64). In my model, I investigated the fraction of MDSC in spleens, DLN, and tumours (TILs) to determine if they were influenced by treatment with anti-CD5 MAb. The percentage of MDSC (Gr-1⁺CD11b⁺) among all cells from spleens and tumours was significantly higher in mice harbouring 4T1 tumour cells compared to naïve mice but was not increased by treatment with anti-CD5 MAb (Figure 34A). The increase in MDSC in tumour-bearing mice was observed only in spleen and in tumours and not in lymph nodes (Figure 34A). T_{regs} (CD4⁺/CD25⁺/FoxP3⁺) were also assessed, with an increase in the fraction of T_{regs} among all CD4⁺ T cells in the draining lymph nodes of mice treated with anti-CD5 MAb (Figure 34B). No differences in the fraction of T_{regs} were observed in spleens or tumours.

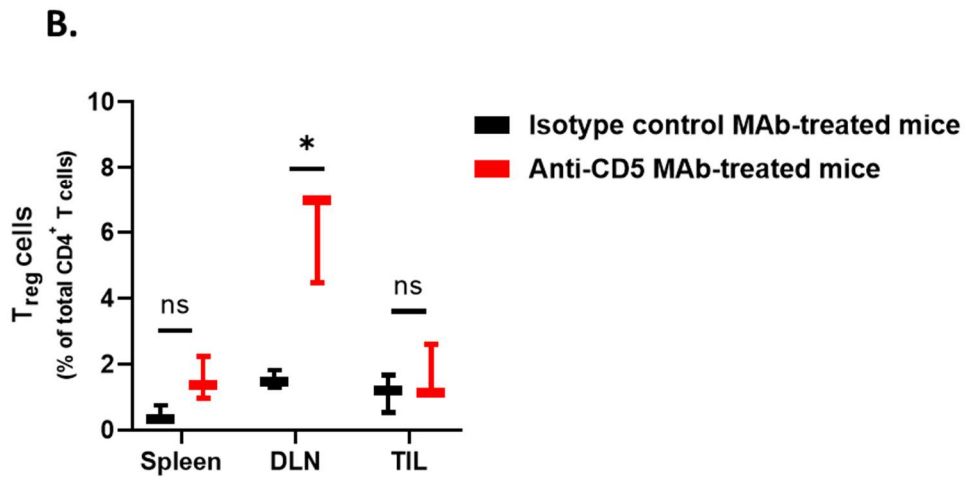
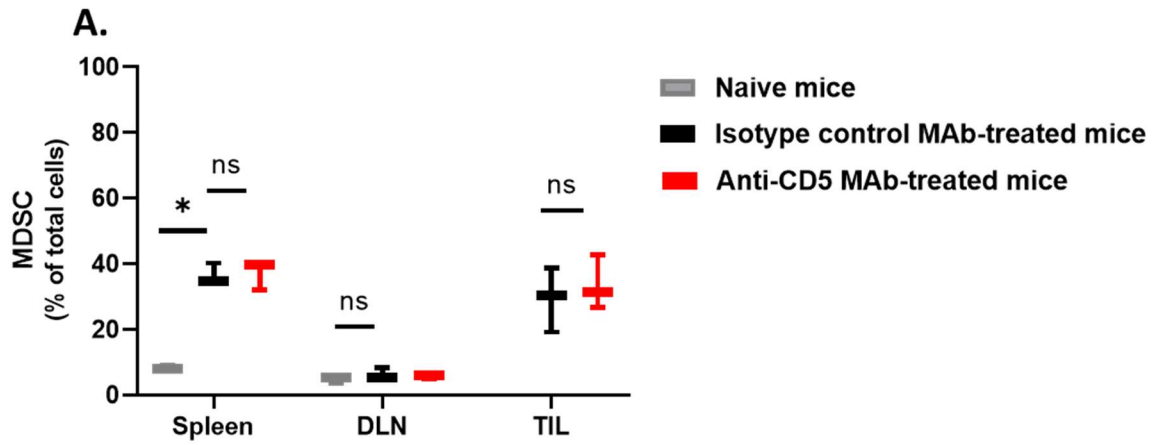


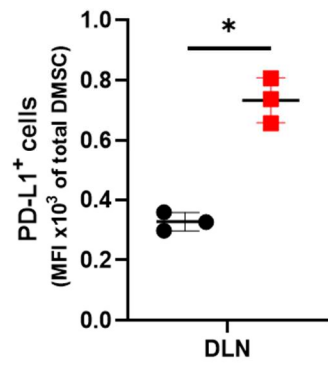
Figure 34. The fraction of T_{reg} and MDSC after treatment with anti-CD5 MAb.

(A) Spleens, lymph nodes, and tumours were collected at day 21 after 4T1 implantation and treatment with anti-CD5 MAb and the fraction of MDSC in those organs assessed by flow cytometry. **(B)** Spleens, lymph nodes, and tumours were collected at day 21 after 4T1 implantation and treatment with anti-CD5 MAb and the fraction of T_{reg} cells was assessed by flow cytometry. Data are mean \pm SD ($n = 3$ mice), one representative experiment of three, ns, not significant, $*p < 0.05$ (Student's unpaired one-tailed t -test).

5.2.8 Myeloid-derived suppressor cells express higher levels of inhibitory ligands in mice treated with anti-CD5 MAb

Although accumulation of MDSC was not significantly enhanced after treatment with anti-CD5 MAb, the levels of inhibitory ligands for PD-1 and Fas receptor were significantly higher in draining lymph nodes in mice treated with anti-CD5 MAb. The MFI of PD-L1 was higher in draining lymph nodes from mice treated with anti-CD5 MAb compared to the mice treated with isotype control MAb (0.3×10^3 versus 0.7×10^3 , respectively, $p < 0.05$)(Figure 35A). In addition, the MFI of Fas ligand on MDSC from draining lymph nodes isolated from mice treated with anti-CD5 MAb was significantly higher compared to mice treated with isotype control MAb (0.14×10^3 versus 0.23×10^3 , respectively, $p < 0.05$)(Figure 35B). No differences were observed in the levels of inhibitory ligands in T_{regs} in the same mice (data not shown).

A.



B.

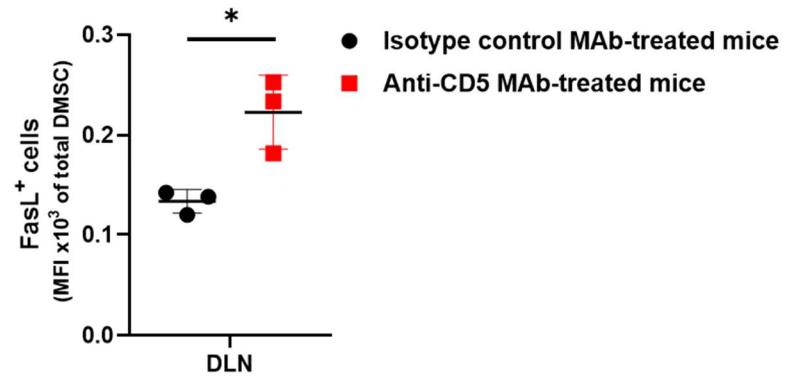
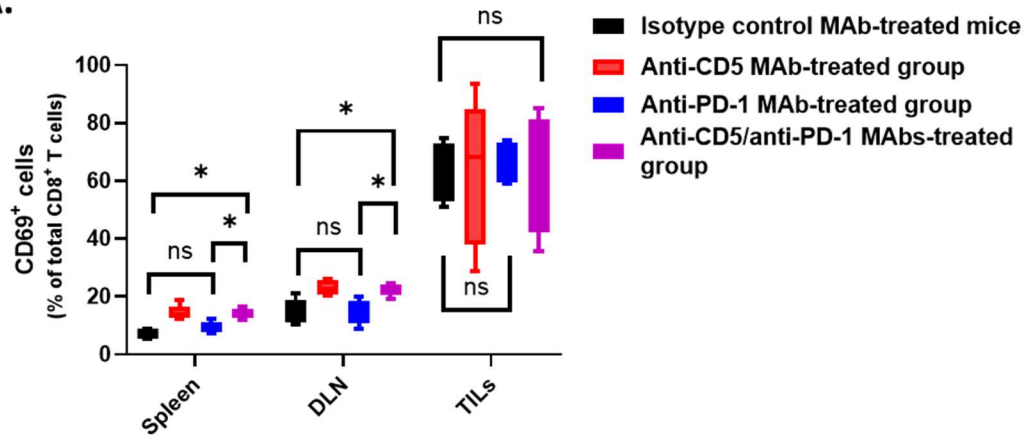


Figure 35. Level of PD-L1 and Fas ligand on MDSC. Draining lymph nodes were collected at day 21 after 4T1 implantation and treatment with anti-CD5 MAb. The level of PD-L1 and Fas ligand on MDSC were assessed by flow cytometry. **(A)** PD-L1 MFI on MDSC isolated from draining lymph nodes. **(B)** Fas ligand MFI on MDSC isolated from draining lymph nodes. Data are mean \pm SD ($n = 3$ mice), one representative experiment of three, $*p < 0.05$ (Student's unpaired one-tailed t -test), MFI: mean fluorescence intensity.

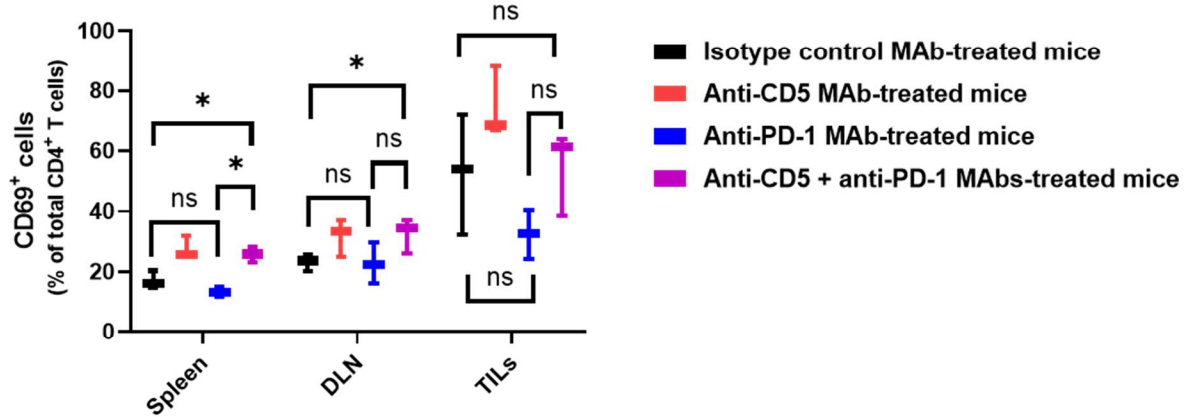
5.2.9 Increased fraction of activated CD8⁺ T cells in mice treated with anti-CD5 + anti-PD-1 MAbs

PD-1 blockade decreases exhaustion in effector CD8⁺ T cells but does not increase activation. To investigate whether *in vivo* administration with anti-CD5 MAb could enhance CD8⁺ T cell activation during anti-PD-1 treatment, I combined anti-CD5 and anti-PD1 treatment of mice (For treatment design see Figure 36C). The results show that addition of anti-CD5 MAb in combination with treatment with anti-PD-1 MAb significantly increased CD8⁺ T cell activation in both spleens and draining lymph nodes compared to single treatment with anti-PD-1 MAb (14.2% vs 9.5% and 22.2% vs 14.7%, respectively; $p < 0.05$; Figure 36A). Furthermore, additional treatment of anti-CD5 MAb in combination with treatment with anti-PD-1 MAb significantly increased CD4⁺ T cell activation in both the spleen and draining lymph nodes compared to single treatment with anti-PD-1 MAb (25.7 vs 13.3% and 32.6% vs 22.7%, respectively; $p < 0.05$; Figure 36B). These data suggest that addition blockade of anti-CD5 enhances CD8⁺ T cell activation during anti-PD-1 blockade.

A.



B.



C.

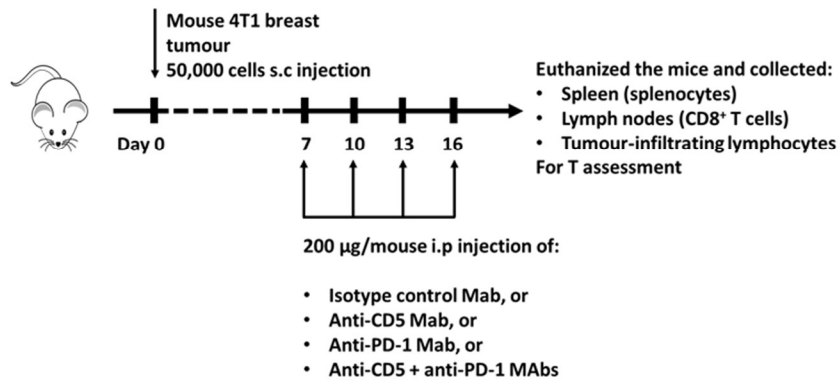
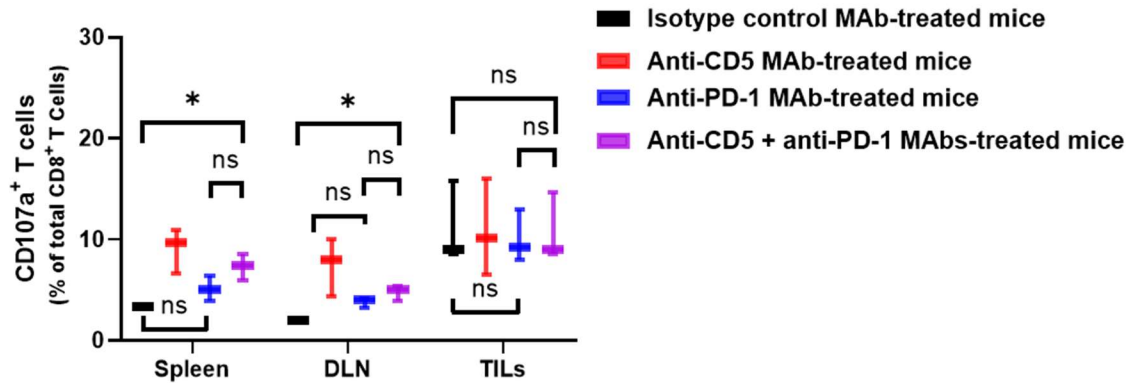


Figure 36. The fraction of CD8⁺CD69⁺ T and CD4⁺CD69⁺ T cells after treatment with anti-CD5 MAb and anti-PD1 *in vivo*. **(A)** The fraction of CD8⁺CD69⁺ T cells in spleens, draining lymph nodes, and tumours (TILs) from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb and anti-PD-1 MAb. **(B)** The fraction of CD4⁺CD69⁺ T cells from spleens, draining lymph nodes, and tumours (TILs) isolated from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb and anti-PD-1 MAb. **(C)** treatment design. Data are mean \pm SD ($n = 3$ mice), one representative experiment of three, * $p < 0.05$, ns, not significant (Student's unpaired one-tailed t -test).

5.2.10 Increased fraction of effector CD8⁺ T cells after anti-CD5 and anti-PD-1 combination therapy

I further assessed whether addition of anti-CD5 MAb to anti-PD-1 MAb could increase effector T cell activation. I observed an increase in the fraction of CD107a⁺CD8⁺ T cells in spleens and draining lymph nodes in mice treated with anti-CD5 + anti-PD-1 MAbs (Figure 37A). Furthermore, the fraction of CD107a⁺CD4⁺ T cells isolated from spleens and draining lymph nodes was higher in mice treated with combination therapy (anti-CD5 + anti-PD-1 MAbs)(Figure 37B). In addition, the surrogate marker for antigen-specific CD8⁺ T cells (CD137) was assessed. The data show that the fraction of CD137⁺CD8⁺ T cells isolated from spleens and draining lymph nodes was significantly higher in mice treated with anti-CD5 MAb and anti-PD-1 MAb compared to single treatment alone (Figure 38A). The fraction of CD137⁺CD4⁺ T cells isolated from spleens and draining lymph nodes was also significantly higher in mice treated with anti-CD5 and anti-PD-1 compared to treatment with each of the MAbs alone (Figure 38B).

A.



B.

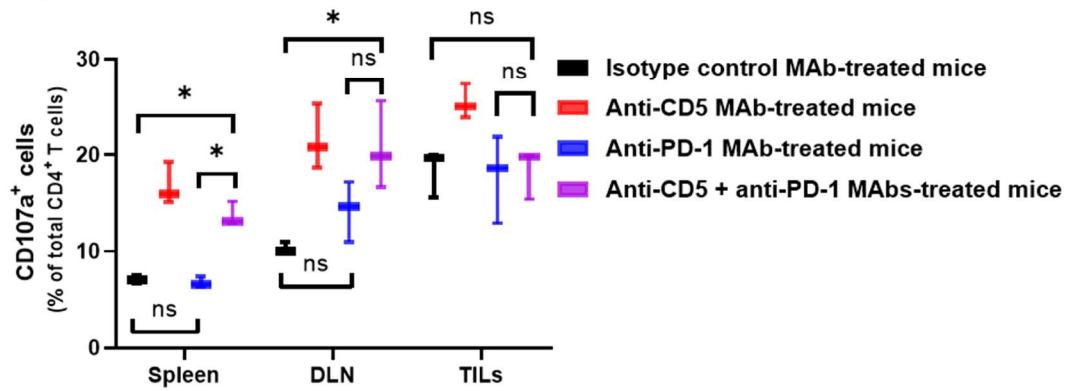
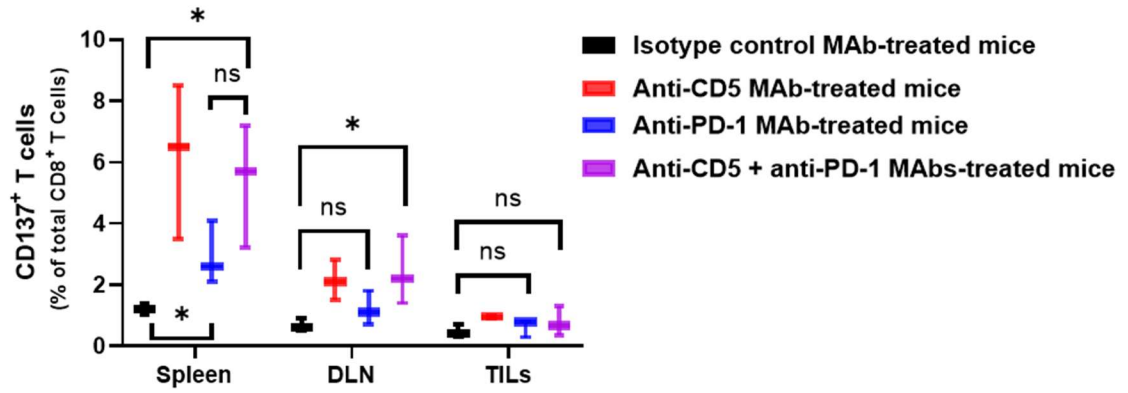


Figure 37. The fraction of CD8⁺CD107a⁺ T and CD4⁺CD107a⁺ T cells after treatment with anti-CD5 MAb and anti-PD1 *in vivo*. (A) The fraction of CD8⁺CD107a⁺ T cells isolated from spleens, draining lymph nodes, and tumours (TILs) isolated from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb and anti-PD-1 MAb. (B) The fraction of CD4⁺CD107a⁺ T cells from spleens, draining lymph nodes, and tumours (TILs) isolated from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb and anti-PD-1 MAb. Data are mean \pm SD ($n = 3$ mice), one representative experiment of three, ns, not significant, $*p < 0.05$ (Student's unpaired one-tailed *t*-test).

A.



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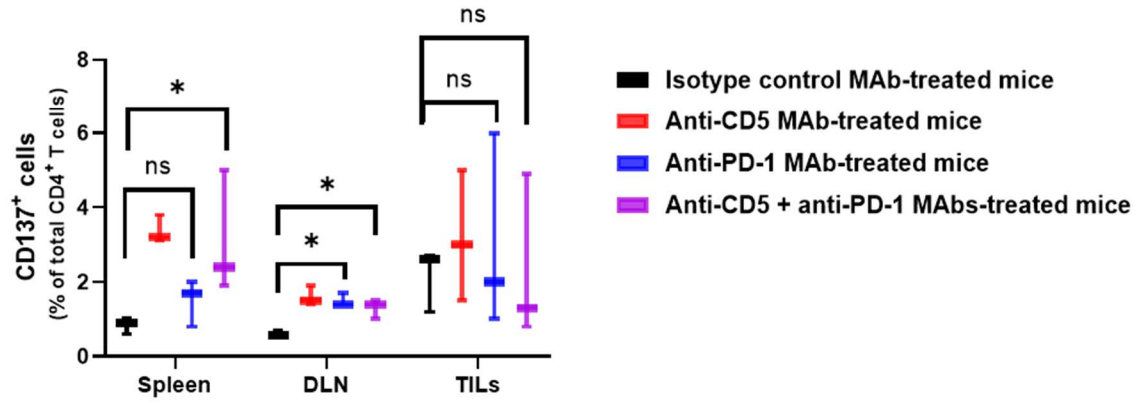


Figure 38. The fraction of CD8⁺CD137⁺ T and CD4⁺CD137⁺ T cells after treatment with anti-CD5 MAb and anti-PD1 *in vivo*. (A) The fraction of CD8⁺CD137⁺ T cells isolated from spleens, draining lymph nodes, and tumours (TILs) isolated from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb and anti-PD-1 MAb. (B) The fraction of CD4⁺CD137⁺ T cells from spleens, draining lymph nodes, and tumours (TILs) isolated from 4T1 tumour-bearing BALB/c mice that were treated with anti-CD5 MAb and anti-PD-1 MAb. Data are mean \pm SD ($n = 3$ mice), one representative experiment of three, * $p < 0.05$, ns, not significant (Student's unpaired one-tailed *t*-test).

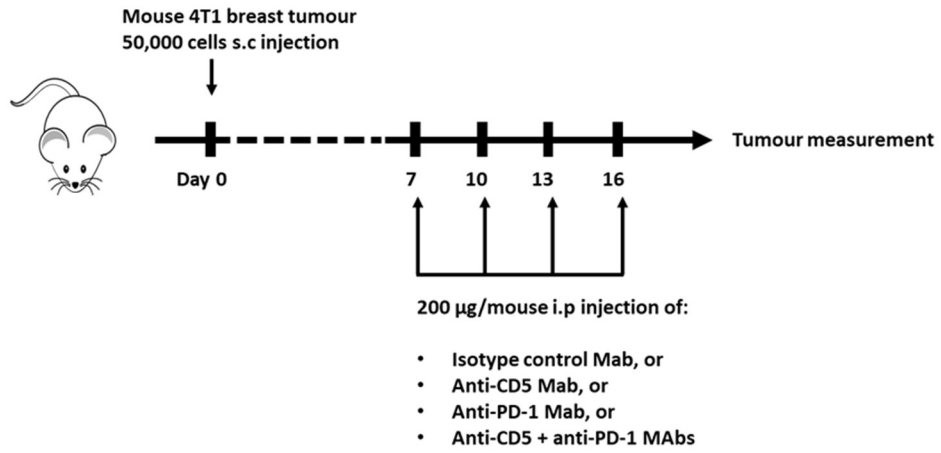
5.2.11 Treatment with anti-CD5 MAb *in vivo* reduced 4T1 tumour growth in mice

To further test the hypothesis, I used anti-CD5 MAb as a single agent, combined with anti-PD-1 MAb, and combined with chemotherapeutic treatment in mice bearing 4T1 tumours to examine their affect in suppressing tumour growth. I found that treatment with anti-CD5 alone did not reduce tumour growth (Figure 39A for treatment design; B for tumour volume). I further tested anti-CD5 in combination with anti-PD-1 MAb and found no significant delay in tumour growth (Figure 39A and B). I then reduced the number of dosages to one injection only of anti-CD5 MAb followed by three dosage of anti-PD-1 MAb and found a trend in tumour delay that was not reproducible (Figure 40A for treatment design; B for tumour volume). I have shown previously that the 4T1 model has increased accumulation of MDSCs in spleens and in 4T1 tumours (Figure 34) which are associated with reduced response to immunotherapeutic agents (64). Therefore, MDSC-depleting drugs such as gemcitabine (190) and 5-FU (70) were next tested in combination with anti-CD5 MAb. The data show delayed tumour growth in mice treated with gemcitabine alone, but anti-CD5 MAb treatment did not enhance gemcitabine-mediated anti-tumour activity (Figure 41). I then tested 5-FU in combination with anti-CD5 MAb and changed the route of administration for anti-CD5 MAb from intraperitoneal (i.p.) to peritumoural injection (p.t) to determine if local administration of anti-CD5 MAb resulted in enhanced tumour regression and prevention of overactivation of T cell that might result in T cell

exhaustion and AICD. Similar to gemcitabine, 5-FU alone reduced tumour growth but anti-CD5 MAb treatment did not enhance 5-FU-mediated anti-tumour activity (Figure 42).

Another experiment was conducted to test the capacity of anti-CD5 MAb to affect tumour homograft growth in mice *in vivo* under different conditions: treatment with lower dosages of anti-CD5 MAb and inoculation with a lower number of 4T1 tumour cells (that, regardless, result in 100% tumour take). On day 0, mice were injected with 5000 4T1 tumour cells. In addition, they were treated with anti-CD5 MAb every 3 to 4 days for a total of 11 injections with MAb (euthanasia of mice when those with the largest tumours reached maximal tumour volume allowable according to humane animal management protocols as required by the Western University Animal Care Committee, the Canadian Council on Animal Care, and the approved protocol under which the experiment was conducted). Tumours volumes were estimated as described in *Materials and Methods*. The resulting data are shown in Figure 44. These data indicate that, anti-CD5 MAb administration *in vivo* did reduce 4T1 tumour growth in mice when administrated frequently at lower dosage.

A.



B.

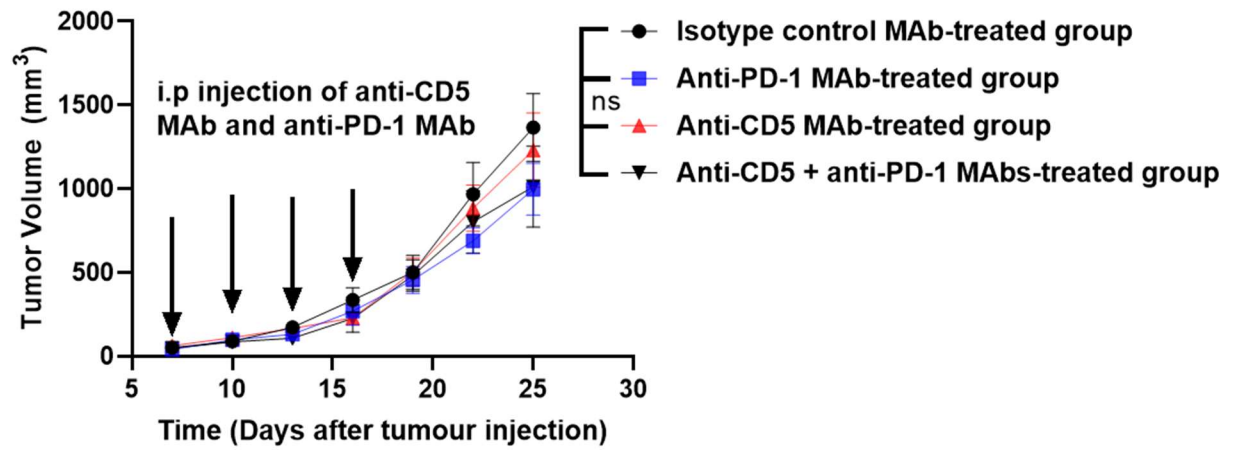
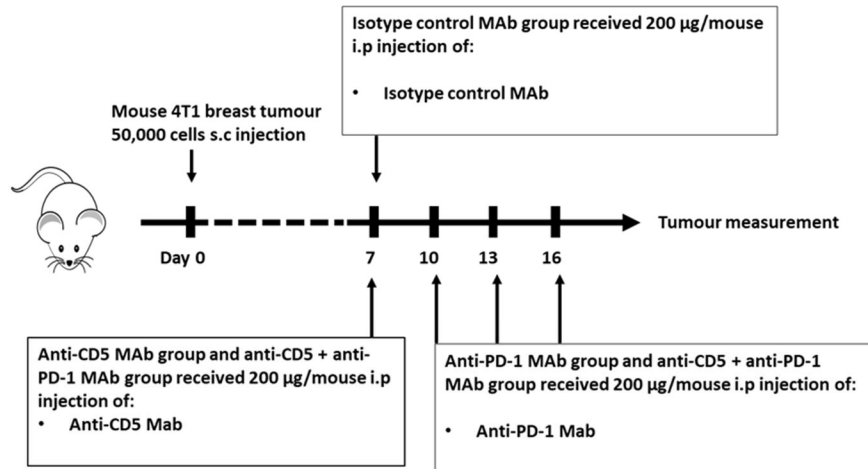


Figure 39: Treatment with anti-CD5 MAb + anti-PD-1 MAb, first treatment design. 4T1 tumour-harboring mice received anti-PD-1 and anti-CD5 MAbs 7 days after tumour inoculation and anti-CD5 MAb every three days. **(A)** Scheme for treatment plan. **(B)** Tumour volume. Data are mean \pm SEM ($n = 5$ mice), one representative experiment of three, ns, not significant (Student's unpaired one-tailed t -test).

A.



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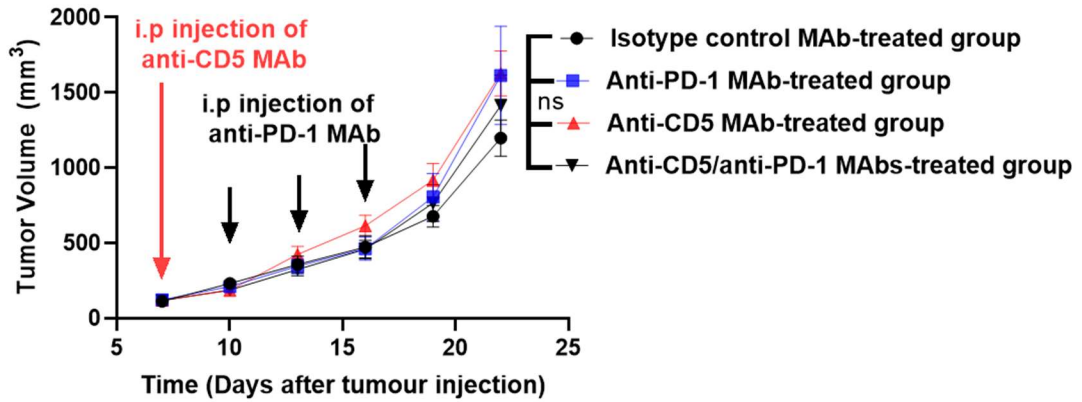
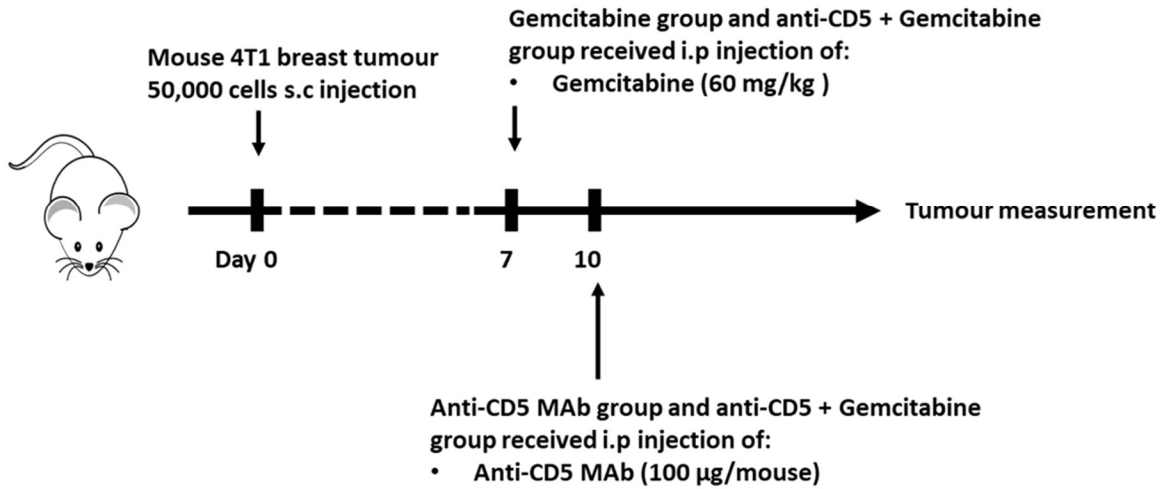


Figure 40: Treatment with anti-CD5 MAb + anti-PD-1 MAb, second treatment design. 4T1 tumour-harboring mice received anti-CD5 MAbs 7 days after tumour inoculation followed by anti-PD-1 MAb every three days. **(A)** Scheme for treatment plan. **(B)** Tumour volume. Data are mean \pm SEM ($n = 6$ mice), one representative experiment of three, ns, not significant (Student's unpaired one-tailed t -test).

A.



B.

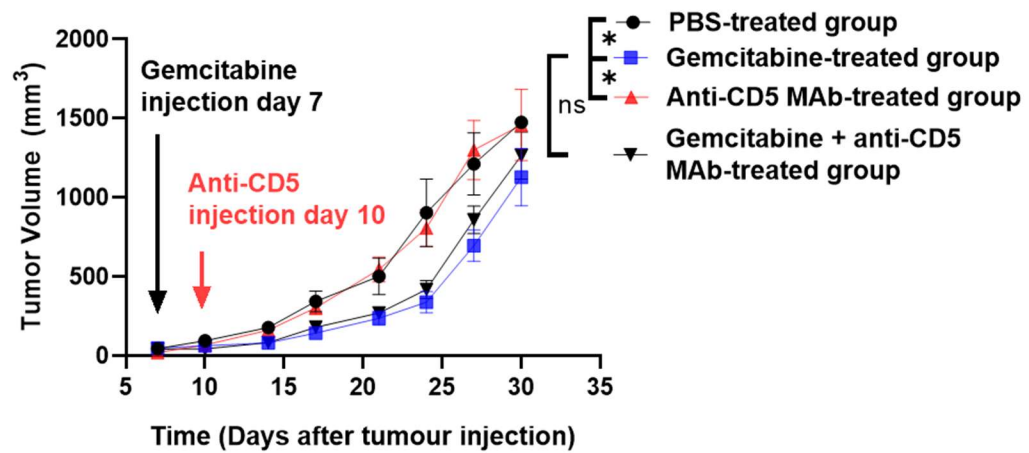
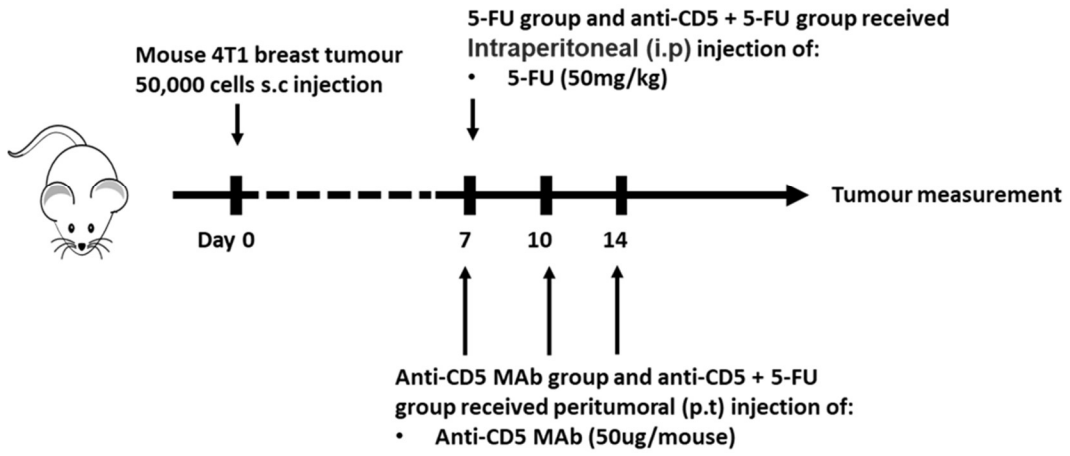


Figure 41: Treatment with anti-CD5 MAb + Gemcitabine. 4T1 tumour-harbouring mice received Gemcitabine 7 days after tumour inoculation and followed by one dosage of anti-CD5 MAb after three days. **(A)** Scheme for treatment plan. **(B)** Tumour volume. Data are mean \pm SEM ($n = 6$ mice), one experiment, ns, not significant, $*p < 0.05$ (Student's unpaired one-tailed t -test).

A.



B.

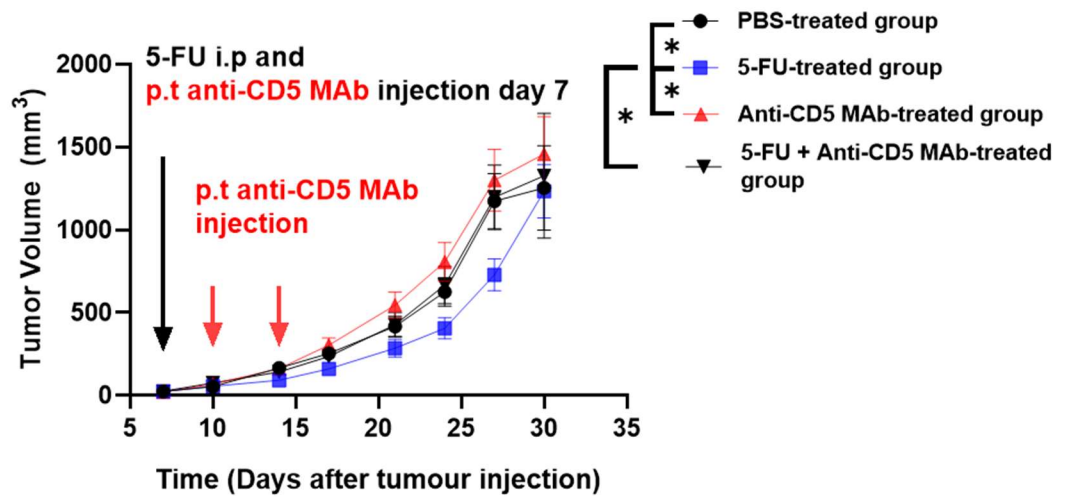


Figure 42: Treatment with anti-CD5 MAb + 5-FU. 4T1 tumour-harboring mice received 5-FU and anti-CD5 MAb 7 days after tumour inoculation and anti-CD5 MAb every three days. **(A)** Scheme for treatment plan. **(B)** Tumour volume. Data are mean \pm SEM ($n = 6$ mice), one experiment, $*p < 0.05$ (Student's unpaired one-tailed t -test).

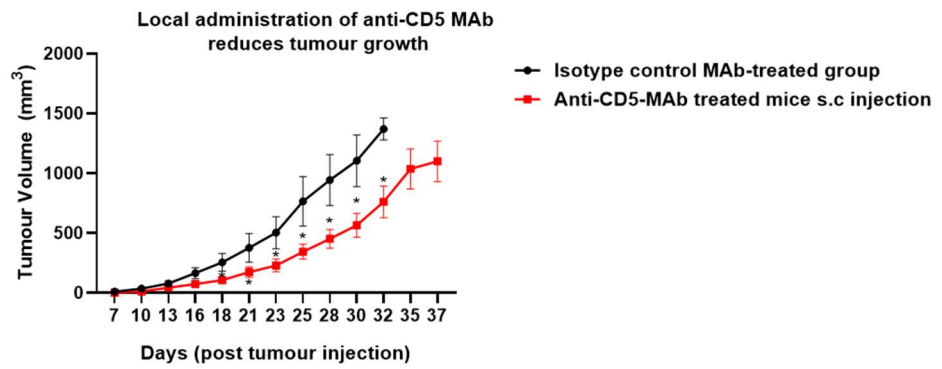
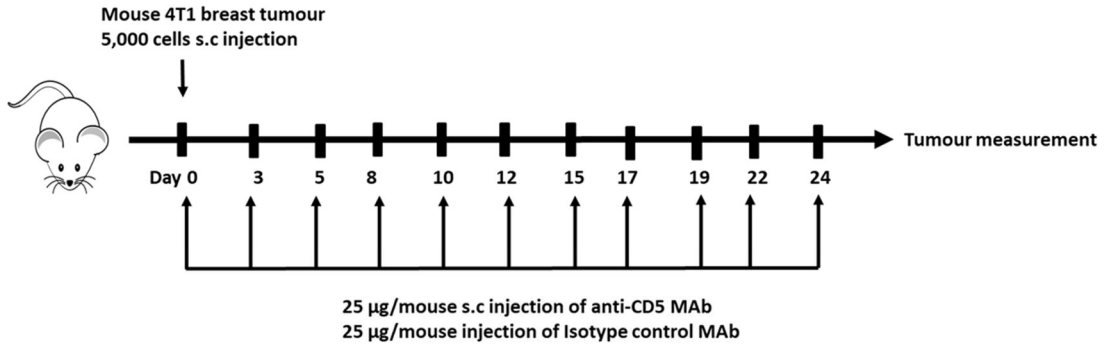


Figure 43: Treatment with low dosage of anti-CD5 MAb. 4T1 tumour-harbouring mice received 25 ug/mouse of anti-CD5 MAb on day 0 along with tumour inoculation and continued to receive the treatment of a total of 11 injections **(A)** Scheme for treatment plan. **(B)** Tumour volume. Data are mean \pm SEM ($n = 7$ mice), one representative experiment of two, $*p < 0.05$ (Student's unpaired one-tailed t -test).

Chapter 6

6.1 Discussion

CD5 is highly expressed on human leukemic cells, including leukemias arising in T and B cell populations (191). As an antigen preferentially and selectively expressed in leukemia, it has been therapeutically targeted in an antibody-drug conjugate approach using anti-CD5 MAb conjugated to toxin (165, 167, 192). More recently, in addition to molecule-based anti-CD5 cancer immunotherapy, cell-based anti-CD5 therapy has been introduced in the form of CAR-T cells engineered to target CD5 on human T and B cells to treat leukemias arising from these immune cell types (129) and a clinical trial has been launched targeting CD5 in CD5⁺ cancer cells using CD5 CAR-T cells (NCT03081910) (193) with recent published result demonstrate the safety and positive outcome with the treatment (illustrated in section 1.8 of this thesis) (168). In addition, CAR-T cells engineered to lack CD5 expression have been generated to prevent fratricide (*i.e.*, CAR T cell-mediated ablation of CD5-expressing effector CAR T cells) and enhance the potential value of anti-CD5 CAR T cell approaches (132).

With respect to CD5 on normal T cells, less is known but data suggest that lack of CD5 activity increases anti-tumour immune surveillance and reduces tumour growth (As illustrated in detail in section 1.6 of this thesis). This suggests that functionally blocking CD5 signalling could lead to increase anti-tumour

immunity and enhanced T cell activation. Thus, CD5 expression on non-solid tumours has been the focus of therapy.

In this thesis, I aimed to understand the relationship between CD5 and T cell activation and exhaustion and investigate the effect of targeting CD5 using monoclonal antibody *ex vivo* and *in vivo* on immune T cell and tumour growth. CD5 has been shown to interact with TCR and impair TCR signaling (89, 105). Previous studies have shown a correlation between CD5 and anti-tumour immunity where CD5 knockout mice inoculated with B16F10 melanoma cells had delayed tumour growth compared to wild type mice (134). However, the correlation between CD5 level and T cell activation and exhaustion in tumours and in peripheral organs is ill-defined and requires further investigation. In Chapter Three of this thesis, I determined the *in vivo* relationship between T cell CD5 level and T cell activation and exhaustion in primary mouse T cells isolated from different organs and syngeneic tumours. I found different patterns of CD5 expression in T cells isolated from different tissues in mice bearing tumour homografts. The differences in CD5 expression patterns correlated with the level of T cell activation and exhaustion in different organs and tumours. First, I determined CD5 levels in T cell subsets isolated from lymphoid organs and the tumour. CD5 expression was observed in T cell subsets including CD4⁺ T cells and CD8⁺ T cells: CD4⁺ T cells were found to express greater amounts of CD5 than CD8⁺ T cells.

CD5 is associated with the TCR/CD3 complex and BCR and has been shown to negatively regulate the TCR signaling pathway (89). Elevated CD5 is

associated with B cell activation (194) and is found to be expressed on certain lymphoid tumours (91). I investigated CD5 levels on mouse CD8⁺ T cells during naïve, *ex vivo* activation and *in vivo* activation to determine their pattern of expression. My data reveal that T cell CD5 levels are enhanced following recognition of specific target (exposure to tumour antigen or stimulation with anti-CD3/anti-CD28 MAbs)(177)). Primary CD8⁺ T cells isolated from mice, activated *ex vivo or in vivo* by exposure to anti-CD3 and anti-CD28 MAbs or tumour cells, had an increased level of CD5 compared to non-activated CD8⁺ T cells. This is consistent with published data linking increased CD5 expression to the strength of T cell receptor signaling and function: a mechanism to fine-tune TCR signaling (89). My results indicate a clear correlation between CD5 and T cell activation. Considering that CD5 is associated with TCR and BCR (76, 195), this agrees with a published report that CD5 is indicator of B cell activation (194). Furthermore, I report that T cell subsets from mouse spleen and lymph nodes display similar CD5 levels. However, the fraction of both CD4⁺ and CD8⁺ T cell subsets infiltrated into tumours had a reduced level of CD5. Although I did not distinguish between the possibilities that T cells with reduced CD5 prior to infiltration into tumours were preferentially recruited into tumours, or that T cells with elevated CD5 infiltrated into tumours but had their CD5 levels reduced once they were within tumours, CD5 levels in T cells have been reported to be induced in correlation with the intensity of antigen recognition by TCR (76). Mouse 4T1 tumours are poorly immunogenic and highly metastatic (64), providing a context conducive to poor antigen recognition leading to low CD5 on T cells within those

tumours. That, along with our observation of reduced CD5 on T cells within the tumour and our previous report that experimental reduction of CD5 leads to increased CD8⁺ T cell activation (177), collectively suggest that T cells with low CD5 within tumours (due wholly or in part to the poorly immunogenic characteristic of 4T1 tumours) may have increased sensitivity to poorly immunogenic 4T1 tumour antigens. These results demonstrated that CD5 may be downregulated in T cells based on the TCR/CD3 signaling intensity within tumours.

I further tested T cell activation in relation to CD5 expression levels in spleens, lymph nodes, and 4T1 tumours. In lymphoid organs, the fraction of CD69⁺CD5^{high} CD4⁺ T and CD69⁺CD5^{high} CD8⁺ T cells were significantly higher than the fraction of CD69⁺CD5^{low} CD4⁺ T and CD69⁺CD5^{low} CD8⁺ T cells, respectively. This suggests that CD5 levels increase on T cells as TCR stimulation proceeds and T cells undergo activation (as evidenced by increased CD69, an early marker of T cell activation). Interestingly, only the CD4⁺ and CD8⁺ T cells in the tumour with low/undetectable CD5 expression exhibited higher levels of activation compared to CD5^{high} TILs: an observation in accord with our previous report that inhibition of CD5 on T cells leads to enhanced T cell activation and proliferation (177) and suggesting that intra-tumoural T cells with naturally-occurring low CD5 are selected for increased presence within 4T1 tumours. Furthermore, CD5 has been reported to protect tumour-reactive circulating T cells from activation-induced cell death (AICD) following recognition of autologous tumour (137). Reduced CD5 levels on T cells within the 4T1

tumour may be evidence of selection of T cells with naturally-occurring low CD5 for enhanced activation and proliferation within tumours: such selection would promote interaction between T cells and tumour cells, enhance the TCR signaling response, and result in increased activation as shown by increased CD69 expression. This is consistent with a report of increased anti-tumour cytolytic activity in T cells with low CD5 levels (136). The mechanism regulating expression *in vivo* is not well understood and it is difficult, with our present level of understanding, to elucidate the reasons underlying our observation of T cells with reduced CD5 within tumours compared to T cells from lymph nodes and spleens. Regardless of that, low CD5 on T cells within tumours can conceivably lead to increased anti-tumour immune activity.

T cell exhaustion is mediated by the duration and magnitude of antigenic activation (196). The expression of PD-1 by T cells is one measurement for T cell exhaustion (197) and binding to its ligand results in limitation of T cell function (198). In this report I show that CD5 might protect T cell from exhaustion in the tumour through downregulation of PD-1. Indeed, our results indicate that the number of PD-1⁺CD5^{-/low}CD8⁺ TILs within tumours was significantly higher than PD-1⁺CD5^{high}CD8⁺ TILs. However, this was not the case with intra-tumoural CD4⁺ TILs, where there was no observation of downregulated CD5 in an increased fraction of this PD-1⁺ subset of T cells in tumours compared to the same subset in lymph and spleen. This may be due to the fact that PD-1 is upregulated when T cells are chronically exposed to non-self tumour antigens (our model of 4T1 breast tumour growth). This role in host defense is fulfilled by

CD8⁺ T cells, making them preferentially susceptible to PD-1 upregulation after exposure to tumour antigen. CD4⁺ T cells, on the other hand, do not recognize tumour cell directly due to the lack of MHC II on solid tumour cells (199).

Therefore, they may not upregulate exhaustion markers when they are activated due to limited interaction with tumour cells.

Taken together, our data suggest that CD5 levels on T cells (particularly CD8⁺ T cells) are correlated with T cell activation and exhaustion, as evidenced by the relationship between CD69 and PD-1: that correlation is evidence supportive of a role for CD5 in T cell survival. CD5 is induced based on the intensity of the TCR-MHC-1 interaction and poorly immunogenic tumour that can lead to T cells with reduced CD5: a situation promoting increased TCR-MHC-I interaction and enhanced T cell activation. A causal role for CD5 in T cell activation, supported by evidence presented here and by published previously (177), forms the basis to propose targeting of CD5 as a therapeutic intervention to enhance anti-tumour immunity. Immune checkpoint blockade is a current immunotherapeutic approach in cancer that is most effective in patients with an increased fraction of PD-1⁺ cells (200). Therapeutic interventions that lead to enhanced activation of T cells in the tumour (for example, interventions to reduce CD5) may be suitable components of combination therapy that include anti-CD5 treatment with immune checkpoint blockade: anti-CD5 would lead to increased PD-1 on tumour-resident T cells that, in turn, would lead to a situation promoting the effectiveness of anti-PD-1 immune checkpoint blockade. The current study provides evidence for a critical role for CD5 in regulating PD-1 expression in

tumour-associated CD8⁺ T cells and suggests a therapeutic approach to sensitize T cell to immune checkpoint blockade.

The first report of anti-CD5 MAb-based therapy of tumours grown as solid tumours involved the effect of administration of anti-CD5 in mouse CD5⁺ leukemias grown as solid tumour homografts (133). Mice injected subcutaneously with CD5⁺ mouse leukemia tumours or CD5⁻ mouse lung cancer cells had increased overall survival when treated with anti-CD5 treatment. This effect was abolished in thymectomized mice, suggesting that the therapeutic effect of anti-CD5 MAb effects was mediated, not by direct effects on CD5⁺ tumour cells, but on thymus-derived CD5⁺ cells including non-tumour host mouse T cells. Further studies have shown that homografted mouse tumours grown in CD5 knockout (CD5^{-/-}) mice grow more slowly than in wild type CD5^{+/+} mice (134). This suggests that reducing CD5 signaling could lead to enhanced anti-tumour immunity. Assessment of the effect of direct administration of anti-CD5 MAb to block CD5 signaling on CD8⁺ T cells will lead to increased understanding of the effect of CD5 blockade in tumour immunity and facilitate the development of CD5-targeted anticancer immunotherapy. In chapter four, I investigated the direct effect of using anti-CD5 MAb on CD8⁺ T cell activation and function *ex vivo*.

To investigate the effect of anti-CD5 MAb of CD8⁺ T cell activation and function I treated primary CD8⁺ T cells isolated from mice homografted (tumour-harboring) and tumour-naïve (non-tumour bearing) with anti-CD5 MAb *ex vivo*. Using flow cytometry, I observed that *ex vivo* activation accompanied by

treatment with an antagonistic anti-CD5 MAb increased the fraction of CD8⁺ T cells expressing markers of activation (CD69 and IFN γ) (201, 202) and increased CD8⁺ T cell proliferation, indicating that anti-CD5 MAb enhances CD8⁺ T cell activation. *Ex vivo* treatment with anti-CD5 MAb alone did not induce CD8⁺ T cell activation suggesting that anti-CD5 treatment mediates enhanced activation by influencing CD5 effects on TCR signaling. In further support of this concept, ERK phosphorylation (indicative of increased TCR signaling) was increased in cells activated by treatment with anti-CD3, combined with treatment with anti-CD5 MAb, compared to activation with anti-CD3 alone. Treatment with anti-CD5 alone did not lead to increased ERK phosphorylation. This is consistent with a report that activation of ERK was greater in thymic cells from mice that express unfunctional CD5 challenged with OVA323-339-peptide to induce T cell activation compared to thymic cells from CD5 wildtype mice (183).

My data show increased AICD (a consequence correlated with increased T cell activation (20, 184)) in activated CD8⁺ T cells treated with anti-CD5 MAb. In addition, and consistent with increased AICD, treatment of spleen cells increased the fraction of FasR⁺ and FasL⁺ T cells; TILs isolated from tumour-bearing mice with CD5 deficiency have been reported to be similarly susceptible to AICD via increased FasR expression (134). AICD can occur as result of overstimulation of TCR (184). CD5 acts to fine-tune TCR responsiveness and, therefore, blockade of CD5 could lead to enhance TCR sensitivity to antigen and increased AICD. Based on these data showing that blocking CD5 on activated T cells by treatment with anti-CD5 blocking MAb enhances CD8⁺ T activation as

shown by elevated CD69, and enhanced production of IFN γ *ex vivo*, and that activation is associated with increased proliferation as shown by CFSE, I proposed that administration of blocking anti-CD5 MAb could enhance anti-tumour immunity. In a cytotoxic T cell lysis (CTL) assay, I observed that CD8⁺ T cells treated with anti-CD5 MAb were more capable of targeting and killing mouse 4T1 tumour cells *ex vivo* than cells treated with control Ab. These data strongly suggest that treatment with anti-CD5 MAb can increase the ability of CD8⁺ T cells to target and eliminate tumour cells, and supports the concept that blocking CD5 to enhance anti-tumour CD8⁺ T cell function has potential as an *in vivo* anti-cancer immunotherapy. Blocking CD5 on CD8⁺ T cells could lead to hyper-activation that, although potentially desirable in increasing anti-tumour action in the short term, could increase inhibitory receptors that, over time, act to suppress CD8⁺ T cell function. Therefore, targeting additional T cell molecules in combination with CD5 blockade (for example, immune checkpoint molecules) may be necessary to prevent CD8⁺ T cell exhaustion and sustain CD8⁺ T cell function. With due consideration of additional effects, my data nevertheless show that blockade of CD5 on T cells *ex vivo* has promise as an anti-tumour immunotherapy.

The data described in Chapter Four reveal that blocking CD5 *ex vivo* results in increased CTL activation and tumour cell cytotoxicity (now published in the *European Journal of Immunology* (177)). In Chapter Five, I aimed to assess the consequences of administration of anti-CD5 MAb to tumour-bearing mice on tumour growth and possible adverse immune-related and other adverse effects,

and to determine whether blocking CD5 *in vivo* could increase activation of normal T cells and enhance detection of poorly immunogenic tumour antigen(s). The triple-negative 4T1 mouse breast tumour cell line was used as a model for poorly immunogenic and highly metastastatic tumours, reflecting similar breast tumours in humans (174). This makes them good candidates to determine whether anti-CD5 MAb delivered *in vivo* could enhance T cell activation and ability to recognize poorly immunogenic tumour antigens. Mice were injected with 4T1 mouse tumour cells and treated with anti-CD5 MAb. The activation and function of T cells isolated from spleens, draining lymph nodes, and tumours (tumour-infiltrating lymphocytes, or TILs) were further assessed using flow cytometry.

The data show that administration of anti-CD5 MAb *in vivo* increases the ratio of CD8⁺ to CD4⁺ T cells in draining lymph nodes and within tumours. The predominant numbers of CD8⁺ T cells and the minority CD4⁺ T cells were significantly activated in spleens, draining lymph nodes, and TILs, suggesting that anti-CD5 treatment mediates enhanced activation by influencing CD5 effects on TCR signalling. As a result of increased activation, the level of the exhaustion marker PD-1 and the fraction of PD-1⁺CD4⁺ T cells and PD-1⁺CD8⁺ T cells were significantly higher after treatment with anti-CD5 MAb. The data was consistent with our previous report showing increased activation-induced cell death (AICD) and early apoptosis in CD8⁺ T cells after treatment with anti-CD5 MAb *ex vivo* (177). I showed significantly increased AICD and early apoptosis in CD8⁺ T cells isolated from draining lymph nodes and TILs and, consistent with increased

AICD, treatment with anti-CD5 blocking MAb increased the level of Fas receptor on the surface of CD8⁺ T cells. Because AICD occurs after chronic stimulation of T cell receptors (TCRs)(184), treatment with anti-CD5 MAb may lead to TCR sensitivity to tumour antigen, resulting in increased AICD. These results suggest that treatment with anti-CD5 MAb could also enhance the effector function of T cells. I found significantly increased T cell effector function in response to anti-CD5 treatment in spleens, draining lymph nodes, and TILs as shown by increased expression of surrogate markers of T cell degranulation and antigen-specific T cell (CD107a and CD137). Together, these data suggest that treatment with non-depleting anti-CD5 MAb *in vivo* resulted in increase T cell activation, AICD, and effector function.

I further tested the fraction of suppressor cell such as T_{regs} and MDSC after treatment with anti-CD5 MAb *in vivo*. I found that the fraction of MDSC did not increase after treatment with anti-CD5, but was influenced by the 4T1 tumour inoculation in the spleen and the tumour bed. T_{regs}, on the other hand, were significantly enhanced in lymph nodes after treatment with anti-CD5 MAb *in vivo*. Although the fraction of myeloid-derived suppressor cells (MDSC) was not altered upon anti-CD5 MAb treatment, the inhibitory ligands FasL and PD-L1 were significantly higher in the draining lymph nodes (DLN) from anti-CD5-treated mice. These data suggest that inhibitory signaling could affect T cell immunity in the 4T1 breast tumour model treated with anti-CD5 MAb. Furthermore, I tested whether combined treatment with anti-CD5 MAb and anti-PD-1 MAb could enhance T cell function and activation compared to single

treatment with anti-PD-1 MAb. The results show that treatment with both anti-CD5 MAb with anti-PD-1 MAb enhanced T cell activation and function compared to single treatment with anti-PD-1 in the spleen and DLN.

Administration of anti-CD5 *in vivo* results in significantly increased numbers of CD8⁺ cells relative to CD4⁺ T cells and enhanced CD8⁺ T cell activation and effector function. To assess whether administration of anti-CD5 MAb can significantly delay in tumour growth, I evaluated several approaches. The first approach was to administer anti-CD5 MAb i.p. on day 7 after 4T1 tumour inoculation (Figures 4,5, 39, and 40). Although there was a trend toward tumour growth delay in one experiment, the result was not reproducible upon repetition. This could be due to several factors. First, there was a significant accumulation of immune suppressor cells in the 4T1 tumour cell model (T_{regs} and MDSC (203) and Figure 34). It has been previously reported that the 4T1 tumour model is resistant to immunotherapeutic approaches (64) and the authors suggested combining several approaches to suppress the growth of 4T1 tumours *in vivo* when treating with immunotherapies: possible additional approaches could include depletion of MDSC or combining immunotherapeutic drugs with chemotherapeutic drugs toxic to immunosuppressive cell populations (64). To address this question, I tested the third approach (Figures 6, 7, 41 and 42) by co-administering two drugs used to deplete MDSCs *in vivo*: 5-fluorouracil (5-FU) and the deoxycytidine analogue and antimetabolite gemcitabine. Although single doses of these drugs resulted in tumour delay, the combination of 5-FU or gemcitabine with anti-CD5 MAb did not significantly reduce tumour growth

compared to single treatment with either of the two drugs. Second, and similar to *ex vivo* results (177), I observed increased AICD in T lymphocytes in response to anti-CD5 MAb treatment: a consequence of increased activation in CD8⁺ T cells from mice treated with anti-CD5 MAb. This observation was associated with increased Fas receptor levels in CD8⁺ T cells. This was expected as increased activation of CD8⁺ T cells has been shown to associate with increased levels of Fas receptor and AICD (204). To overcome this limitation, I tested the fifth approach: that is, by reducing the dose of anti-CD5 MAb used to treat homograft tumors *in vivo* and administered CD5 MAb as early as day 0. In addition, the route of CD5 MAb administration was peritumoural to limit systemic overactivation of T cells and reduce the chance of developing AICD (Figure 8 and 43). This approach gave the best result in delaying tumour growth *in vivo* as a single treatment of anti-CD5 MAb (Figure 43).

Considering that CD5 is expressed on all T cells, treatment with anti-CD5 MAb could have multiple effects, including adverse consequences: such possible consequences require further investigation. It has been reported that B6 polyclonal T cells with no or low CD5 expression are less capable of generating induced T_{regs} (iT_{regs}) under certain conditions (205) which play a role in suppressing immune cells: anti-CD5 MAb administration could, therefore, result in adverse auto-immune effects. On the other hand, other have reported that generation of T_{regs} is not affected by the absence of the CK2 binding domain in CD5, suggesting that reduced CD5 activity have little or no T_{reg} effects (182). It is possible that the presence of the whole CD5 receptor could affect generation of

T_{regs}, but that deletion of part of the CD5 molecule functional in affecting TCR signaling, or temporary blockade of the CD5 signaling pathway, would have little effect on generation of T_{regs} and reduced danger of induction of autoimmune effects. Nevertheless, multiple immune cell types (B cells, dendritic cells, and others) have been reported to express CD5 (146, 161, 206) and the consequences of systemic administration of anti-CD5 capable of blocking CD5 function on both anti-tumour CD8⁺ T cells (with potential to enhance anti-tumour immunity) and other immune cells (where blockade may have adverse effects) requires further study.

The use of toxic molecule-conjugated anti-CD5 depleting MAb was studied to treat CD5⁺ non-solid tumours. However, the impact of CD5 blocking antibody on normal T cells in CD5⁻ solid tumours is not well illustrated. Despite the study that was conducted 1984 that shows continues administration of CD5 polyclonal antibodies to slow the growth of EL lung cancer (133) there has been no further reports of the consequences of administration of anti-CD5 MAb for normal T cells *in vivo* to enhance its function. This thesis is the first to describe the correlation between CD5 and T cell activation and exhaustion in lymphoid organs and tumour and assess the affect of blocking CD5 function *ex vivo* in normal T cell to increase their activation and function. In addition, it illustrates the phenotypic changes in immune cell subsets in lymphoid organs as well as within tumours after *in vivo* administration of anti-CD5 MAb. These results require further investigation, particularly in combination with current anti-tumour immunotherapies. The dosage of anti-CD5 MAb should be taken into

consideration as high dosage could results in unwanted high activation of CD8⁺ T cell and induced AICD.

Chapter 7

7.1 References

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200. Hamid O, Robert C, Daud A, Hodi FS, Hwu W-J, Kefford R, et al. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *NEW ENGLAND JOURNAL OF MEDICINE*. 2013;369(2):134-144.
201. Simms PE, Ellis TM. Utility of flow cytometric detection of CD69 expression as a rapid method for determining poly- and oligoclonal lymphocyte activation. *CLIN. DIAGN. LAB. IMMUNOL*. 1996;3(3):301-304.
202. Ghanekar SA, Nomura LE, Suni MA, Picker LJ, Maecker HT, Maino VC. Gamma interferon expression in CD8+ T cells is a marker for circulating cytotoxic T lymphocytes that recognize an HLA A2-restricted epitope of human cytomegalovirus phosphoprotein pp65. *CLIN. DIAGN. LAB. IMMUNOL*. 2001;8(3):628-631.
203. Bunt SK, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. *THE JOURNAL OF IMMUNOLOGY*. 2006;176(1):284-290.
204. Alderson MR, Tough TW, Davis-Smith T, Braddy S, Falk B, Schooley KA, et al. Fas ligand mediates activation-induced cell death in human T lymphocytes. *THE JOURNAL OF EXPERIMENTAL MEDICINE*. 1995;181(1):71-77.
205. Henderson JG, Opejin A, Jones A, Gross C, Hawiger D. CD5 instructs extrathymic regulatory T cell development in response to self and tolerizing antigens. *IMMUNITY*. 2015;42(3):471-483.
206. Moreau M-F, Thibaud J-L, Miled LB, Chaussepied M, Baumgartner M, Davis WC, et al. *Theileria annulata* in CD5+ macrophages and B1 B cells. *INFECTION AND IMMUNITY*. 1999;67(12):6678-6682.

Curriculum Vitae

Name: Faizah Alotaibi

Post-secondary Education and Degrees:

- The University of Western Ontario

London, Ontario, Canada

2016-2020 PhD (Dr. James Koropatnick and Dr. Wei-Ping Min, thesis supervisors)

- Queen's university

Kingston, Ontario, Canada

2013-2015 MSc (Dr. Peter Greer, thesis supervisor)

- Taibah University

Madinah, Saudi Arabia

2007-2011 B.Sc. with honors.

Honors and Awards:

- Graduate Student Innovation Scholars (GSIS).
2020 cohort - I have been selected with other 11 candidates from 90 applicants
- Nominated by the Chair of the Graduate Studies Committee for the Drs. Madge and Charles Macklin Fellowship award for Teaching & Research in Medical Sciences | 2020
- UWO Department of Microbiology and Immunology travel award 2019 | Departmental level award based on academic merit
- King Abdullah Doctoral Scholarship
2017- 2020 | From the High Ministry of Education in Saudi Arabia to support my PhD training

- CIHR Training Program in Cancer Research and Technology Transfer (CaRTT, a CIHR Strategic Training Initiative in Health Research [STIHR] program) Scholarship.
2017 – 2018
- Western Graduate Research Scholarship (WGRS),
2016 – 2017 | Western Ontario University
- High Academic Achievement Award
2020 | From Saudi Cultural Bureau in Canada for publishing a first author paper.
- King Abdullah scholarship for MSc training
2012- 2015
- High Academic Achievement Award
2012 | From Saudi Cultural Bureau in Canada for achieving high academic record
- Undergraduate research project award
2011 | From Taibah University for achieving 98% in the final year research project
- High Academic Achievement in undergraduate
2010 | Award from Taibah University for achieving high GPA score during the last two years.
- Red Crescent Award for Volunteers
2010 | Red Crescent

Peer-reviewed published papers

- **Alotaibi F**, Min WP and Koropatnick J. Reduced CD5 on CD8+ T cells in tumors but not lymphoid organs is associated with increased activation and effector function. Journal Frontiers in Immunology. Impact factor (3 Yrs Avg) 5.65. Accepted 8th of December 2020.
- Zhang M, Shi Y, Zhang Y, Wang Y, **Alotaibi F**, Qiu L, Wang H, Peng S, Liu Y, Li Q, Gao D, Wang Z, Yuan K, Dou FF, Koropatnick J, Xiong J, Min W. miRNA-5119 regulates immune checkpoints in dendritic cells to

enhance breast cancer immunotherapy. *Cancer Immunol Immunother*. 2020 Jun;69(6):951-967. doi: 10.1007/s00262-020-02507-w. Epub 2020 Feb 20. PMID: 32076794. Impact factor 4.9 (2018/2019)

- **Alotaibi F**, Rytelowski M, Figueredo R, Zareardalan R, Zhang M, Ferguson PJ, Maleki Vareki S, Najajreh Y, El-Hajjar M, Zheng X, Min WP, Koropatnick J. CD5 blockade enhances *ex vivo* CD8⁺ T cell activation and tumour cell cytotoxicity. *Eur J Immunol*. 2020 May;50(5):695-704. doi: 10.1002/eji.201948309. Epub 2020 Feb 25. PMID: 31943150. Impact factor 4.7 (2018/2019)
- Zhu C, Su Y, Juriasingani S, Zheng H, Veramkovich V, Jiang J, Sener A, Whiteman M, Lacefield J, Nagpal D, **Alotaibi F**, Liu K, Zheng X. Supplementing preservation solution with mitochondria-targeted H2 S donor AP39 protects cardiac grafts from prolonged cold ischemia-reperfusion injury in heart transplantation. *Am J Transplant*. 2019 Nov;19(11):3139-3148. doi: 10.1111/ajt.15539. Epub 2019 Sep 3. PMID: 31338943. Impact factor 7.1 (2018)
- Zhang M, Gao D, Shi Y, Wang Y, Joshi R, Yu Q, Liu D, **Alotaibi F**, Zhang Y, Wang H, Li Q, Zhang ZX, Koropatnick J, Min W. miR-149-3p reverses CD8⁺ T-cell exhaustion by reducing inhibitory receptors and promoting cytokine secretion in breast cancer cells. *Open Biol*. 2019 Oct 31;9(10):190061. doi: 10.1098/rsob.190061. Epub 2019 Oct 9. PMID: 31594465; PMCID: PMC6833224. Impact factor 3.9 (2018)
- **Alotaibi, F**. An immune modulatory role for the fes proto-oncogene. MSc Dissertation. 2015.

Book chapter:

- **Alotaibi F**. Book title: Exosomes in biological communication system. Chapter 16: Exosomal microRNAs: Potential Biomarkers for Cancer Diagnosis, Treatment Response, and Prognosis. Springer Nature. Doi: 10.1007/978-981-15-6599-1. 2020.

Papers in Preparation

- **Alotaibi, F** and Koropatnick, J. CD5 as a targetable antigen in solid and non-solid tumours. Review article in preparation
- **Alotaibi, F**, Min, WP and Koropatnick, J. Administration of anti-CD5 MAb *in vivo* enhances CD8⁺ T cell activation and function in poorly immunogenic 4T1 breast tumour model. Article in preparation
- **Alotaibi, F** and Koropatnick, J. Destabilizing the genome: a therapeutic strategy to enhance immune checkpoint blockade. Review article in preparation

Accepted abstracts and posters at international and local conferences

- **Alotaibi, F**. Figueredo, R. Zareardalan, R. Ferguson, P. Zheng, X. Min, WP. And Koropatnick, J. (2020). Abstract: Enhancing efficacy of PD-1 blockade in 4T1 breast tumour model. The American Association for Cancer Research annual meeting. San Diego, CA, USA which turned to virtual meeting due to COVID-19.
- **Alotaibi, F**. Figueredo, R. Zareardalan, R. Min, WP. And Koropatnick, J. (2020). Abstract: Enhancing T cell tumour immunity. Immune-oncology symposia. London, Ontario, Canada
- **Alotaibi, F**. Figueredo, R. Zareardalan, R. Min, WP. And Koropatnick, J. (2019). Abstract: Downregulation of CD5 enhances immune T cell activation *ex vivo*. Infection and immunity research day. London, Ontario, Canada
- **Alotaibi, F**. Figueredo, R. Zareardalan, R. Zheng, X. Min, WP. And Koropatnick, J. (2019). Abstract: CD5 blockade as a novel immune-therapy. London health research day, London, Ontario, Canada.
- **Alotaibi, F**. Min, WP. And Koropatnick, J. (2019). Abstract: CD5 blockade enhances CD8⁺ T cell activation and tumour cell cytotoxicity. the American Association for Cancer Research for Tumour Immunology and Immunotherapy, Boston, Massachusetts, USA.
- Wang, Y. **Alotaibi, F**. Zhang, M. Joshi, R. & Min, W. (2019). Abstract: microRNA-5119 engineered DC Vaccine for breast cancer

immunotherapy. Oncology Research and Education Day, London, Ontario, Canada.

- **Alotaibi, F.** Figueredo, R. Zareardalan, R. Min, WP. And Koropatnick, J. (2018). Abstract: Downregulation of CD5 and FasR on CD8⁺ immune T cells to suppress tumour growth. Infection and immunity research day. London, Ontario, Canada
- **Alotaibi, F.** Min, WP. And Koropatnick, J. (2018). Abstract: Targeting CD5 receptor on CD8⁺ immune T cells to improve anti-tumour immunity. The Canadian Society for Immunology, London, Ontario, Canada.
- **Alotaibi, F.** Min, WP. And Koropatnick, J. (2018). Abstract: Targeting CD5 on CD8⁺ immune T cells to improve anti-tumour immunity. Keystone Symposia sponsored meeting entitled "Regulation and Dysregulation of Innate Immunity in Disease (B9). Vancouver, British Columbia.
- **Alotaibi, F.** Figueredo, R. Zareardalan, R. Min, WP. And Koropatnick, J. (2017). Abstract: Combine siRNA targeting of CD5 and FasR on CD8⁺ immune T cells to improve anti-tumour immunity. Infection and immunity research day. London, Ontario, Canada
- **Alotaibi, F.** Ferguson, P. And Koropatnick, J. (2016). Abstract: Combined targeting of IDO and BRCA2 for anticancer therapy. London health research day, London, Ontario, Canada.
- **Alotaibi, F.** Basta, S., & Greer, P. A. (2015). Abstract: The role for the fes oncogene in immunity. Health Science Research Trainee Day, Kingston, Ontario, Canada
- **Alotaibi, F.** M., Zhang, C., Basta, S., & Greer, P. A. (2015). Abstract B05: An immune modulatory role for the Fes protein tyrosine kinase. the American Association for Cancer Research for the Tumour Angiogenesis and Vascular Normalization conference, Orlando, Florida, USA.

Invited talks, lectures, and delivered papers

- Invited oral presentation. Title: Novel strategy to improve cancer immune checkpoint inhibitors efficacy. Presented at Lawson's Talk on Mondays (TOMs). Feb 10th, 2020. London, Ontario, Canada
- Invited lecture. Title: Pathogenic bacteria part of Microbiology and Immunology 3820A course for nursing students. The course size was 400 students. Nov 15th, 2019. London, Ontario, Canada
- Invited poster presentation. Title: Targeting CD5 and FasR to improve tumour immunity. Presented at Western University Health and Research Conference (WUHRC). March 22nd, 2019.
- Invited review paper. Title: Destabilizing the genome: a therapeutic strategy for treatment of cancer. In preparation for the journal *Biomolecules*.

Professional experience

Teaching experience

- **Teaching Assistant | Western university | 2019-2020**
TA in the microbiology and immunology course for nursing students (MICROIMM 3820A), my role was to help in the pathological bacteria part of the course, answering students questions and writing case studies and marking them. As well as holding a review session to 400 students.
- **Teaching Assistant | Western university | 2016-2017**
TA in the Infection and immunology course (MICROIMM 2500A), the course design to tough students concept in immunity including: innate and adaptive immunity, T and B cell immunity, pathogen recognition, and more.

Research experience

- **Graduate research Assistant | Western university | 2016-2020**
Conducting research for projects investigating the effect of targeting CD5 and Fas receptors to improve cancer immunity.
- **Research Assistant | London Regional Cancer Program, Cancer Research Laboratory Program | 2016-2017**

Conducting research for projects investigating the effect of IBR2 drugs targeting RAD51 on human lung cancer cell lines.

- **Graduate Research assistant | Queen's university | 2013-2015**

Conducting research for projects investigating the effect of fes/fps oncogene in Macrophages and T cells in the immune system.

Work-related experience

- **Judge | Thames Valley Science & Engineering Fair | 2019.**

The Thames Valley Science & Engineering Fair is an annual event that provides an opportunity for Grades 4 to 12 students in the city of London and Middlesex, Oxford and Elgin counties to showcase their knowledge, talents and ingenuity in science, engineering and technology. My work involved reviewing and evaluating the student's projects in science as well as exam them.

- **Judge | The Frontenac, Lennox and Addington Science Fair (FLASF) | 2015.**

The Frontenac, Lennox and Addington Science Fair (FLASF) is an annual event that provides an opportunity for Grades 5 to 12 students in Kingston, Ontario to showcase their knowledge, talents and ingenuity in science, engineering and technology. My work involved reviewing and evaluating the student's projects in science as well as exam them.

- **Member of the organization committee of the UWO Department of Microbiology and Immunology Infection and Immunity Research Forum (IIRF) | 2018 – 2020**

- **Member of the Program Advisory Committee of the Cancer Research and Technology Transfer (CaRTT) program (trainee representative) | 2017-2018.**

Helped in organizing its most recent Annual Retreat to consider and recommend improvements to the CaRTT program as well as other tasks related to the committee, including designing and analyzing a survey and presenting data at the Annual Retreat to improve the CaRTT program for the upcoming year.

- **Lab internship | Maternity and Children Hospital | 2011-2012**
Helped in collecting samples from patients and perform sample analysis including blood samples, tissue samples. Report collected data to system.

Workshop and professional training

- **Western certificate in university teaching and learning | 2020**
The goal of the Western Certificate in University Teaching and Learning is to enhance the quality of teaching by graduate students and postdoctoral scholars, and to prepare them for a future faculty or professional career.
 - **Teaching Assistant Training Program | 2016**
The Teaching Assistant Training Program (TATP) is a 2½ day intensive training program designed for new TAs, including workshops on building lessons, providing feedback, grading fairly, running tutorials/labs, managing TA duties, and intercultural competence.
 - **Netiquette: Communicating with Your Students workshop**
2016 | western university, London, Ontario
 - **Open Educational Resources for Teaching and Learning: Trends and Opportunities workshop**
2016 | western university, London, Ontario
 - **Designing Your Own Course: Components of a Great Syllabus workshop**
2016 | western university, London, Ontario
 - **Privacy and confidently training**
2016 | London health science center, London, Ontario
 - **Workplace Hazardous Materials Information System (WHIMS) Advanced level training**
2016 | London health science center, London, Ontario
 - **Writing Advanced course**
2013 | Algonquin college, Ottawa, Canada
- Memberships**
- Member of The Society for Immunotherapy of Cancer's (SITC). 2020 – Present

- Member of the American Association for Cancer research (AACR). 2019 - present
- Member of the Canadian society for Immunology. 2017 – 2019
- Member of the Saudi Cancer Society. 2011 - 2015